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Inhibition of secondary caries *in vitro* by addition of chlorhexidine to adhesive components

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ABSTRACT

Objective. To investigate secondary caries inhibition after dentine pre-treatment with 2% CHX, experimental addition of CHX in primer and adhesive of a 3-step adhesive system, and industrial addition od CHX in a 2-step adhesive system.

Materials and method. Sixty Class-V cavities were restored according to the adhesive protocol (n = 12): 1) control group, Scotchbond Multi-Purpose, 3M (CTRL), 2) 2% CHX dentine pre-treatment (DENT), 3) 0.1% CHX in primer (PRIM), 4) 0.1% CHX in bonding agent (BOND), 5) Peak Universal Bond including 0.2% CHX (PEAK). Specimens were thermocycled (10,000 cycles) and inserted into a *Streptococcus mutans* biofilm artificial mouth (caries model). The 10-day biological loading protocol consisted of consecutive phases of demineralisation (1 h) and remineralisation (5 h). Evaluation under a fluorescence microscope (demineralisation) and an SEM (marginal gap) followed, at restoration margins, and at 0.3 mm and 0.5 mm distance from the margins, in enamel and in dentine. Total demineralization was calculated as the sum of demineralisation and substance loss due to demineralisation.

Results. PRIM ($p = 0.007$, mod. LSD), BOND ($p = 0.012$, mod. LSD) and PEAK ($p = 0.008$, mod. LSD) exhibited significantly higher total demineralisation values in enamel margins than CTRL. No significant differences were noted for total demineralisation in dentine. Regarding marginal gaps, DENT exhibited significantly lower enamel gap values compared to all other groups ($p = 0.001$).

Conclusions. 2% CHX as dentine pre-treatment, 0.1% or 0.2% CHX added in adhesives did not provide any antibacterial effect regarding secondary caries in dentine. On the other hand, 2% CHX dentine pre-treatment managed to limit marginal gap formation in enamel compared to the other adhesive protocols in the study.

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1. Introduction

Resin composites gradually became the most used restorative material, also for posterior teeth in stress-bearing areas [1,2]. However, facing a 2.2–3.6% annual failure rate [2], resin composite restorations still suffer suboptimal durability having been always an important clinical issue. Whereas in the 1970s early bonded restorations have been failing due to material wear, nowadays the main reason for restoration failure is bulk fracture as well as secondary caries [2]. Especially for Class-V restorations, it has been reported that up to 62.5% fail after 5 years due to reasons associated with marginal integrity [3]. Despite the fact that restorative materials have been evolved regarding their basic mechanical, physical and bonding properties, any incorporated "therapeutic effect" would be a true innovation.

Both hydrolytic degradation and enzymatic activity in dentine are the main reasons for adhesive failure of resin composites [4]. Moreover, a further challenge for the restorations are bacteria from the oral cavity, being able to adhere on restorative materials, especially on composite restorations or be accumulated in bonded interfaces [5]. This furthermore threatens the restoration's viability, through microleakage of fluids, bacteria, toxins and ions through the material–tooth interface potentially leading to secondary caries [6]. Bonded resin composite restorations accumulate more biofilm and they are subject to faster bacterial degradation with the extent of degradation of the restorative or the adhesive being primarily dependent on their chemical formulation [7]. Since this is localized around restoration margins [7], the adhesive interface is considered the Achilles' heel of adhesive restorations. This space varies between 2–20 μm at the cavity floor and 1–10 μm at the lateral restoration walls and the bacterial biofilm gathered in up to 8 weeks, may be 2–15 μm [8]. Even though a threshold marginal gap size for clinical failure of the restorations has not been established [9], restorations with marginal defects fail more frequently [10].

Cariogenic properties of *S. mutans* [11] have early led to the perception that mutans streptococci are the main pathogen which causes dental caries. Chlorhexidine (CHX) is a cationic-bisguanide ($C_2H_7N_5$), with antiseptic action against *S. mutans* and is therefore widely used in oral hygiene products and in preventive dentistry. CHX possesses both bacteriostatic and bactericidal effects against Gram+ and Gram- depending on its concentration and acts by disruption of the cell membrane [12]. The importance of CHX in restorative dentistry and its benefit in adhesive restorations has however recently been discussed [13–15] and was at first used as a dentine disinfectant and re-wetting agent prior to adhesive bonding, since it did not influence the immediate bond strengths [16]. CHX is able to bind to acid etched dentine and be slowly released overtime because of its substantivity [17]. It can be delivered as dentine pre-treatment or admixed with the adhesives, however issues are raised regarding its potential interference with the mechanical properties and the bonding efficiency of the adhesives used as carriers [18,19]. Inhibition of bacterial action via incorporation of antibacterial substances in many dental materials, such as composite resins, resin cements, glass-ionomer cements, provisionary cements and adhesives is extensively discussed [20,5]. However, a review of the Cochrane Collaboration concludes that there is not enough clinical data to assess the ability of antibacterial restorative materials to prevent dental caries [21]. Despite the fact that adhesives possess an antibacterial effect themselves due to their low pH, this is limited to 24–48 h and their acidity is neutralized by their contact with the tooth tissues [20]. Therefore, materials with a longer-lasting antibacterial effect were developed, mainly by incorporation of antibacterial substances in their composition, which can be (i) releasing, soluble antibacterial agents, (ii) non-releasing co-polymerized antibacterial agents and (iii) inorganic fillers [5,20]. The main advantage of soluble antibacterial agents, such as CHX, is that they can easily be released from the restorations to the oral environment. The antibacterial substances which are released from the materials used as carriers, may challenge their kinetics of release or affect the physical properties of their carriers. Inevitably, their antibacterial activity decreases over time [5]. Issues concerning the physical properties of the carrier-materials and their release potential also arise for the non-releasing antibacterials that are *in situ* polymerized. On the other hand immobile antibacterials can only kill bacteria which come in contact with the adhesive [20]. Resin cements containing 3–4% CHX exhibited CHX release for 5 weeks and antibacterial action for 2 weeks, while no CHX release was detected when its concentration in the cement was 2% or lower [18]. No inhibition zone against bacteria was produced by CHX-containing resin after 2-week storage [22], showing that CHX concentration was not enough. When CHX was incorporated in ion-exchanging materials, such as glass-ionomer cements, its antibacterial activity against *S. mutans* increased to 90 days [23] and their inhibition zones were not dependent upon CHX content [24]. *In vivo*, CHX-containing glass-ionomer cements decreased the microbial count in dentine under the restoration after 3 months [25], but a CHX-containing glass-ionomer cement pit-and-fissure sealant did not increase caries reduction in 12 months [26]. Clinical trials provide controversial data, as some studies suggest that 2% CHX application on Class V dentine, provides after 36 months reduced retention – however not significantly – [27] and after 6 or 18 months delivered same retention rates [28–30] as without CHX. Up to date, there is no published study investigating the anticariogenic effect of CHX adhesives.

Due to ethical problems with clinical studies, *in vitro* models have been developed to generate fundamental aspects of carious process by simulating the oral microcosm. Bacterial caries model provide a realistic simulation of secondary caries formation [31–33], several factors implicated in caries aetiology can be separately investigated, a large number of samples can be studied and ethical issues connected to animal trials are set aside [34]. Fundamental requirements for an effective caries model are: pH control, pH cycling reproducing de- and remineralisation phases, simulation of intra-oral sugar effects for the demineralisation phase, adjustment of saliva effect and of sugar clearance for the remineralisation phase and choice of nutrition medium being exactly adjusted to the bacteria under investigation [35]. It is demonstrated that dentine caries produced by caries models resembles natural dentine caries both histologically and microradiographically, since both include subsurface mineral loss, producing different zones [36]. Bac-

terial caries model are also able to create an infected outer layer and an affected inner layer like in natural dentine caries [37]. An optimal period of 8 days is suggested for producing non-cavitated caries-like lesions [38].

Only one adhesive system with industrially incorporated 0.2% CHX is commercially available [39,40] and a direct comparison between the different ways of CHX addition into the different steps of the adhesive procedure (primer or bonding agent) has never been made in a single study nor has it been directly compared with the use of CHX as a cavity pre-treatment agent, under biological loading. The aim of the study was to test CHX adhesives for their ability to arrest secondary caries-like lesions around Class V composite restorations when biologically loaded in a fully automated artificial caries model with *S. mutans*. Null hypothesis was that addition of CHX in the adhesives is not able to inhibit secondary caries formation around Class V composite restorations.

2. Materials and methods

Sixty freshly extracted human third molars collected under the informed consent of the patients upon approval of the Ethical Committee of the Medical Faculty of Justus Liebig University Giessen (AZ 143/09). Teeth were cleaned, examined with 3X magnification loupes for caries, fractures or defects and stored in 0.5% Chloramin-T solution (Chloramin T trihydrate, Carl Roth, Karlsruhe, Germany) in 5–7 °C for up to 30 days. In case longer storage was needed, teeth were refrigerated (−15 °C) in distilled water until further use. Standardized buccal Class V cavities (4–5 mm in width mesiodistally, 2–3 mm height, 2 mm depth) with margins located 50% in enamel and 50% in dentine or cementum were prepared with a cylindrical round-end diamond bur (Revelation Diamond #881-014C, SS-White Burs, Pennsylvania, USA) and a 2-mm depth-indicator bur (Diamond Bur FG 2 mm, Meisinger, Neuss, Germany), and were randomly divided into 5 groups (n = 12), according to the adhesive protocol used.

Five adhesive bonding protocols were used, including a 3-step bonding system, two experimental CHX adhesives and a commercially available 2-step etch-and-rinse CHX adhesive (Table 1), in order to compare experimental and industrial CHX addition The experimental adhesives were built on the basis of the 3-step bonding system (Adper Scotchbond MP, 3M Oral Care, Seefeld, Germany) [41,42], using 2% chlorhexidine digluconate (Gluco-Hex 2% Solution, Cerkamed). Application procedure is demonstrated in Table 2. Primer was air-dried in order to allow for sufficient solvent evaporation and air-thinning was performed for the bonding agent, until no visible liquid movement. No rinsing was performed after application of 2% CHX as dentine surface pre-treatment. For the experimental adhesives CHX was separately admixed into the primer or bonding agent (Table 2), so as to isolate its effect in every step. Final solutions contained 5% of 2% CHX and 95% of primer or bonding agent, reaching a CHX concentration of 0.1%. The ingredients were thoroughly mixed with a 2-mm sized brush applicator for 20 s and the mixture was allowed to set for 10 s. Fresh quantity was prepared for each tooth. Following groups were formed: (1) control group (CTRL), (2) 2% CHX dentine pre-treatment (DENT), (3) 0.1% CHX in primer (PRIM), (4) 0.1% CHX in bonding agent (BOND), (5) Peak Universal Bond with 0.2% CHX (PEAK).

Cavities were finally restored with composite resin (Filtek™ Z250, 3M-ESPE), placed in two diagonal layers and polymerized for 40 s each with a LED polymerization unit (Elipar S10, 3M-ESPE, light intensity 1200 mW/c^2). Excess material at restoration margins was removed with a scaler (H5 Hygienist/U15 Towner Scaler, Hu-Friedy, Frankfurt, Germany) and restorations were polished with Al_2O_3-coated polishing discs in successive roughness (Sof-Lex™ Discs and Sof-Lex™ Wheels, 3M-ESPE), in order to obtain an absolutely composite-free margin. Specimens were stored in an incubator for 2 weeks in distilled water at 37 °C (Incubator Function Line, Heraeus Holding, Hanau, Germany). Thermocycling followed for 10 000 cycles (±5 °C and ±55 °C with 15″ dwell time and 15″ transfer time) (Thermocycler, Thermo Fisher Scientific™, Waltham, Massachusetts, USA). After that a first set of replicas (Impression material: Panasil Putty, Kettenbach and Panasil Initial Contact Light, Kettenbach, Impression Trays: Miratray-Mini, Hager Werken, Die cast material: AlphaDie MF, Schütz Dental Group) was manufactured and analyzed under a scanning electron microscope (SEM) (SEM Amray Model 1610 Turbo, Amray, Bedford, MA, USA). Roots of specimens were then shortened at a slow-speed diamond saw (Isomet 1000, Buehler, Lake Buff, IL, USA), teeth were attached with glue wax (Supradent-Wachs, Chemisches Dental-Labor Oppermann-Scwedler, Pluradent, Frankfurt, Germany) to chewing simulator plates (custom-made plates, Festo Systemtechnik, Denkendorf, Germany) and were disinfected for 2 h in 70% ethanol solution. Following to that, specimens were transferred into the sterilized reaction chamber (300–4100 Reusable Filter Holder with Receiver, Thermo Fisher Scientific™ Nalgene ™ Labware, Rochester, NY, USA) under a Clean Bench (Clean bench, Thermo Fisher Scientific™).

Artificial secondary caries was produced around restorations during a 10-day biological protocol in a fully automated caries model (Fig. 1), with consecutive demineralisation (1 h) and remineralisation phases (5 hours) [31,43,33,35]. For one 10-day experimental cycle, 40 demineralisation phases, i.e. four per day were produced. As bacteria for biofilm generation freeze-dried *S. mutans* was chosen (DSMZ 20523). *S. mutans* were cultured on Columbia Agar plates for 48 h. The overnight culture followed for 12 h at 37 °C, attenuated 1:10 and after another 9 h incubation, was inoculated. Average microbial load at the end of each cycle was 10^6 microbes/mL. Each demineralisation was induced after 6 h incubation of the bacterial solution in the nutrition medium (Schaedler Broth, Becton Dickinson, Franklin Lakes, USA) and remineralisation was achieved with artificial saliva solution [31,43,33,35]. During each remineralisation phase, the reaction chamber was rinsed four times with artificial saliva and saliva of the final rinse process remained in the chamber. Navigation of the involved pumps (Cyclo II, Roth, Karslruhe, Germany) was managed with a specially designed software (LeC Operating Software for Relay Module 8X-serial, Conrad Electronics SE, Hirschau, Germany). Temperature (37 °C) and pH (7 during remineralisation and ˜4,3 during demineralisation) were monitored

Table 1 – Information of materials tested. Adhesives were used according to manufacturers' directions.

Product-manufacturer	Type	Composition (% by wt.)	LOT
Adper scotchbond multipurpose adhesive system, 3M-ESPE	3-step etch-and-rinse bonding system	Scotchbond etchant: 55–65% Water, 30–40% phosphoric acid, 5–10% synthetic amorphous silica	516827
		Primer: 40–50% water, 35–45% 2-HEMA, 10–20% copolymer of acrylic and itaconic acids	N510460
		Bonding: 60–70% BISGMA, 30–40% 2-HEMA, <0.5% triphenylantimony	N515442
Peak universal bond with 0.2% chlorhexidine, ultradent, cologne	2-step etch-and-rinse bonding system	Ultra-etch: <45% phosphoric acid Adhesive: <20% ethyl alcohol, ≤16% 2-HEMA, ≤6% methacrylic acid, <0.3% chlorhexidine di(acetate), 7.5% Fillers	B8ZG1
Gluco-CHeX 2%, Cerkamed, Stalowa Wola, Poland	Chlorhexidine digluconate	2% Chlorhexidine gluconate	1806131
Filtek™ Z250, 3M-ESPE	Composite resin	75–85% silane treated ceramic, 1–10% BISEMA6, 1–10% UDMA, 1–10% BISGMA, <5% TEGDMA, <5% aluminum oxide, <0.5% benzotriazol, <0.2% EDMAB	N512895 N561790 N608865 N635023

Table 2 – Application directions of the adhesives used.

Bonding system used	Group	Preparation of CHX adhesives	Application steps
	CTRL	–	1–7
	DENT	–	1, 2, 9, 3, 4–7
Adper scotchbond multipurpose adhesive system, 3M-ESPE	PRIM	Mix 0.5 µL of 2% CHX digluconate and 9.5 µL scotchbond primer =5% v/v CHX PRIMER	1–3 (CHX PRIMER), 4–7
	BOND	Mix 0.5 µL of 2% CHX digluconate and 9.5 µL scotchbond bonding =5% v/v CHX BOND	1–5 (CHX BOND), 6, 7
Peak universal bond with 0.2% chlorhexidine, ultradent	PEAK	CHX industrially admixed	1, 2, 8, 4, 7

[1]Etch enamel (30 s) and dentine (15 s) with phosphoric acid, [2]rinse for 30 s and dry, [3]apply primer with an applicator brush to enamel and dentine for 10 s, [4]air-dry gently for 5 s from 10 cm distance, [5]apply bonding with an applicator brush to enamel and dentine for 10 s, [6]air-thinning, [7]light-cure for 20 s, [8]apply adhesive with applicator sponge and scrub for 10 s, [9]apply 2% CHX on dentine with an applicator sponge for 10 s and air-dry.

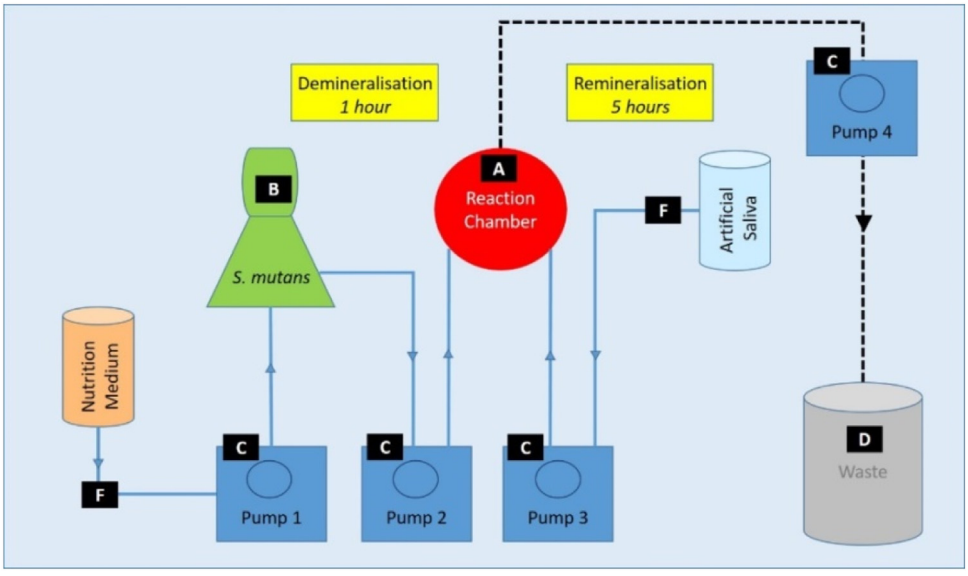

Fig. 1 – Schematic demonstration of experimental steps for Class V caries model.

throughout the whole experiment (Ph Electrode Blueline, SI Analytics, Mainz, Germany; Ph Measuring Instrument Lab870, SI Analytics, Mainz, Germany; MultiLab Pilot Freeware). Parts of caries model, nutrition medium and artificial saliva were autoclaved at 121 °C for 15 min and the pH electrode was separately disinfected with 70% ethanol and rinsed with sterile water. Clarity control and estimation of average microbial load was performed before inoculation and after the end of the experiment, in order to exclude the possibility of external contamination.

After the experimental cycle, teeth were disinfected with 70% ethanol and a second set of replicas was made and analyzed under SEM. Specimens were then cut longitudinally in the middle of the restoration, using a slow-speed diamond saw (Isomet 1000, Buehler). Restoration margins at enamel and at dentine were analyzed under a fluorescence microscope (AZ 100, Nikon, Tokyo, Japan) using a FITC filter (excitation filter 450–490 nm, blocking filter 515–565 nm) at 72× magnification (objective 3× * zoom 4× * ocular 10× * tube factor 0.6*) and demineralisation was measured using NIS-Elements AR 4.00.07 (64 bit) for Windows XP at 0.9 μm/px. Total demineralization (TOTAL) was calculated as sum of demineralization (DEM) and substance loss due to demineralization (SUB), at marginal, 0.3 mm and 0.5 mm away (Fig. 2).

Statistical analysis was performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Level of significance was set at $p < 0.05$. Normal distribution was checked with Kolomogorov–Smirnov test. Differences between the 5 randomized caries model series, were checked with Mann–Whitney *U* test. Comparisons between enamel and dentine, margins and 500 μm away from the margins, or between 300 μm and 500 μm away from the margins, were performed with Student's T-Test. Analysis of variance of fluorescence microscope values was performed with One-way ANOVA and *post-hoc* analysis Fisher's Least Significant Difference (LSD) test was conducted to explore the presence of significant differences between tested adhesives. Comparisons between SEM values before and after caries model were performed pairwise for every adhesive and every variable with the non-parametric Friedman Test. Differences between the tested adhesives regarding SEM values were checked with One-way ANOVA followed by *post-hoc* analysis with LSD.

3. Results

During biological loading in caries model, mean demineralisation time was 40 ± 0 h and average pH value was 4.22 (4.2–4.3). Respectively, specimens were remineralised for 203.6 ± 8.05 h at pH levels 7.08 (7–7.2). No statistical difference was reported between caries model series ($p > 0.05$, Mann–Whitney). Restoration margins presented significantly higher values compared to 500 μm away from the margins ($p < 0.05$, T-Test) (Figs. 5–9). No significant difference was noted for between 300 μm and 500 μm away from the restoration margins for all tested adhesives ($p > 0.05$, T-Test). As expected, enamel showed lower total demineralization (TOTAL) than dentine ($p < 0.05$, T-Test) (Figs. 5–9). No significant differences between the adhesives were noted for total demineralisation (TOTAL) at 300 μm and at 500 μm distance from enamel (Table 3, Fig. 3) or dentine margins (Table 4, Fig. 4) ($p > 0.05$, ANOVA).

3.1. *Enamel margins*

Significant differences were exhibited between the tested adhesives for the variables total demineralization (TOTAL) at enamel margins ($p = 0.03$, ANOVA), for demineralisation and substance loss at dentine margins ($p = 0.003$, ANOVA), and for marginal gap depth in enamel ($p = 0.029$, ANOVA). Further analysis with *post hoc* test LSD showed that PRIM ($p = 0.007$, mod. LSD), BOND ($p = 0.012$, mod. LSD) and PEAK ($p = 0.008$, mod. LSD) exhibited total demineralisation values in enamel margins, which were significantly higher than the CTRL. DENT showed however no statistical difference with any of the groups ($p > 0.05$, mod. LSD) (Table 3).

3.2. *Dentine margins*

No significant differences were noted for total demineralization (TOTAL) in dentine. However, adhesives showed significantly worse demineralization (DEM) in dentine margins in comparison to CTRL; DENT ($p = 0.001$, mod. LSD), PRIM ($p = 0.07$, mod. LSD), BOND ($p = 0.000$, mod. LSD) and PEAK ($p = 0.006$, mod. LSD). On the contrary, substance loss due to demineralization (SUB) at dentine margins was significantly higher for CTRL in comparison to BOND ($p = 0.004$, mod. LSD) and PEAK ($p = 0.023$, mod. LSD) (Table 4).

3.3. *SEM marginal analysis*

The percentage of perfect margins in enamel decreased significantly for all adhesives after biological loading ($p < 0.05$, Friedman) (Table 5). On the contrary, no such difference was demonstrated for dentine, except for adhesives BOND ($p = 0.021$, Friedman) and PEAK ($p = 0.001$, Friedman) (Table 6). DENT exhibited significantly lower enamel gap values compared to all other groups ($p = 0.001$, ANOVA) (Table 5). Regarding dentine margins, DENT showed significantly lower gap percentage compared to the CTRL ($p = 0.025$, mod. LSD) (Table 6).

4. Discussion

There are hints in the literature of the field that CHX may stablize long-term bonding to dentin, especially when the etch-and-rinse-approach is followed. However, this requires a separate step and in many clinical cases it is not clear what kind of CHX material is used. Incorporation of CHX in dental materials has been investigated in order to avoid addition of one more step of separate CHX application on dentine, during the adhesive procedure. Since addition of CHX in restorative composites is related to serious side-effects in their physicomechanical properties [18], it was decided to alternatively test CHX addition in adhesive systems [41,42].

Adding CHX to commerically available adhesives may convert then to CHX carriers being able to penetrate deeper into the adhesive zone. Hypothetically, a potentially slower CHX release due to its increased depth and deeper localization,

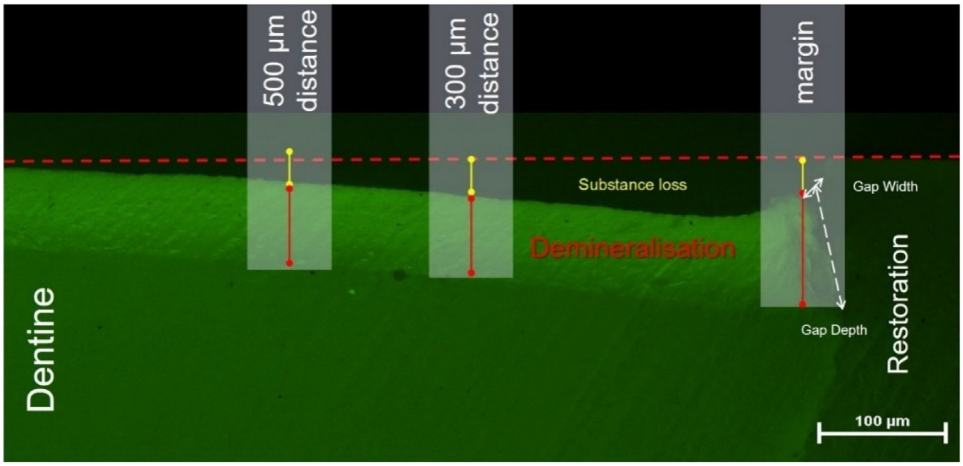

Fig. 2 – Evaluation of fluorescence microscope capture at restoration margins. The following parameters are determined: substance loss due to demineralisation (yellow), demineralisation depth (red), marginal gap width (white), marginal gap depth (white-stripped). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3 – Results of enamel demineralisation (µm, [SD]) after biological loading of Class V restorations for 10 days in the caries model.

Enamel			
Demineralisation µm, [SD]	DEM	SUB	TOTAL
	At restoration margins		
CTRL	55 [21]	6 [8]	61 [19] ^{A,B,C}
DENT	66 [18]	7 [15]	73 [23]
PRIM	69 [16]	13 [17]	81 [16] ^{A}
BOND	74 [12]	6 [12]	80 [14] ^{B}
PEAK	68 [15]	15 [20]	82 [19] ^{C}
	300 µm away from the margins		
CTRL	48 [16]	7 [13]	55 [12]
DENT	36 [17]	24 [34]	60 [28]
PRIM	39 [23]	20 [21]	58 [14]
BOND	51 [15]	18 [14]	69 [10]
PEAK	41 [25]	14 [17]	54 [21]
	500 µm away from the margins		
CTRL	50 [15]	3 [11]	53 [13]
DENT	38 [18]	15 [27]	54 [25]
PRIM	40 [16]	22 [28]	62 [19]
BOND	47 [19]	18 [17]	65 [23]
PEAK	37 [23]	13 [22]	50 [32]

Demineralisation (DEM), substance loss due to demineralisation (SUB) and total demineralisation (TOTAL) in enamel after biological loading in the caries model for 10 days. TOTAL = DEM + SUB. Adhesives exhibiting statistically significant differences are marked with the same capital letters.

and thus longer duration of antimicrobial action could be possible. Moreover, the adhesive interface still is the weak link in bonded resin-based composite restorations and thus more vulnerable to bacterial attack. Therefore, loading adhesives with antimicrobials instead of loading restorative materials themselves could be beneficial for these restorations by providing a localized effect.

The literature in the field, however, reports controversial results regarding the degree of conversion [44,45], modulus of elasticity [45], water sorption [46,47] and bond strength [48,41,49,47,50] when CHX is mixed into adhesives in concentrations up to 5%, a safe concentration of 0.1% CHX was chosen as one of the lowest evaluated in the literature [49,44,48,42,41,39,51,50,46,47,45]. On the other hand, it remains unclear whether this very low – but safe – CHX concentration shows an antibacterial effect at all. On the contrary, after application of CHX on dentine or after its release from the adhesive, it binds to dentine due to its excellent substantivity, which is not affected by its concentration either being 0.2% or ten times higher (2%) [17]. Since CHX is classified as a soluble agent [5,20], CHX release could be monitored for up to 5 weeks [18] and decrease in bacteria counts up to 3 months

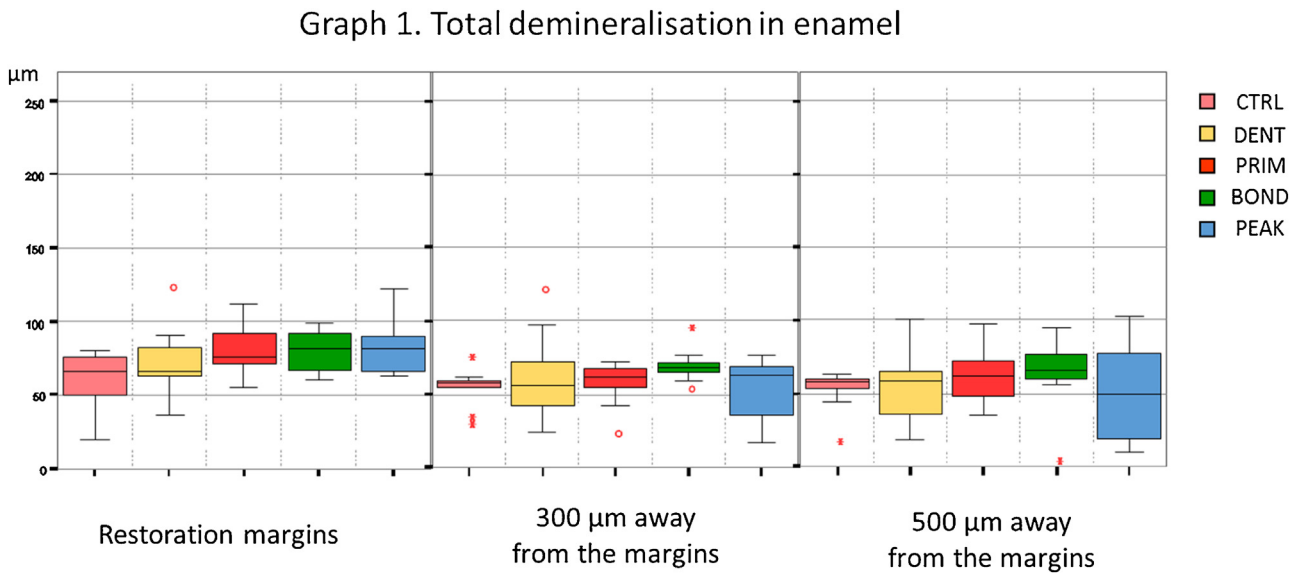

Fig. 3 – Graphic presentation with boxplot of total demineralization (TOTAL) in enamel after 10 days biological loading in caries model. Total demineralization (TOTAL) = demineralization (DEM) + substance loss due to demineralization (SUB). Evaluation took place at restoration margins, 300 µm and 500 µm away from the margins. Values displayed in Table 3.

Table 4 – Results of dentine demineralisation (µm, [SD]) after biological loading of Class V restorations for 10 days in the caries model.

Dentine			
Demineralisation µm, [SD]	DEM	SUB	TOTAL
	At restoration margins		
CTRL	34 [25] $^{A, B,C,D}$	105 [38] ^{E,F,G}	144 [21]
DENT	74 [19] ^{A}	76 [40] ^{G}	150 [37]
PRIM	65 [29] ^{B}	93 [34] ^{K}	158 [28]
BOND	77 [18] ^{C}	58 [34] ^{E,K}	135 [31]
PEAK	68 [39] ^{D}	67 [47] ^{F}	134 [39]
	300 µm away from the margins		
CTRL	48 [21] ^{G,H,J}	65 [26]	113 [33]
DENT	75 [21] ^{G}	46 [31]	122 [38]
PRIM	69 [16] ^{H}	62 [19]	131 [22]
BOND	61 [20]	50 [24]	111 [33]
PEAK	70 [30] ^{J}	56 [38]	126 [32]
	500 µm away from the margins		
CTRL	52 [16]	52 [24]	104 [21]
DENT	71 [35]	42 [36]	114 [63]
PRIM	66 [10]	53 [27]	119 [25]
BOND	64 [14]	51 [14]	116 [24]
PEAK	65 [31]	35 [34]	101 [50]

Demineralisation (DEM), substance loss due to demineralisation (SUB) and total demineralisation (TOTAL) in dentine after biological loading in the caries model for 10 days. TOTAL = DEM + SUB. Adhesives exhibiting statistically significant differences are marked with the same capital letters.

[25], both observation times being within the timeframe of the present experiment. However, since chemical integrity of the adhesive is a critical factor in the adhesive procedure, it seems logical not to overload adhesives with CHX in order to achieve higher release. Industrially added CHX (in PEAK) reached a higher final concentration of 0.2% according to the manufacturer, compared to the experimental CHX adhesives, PRIM and BOND (Table 1). However, 0.1% CHX was chosen for the experimental adhesives in terms of safety, as discussed above. In order to comply with literature [14,13,52,28,15,53] 2% CHX as dentine pre-treatment was used. CHX was separately added primer and in bonding in order to isolate the effect of the antimicrobial in every step.

A mono-bacterial, automated artificial mouth was used, having been established in previous studies [31,43,33] in the present investigation, loading specimens with 40 demineralisation phases. For a appropriate demineralisation phase, the pH of the bacterial solution should be 4.2–4.3. This enables

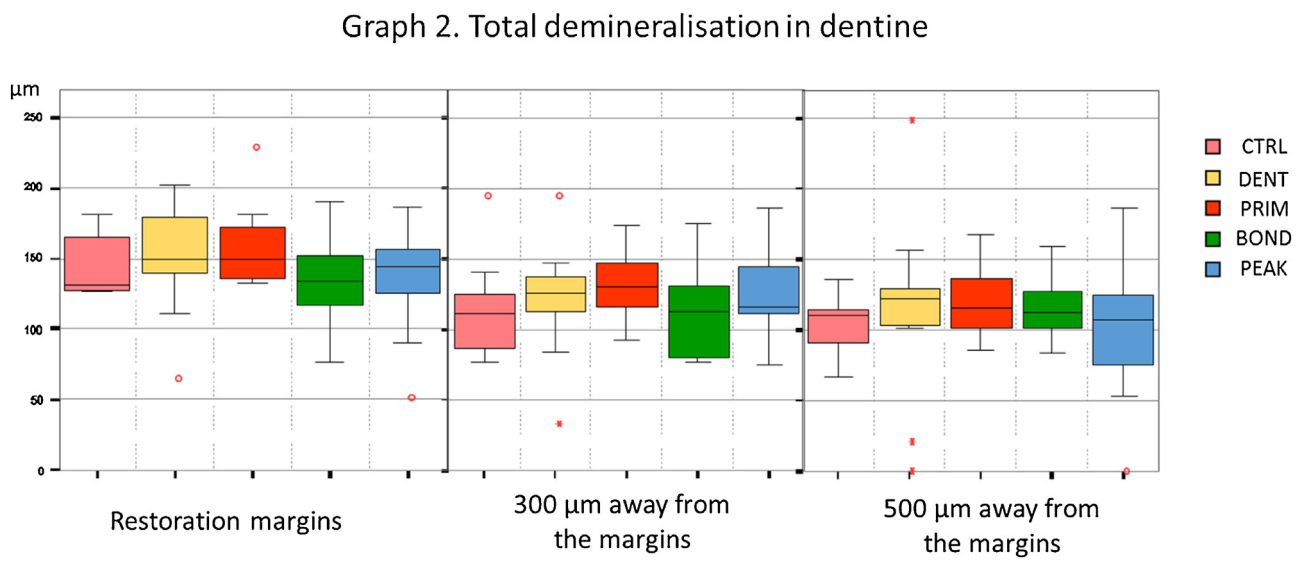

Fig. 4 – Graphic presentation with boxplot of total demineralization (TOTAL) in enamel after 10 days biological loading in caries model. Total demineralization (TOTAL) = demineralization (DEM) + substance loss due to demineralization (SUB). Evaluation took place at restoration margins, 300 µm and 500 µm away from the margins. Values displayed in Table 4.

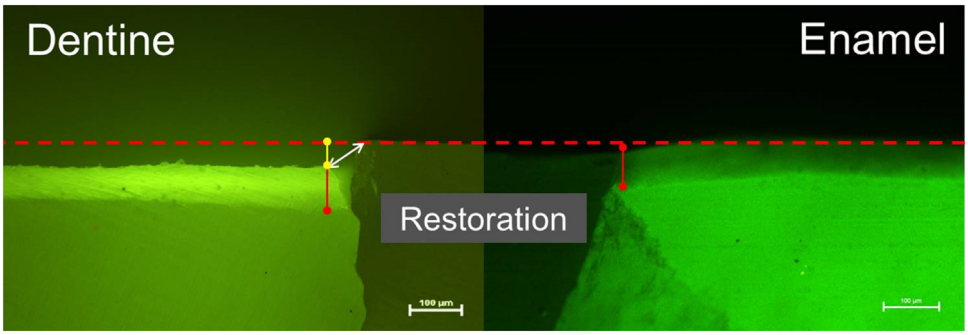

Fig. 5 – Fluorescence microscope evaluation of CTRL in 4× magnification after biological loading, in dentin (left) and in enamel (right). Evaluated parameters are: substance loss due to demineralisation (yellow), demineralisation depth (red) marginal gap width (white). Restoration level is marked with stripped line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

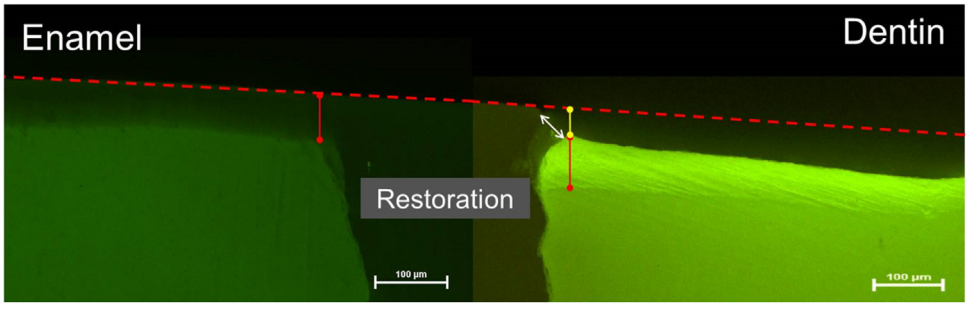

Fig. 6 – Fluorescence microscope evaluation of DENT in 4× magnification after biological loading, in dentin (left) and in enamel (right). Evaluated parameters are: substance loss due to demineralisation (yellow), demineralisation depth (red) marginal gap width (white). Restoration level is marked with stripped line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

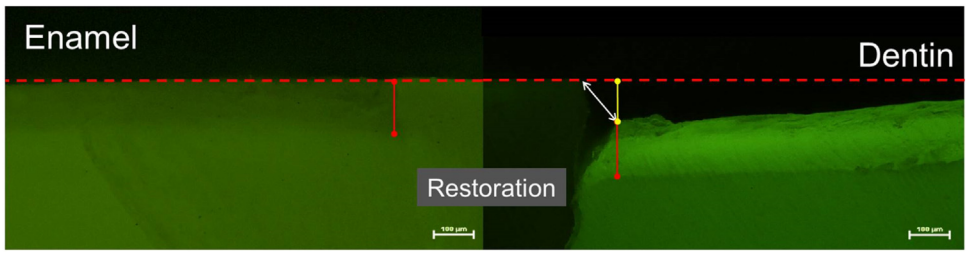

Fig. 7 – Fluorescence microscope evaluation of PRIM in 4× magnification after biological loading, in dentin (left) and in enamel (right). Evaluated parameters are: substance loss due to demineralisation (yellow), demineralisation depth (red) marginal gap width (white). Restoration level is marked with stripped line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

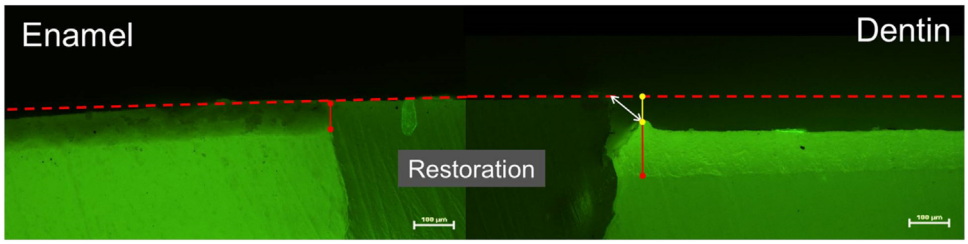

Fig. 8 – Fluorescence microscope evaluation of BOND in 4× magnification after biological loading, in dentin (left) and in enamel (right). Evaluated parameters are: substance loss due to demineralisation (yellow), demineralisation depth (red) marginal gap width (white). Restoration level is marked with stripped line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

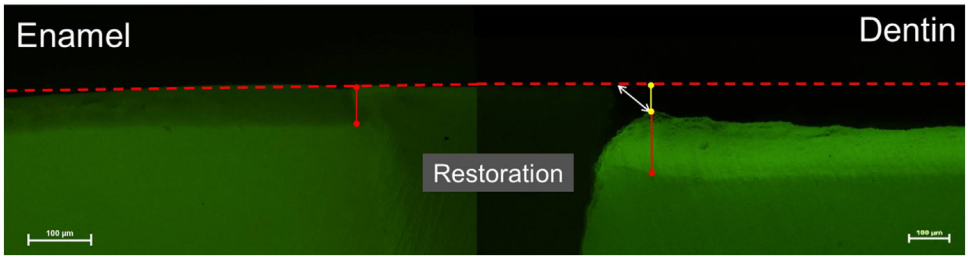

Fig. 9 – Fluorescence microscope evaluation of PEAK in 4× magnification after biological loading, in dentin (left) and in enamel (right). Evaluated parameters are: substance loss due to demineralisation (yellow), demineralisation depth (red) marginal gap width (white). Restoration level is marked with stripped line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5 – Marginal analysis before and after caries model in enamel for variables perfect margin, overhand and gap, demonstrated in % percentage mode.

Mean value % of total margin length [SD]	Before caries model			After caries model		
	Perfect	Overhang	Gap	Perfect	Over hang	Gap
CTRL	83 [14] ^{A}	1 [0]	14 [4] ^{A}	8 [2] ^{A,a}	0 [0]	98 [6] ^{A,a}
DENT	95 [7] ^{A}	1 [0]	2 [0] ^{A}	78 [4] ^{A,a,b,c,d}	4 [0]	52 [5] ^{A,a,b,c,d}
PRIM	81 [20] ^{A}	1 [0]	17 [2] ^{A}	6 [2] ^{A,b}	4 [0]	93 [14] ^{A,b}
BOND	87 [19] ^{A}	0 [0]	12 [1] ^{A}	2 [2] ^{A,c}	0 [0]	100 [0] ^{A,c}
PEAK	78 [27] ^{A}	4 [1]	16 [2] ^{A}	19 [12] ^{A,d}	18 [14]	81 [18] ^{A,d}

Variables Underfilled, fracture and not evaluable are not included since they range ≈0% for all tested adhesives. Adhesives exhibiting statistically significant differences before and after caries model (horizontal) are marked with the same capital letters, while significant differences between the adhesives (vertically) are marked with same lowercase letters.

Table 6 – Marginal analysis before and after caries model in dentine for variables perfect margin, overhand and gap, demonstrated in % percentage mode.

Mean value % of total margin length [SD]	Before caries model			After caries model		
	Perfect	Over hang	Gap	Perfect	Over hang	Gap
CTRL	49 [2] [a]	19[6]	46 [2] [a]	33 [6]	0 [0]	63 [2]
DENT	69 [2] [a]	5 [1]	25 [2] [a]	45 [2]	7 [1]	44 [2]
PRIM	60 [3]	8 [1]	31 [2] [A]	29 [2]	2 [0]	67[19] [A]
BOND	68 [9] [A]	1 [0]	30 [2]	44 [19] [A]	5 [0]	50 [19]
PEAK	76 [17] [A]	6 [1]	36 [14] [A]	27 [21] [A]	9 [1]	63 [22] [A]

Variables Underfilled, fracture and not evaluable are not included since they range ≈0% for all tested adhesives. Adhesives exhibiting statistically significant differences before and after caries model (horizontal) are marked with the same capital letters, while significant differences between the adhesives (vertically) are marked with same lowercase letters.

sufficient demineralisation of both enamel and dentine. In the experiment, each demineralisation lasted one hour, and every day specimens were incubated with *S. mutans* for 4 h. This induces secondary caries-like lesions comparable to clinical situations, while smooth surface caries like secondary caries around Class V restorations, could be induced at even shorter demineralisation periods [35]. Finally, the effect of intraoral sugar clearance was simulated by rinsing the reaction chamber three times with artificial saliva after each demineralization, removing bacterial remnants which would otherwise impede pH rise. In order to mimic the aging process in the intraoral environment and provoke marginal gaps allowing for sufficient bacterial concentration, restorations were thermocycled prior to biological loading. It has been demonstrated that when thermocycling is used in combination with long-term water storage, it may be useful in forecasting *in vivo* outcomes [54]. No evidence of the number of cycles likely to be experienced *in vivo* was found, but an estimate of approximately 10,000 cycles per year was suggested [55].

Despite the fact that CHX reduces the number of *S. mutans* when applied as dentine pre-treatment [56], CHX adhesives were only partially able to protect restoration margins from demineralisation in the artificial mouth. Therefore, the null hypothesis was partially accepted for demineralisation values, but not for marginal gap formation. CHX addition in the adhesives (PRIM, BOND, PEAK) resulted in significantly higher demineralisation in enamel, compared to the control group without CHX ($p < 0.05$, mod. LSD), showing that it did not arrest secondary caries, it it made the situation even worse — either when experimentally or industrially added. This could be explained in two ways, which represent two totally different directions; either due to the inability of the CHX adhesives to bond efficiently to enamel due to their altered chemistry, thus initially leaving a greater marginal gap for further biological degradation by *S. mutans* or due to the hermetic closure between enamel and composite [57], which does not allow CHX to be released outside the restoration. SEM marginal analysis (Table 5) shows a definite deterioration of enamel margins after caries model for PRIM, BOND and PEAK ($p < 0.05$, Friedman), therefore the first scenario is confirmed. Moreover, negative results for PEAK can be explained by the fact that as demonstrated in a recent study, this adhesive could only inhibit anaerobic bacteria and not facultative anaerobic bacteria, such as *S. mutans* [39]. Another possible explanation would be the short duration of CHX release, as shown for CHX-containing copolymers [19]. This deterioration, however, was not noted for dentine pre-treatment with CHX (DENT), ($p = 0.001$, ANOVA), indicating that 2% CHX as dentine pre-treatment offers an advantage in protection of enamel margins against secondary caries. Despite the fact that none of the CHX adhesives inhibited secondary caries formation in enamel, 2% CHX dentine pre-treatment (DENT) obviously limited marginal gap formation in enamel compared to the other adhesive protocols in the study. Regarding dentine margins, CHX addition in adhesives did not affect demineralisation compared to the control group (CTRL) ($p > 0.05$, ANOVA) (Table 4), thus showing no secondary caries inhibition in dentine.

According to the results of the present study and within its limitations, neither 2% CHX as dentine pre-treatment, nor 0.1% or 0.2% CHX added in adhesives provideed any effect against secondary caries in dentine. Neither experimental nor industrial addition of CHX in the adhesive procedure could provide protection against secondary caries in dentine. On the other hand, 2% CHX dentine pre-treatment managed to limit marginal gap formation in enamel compared to the other adhesive protocols in the study. Future research should be directed towards testing higher CHX concentrations in the adhesives, in a form of delivery providing controlled release without affecting material's properties.

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