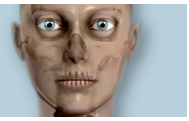
Orthodontics & Craniofacial Research



ORIGINAL ARTICLE

H. Slutzky
O. Feuerstern
K. Namuz
N. Shpack
I. Lewinstein
S. Matalon

The effects of *in vitro* fluoride mouth rinse on the antibacterial properties of orthodontic cements

Authors' affiliations:
H. Slutzky, I. Lewinstein, S. Matalon, Department of Oral Rehabilitation, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel
O. Feuerstern, K. Namuz, Department of Prosthodontics, Faculty of Dental Medicine, Hebrew University, Jerusalem, Israel
N. Shpack, Department of Orthodontics, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel

Correspondence to:
H. Slutzky
Department of Oral Rehabilitation
The Maurice and Gabriela Goldschleger School of Dental Medicine
Tel Aviv University
Israel
E-mail: slutsky@netvision.net.il

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Structured Abstract

Objectives – To investigate the ability of orthodontic cements to regain their antibacterial effect after aging for 1 month, followed by 2 weeks of fluoride 'recharging' through daily fluoride rinse.

Materials and Methods – Four orthodontic cements were tested: composite resin-based materials (Transbond XT and Transbond Plus), a conventional glass ionomer cement (CX Plus) and dual-cured resin-reinforced glass ionomer cement (Fuji ORTHO LC) by direct contact test. After polymerization and a 30-day aging process, the samples were rinsed daily with 0.05% NaF solution for 14 days. Twenty-four hours after the last fluoride rinse, *Streptococcus mutans* cells (approximately 1×10^6) were placed on the surface of each sample for 1 h at 37°C to establish direct contact. Bacterial growth was monitored for 24 h by temperature-controlled spectrophotometry. Similar experiments were conducted after aging for 48 h and 72 h after the last fluoride rinse. One-way ANOVA, two-way ANOVA, and Tukey's multiple comparison test were applied to the data.

Results – Twenty-four hours after the last fluoride rinse, the resin-modified glass ionomer and the glass ionomer showed potent antibacterial properties, whereas the composite-based material Transbond XT encouraged bacterial growth. After 72 h, only Fuji Ortho LC maintained its bacteriostatic properties while all the other tested materials showed no antibacterial activity.

Conclusions – Fourteen days daily fluoride rinse temporarily revives the antibacterial properties of the resin-modified glass ionomer cement and glass-ionomer-based cement.

Key words: fluoride rinse; glass ionomer; orthodontic cements

Introduction

Orthodontic patients are at a high risk of developing initial carious lesions (1). The use of topical fluoride or fluoride-containing bonding materials during orthodontic treatment reduces the occurrence of white spot lesions. Thus, it is recommended that patients with fixed braces rinse daily with a 0.05% sodium fluoride mouth solution (2). There are several anticariogenic mechanisms of fluoride, including the reduction in tooth demineralization, the enhancement of remineralization, and the inhibition of microbial growth and metabolism (3–6).

Fluoride-releasing orthodontic cements based on different generic materials such as glass ionomer cements (GICs) that are composed of fluoride-containing silicate glass and polyalkenoic acids, which are set via an acid-base reaction, have been proposed. Fluoride may be released from the glass ionomers into an aqueous environment by a rapid dissolution from the outer surface or during a gradually sustained diffusion of ions through the bulk cement (7). A fluoride-releasing resin composite orthodontic cement has been developed and presented small concentrations of fluoride release over time, with significantly lower tensile bond strengths than conventional composite cement (8). The use of resin-modified glass ionomers (RMGIs) have been proposed to overcome the problems of initial moisture sensitivity and low mechanical strength that are typical for conventional GICs. Light-cured RMGIs, basically formed by adding methacrylate components, were found to have the potential for releasing fluoride equivalent to that of GIC, with a considerable variation in the fluoride release between materials (9, 10). There is poor evidence that the use of fluoride-releasing GIC for orthodontic bracket bonding is more effective at preventing enamel demineralization than a conventional composite resin (3). As fluoride discharge from those materials decreases over time, 'recharging' them with fluoride has been suggested to maintain a continuously increased level of fluoride release (11, 12). Indeed, glass-ionomer-based materials have demonstrated a high ability to recharge from daily exposure to fluoridated dentifrices (13–15). A previous study showed that glass-ionomer-based orthodontic cements exhibited potent antibacterial activity on *S. mutans*, which diminished over 1 month of aging in phosphate-buffered saline (PBS) (16). The objective of this *in vitro* study was to investigate the potential of four orthodontic cements to regain their antibacterial effect after aging for 1 month, followed by 2 weeks of fluoride 'recharging' by daily exposure to a fluoride rinse.

Materials and methods

Tested materials

Four orthodontic cements were tested: two composite resin-based materials, Transbond XT and Transbond Plus (3M Unitek Dental Product, Monrovia, CA, USA); a conventional glass ionomer cement, CX Plus (SHOFU Inc., Kyoto, Japan); and a dual-cured resin-reinforced glass ionomer cement, GC Fuji ORTHO LC (GC Corporation, Tokyo, Japan).

Test microorganism and growth conditions

Streptococcus mutans, the primary etiological agent of caries and a frequent caries lesion isolate, has been widely used to test the antimicrobial activity of restorative materials (17–19).

Streptococcus mutans (ATCC# 700610) was grown aerobically from frozen stock cultures in a brain heart infusion (BHI) broth which contained 0.5% bacitracin and 5% glucose at 37°C for 24 h, with automixing every 15 min.

The 30-day aging process was conducted in the following manner; the orthodontic cements were evenly coated in equal amounts [surface area of 19.37 ± 0.04 mm^2] on the sidewall of the of eight wells of a 96-well flat bottom microtiter plate (Nunclon; Nunc, Copenhagen, Denmark), that was held vertically, and were polymerized by using either FreeLight 2 (3M-ESPE, St. Paul, MN, USA) or by mixing according to the manufacturer's recommendations. The plate was then positioned horizontally, and

the samples were covered completely with 275 μl of PBS. For the next 30 days, the PBS was replaced every 24 h with a fresh solution. The 30-day aging process allowed for a complete depletion of all fluoride ions from the tested materials as shown in a previous study (16). After the depletion of fluoride, an attempted was made to emulate the clinical instructions for use of fluoride-containing mouth rinse, for a reasonable duration of time. The samples were rinsed for 30 s' twice a day with a 12-h intervals, with 275 μl of NaF 0.05% for 14 days. Following each rinse, the fluoride solution was replaced with 275 μl of PBS.

The direct contact test (DCT) quantitatively measures the effect of direct and close contact between the test microorganism and the tested materials, regardless of solubility and diffusiveness of their components (16, 19, 20). The DCT is based on the turbidimetric determination of bacterial growth in 96-well microtiter plates (20). The kinetics of the outgrowth in each well was recorded at 650 nm every 30 min for 24 h using a temperature-controlled spectrophotometer set at 37°C (Versamax; Molecular Devices Corporation, Menlo Park, CA, USA). Automixing of 30 s' prior to each reading ensured a homogeneous bacterial cell suspension. The experiment was set up as follows: A 96-well flat bottom microtiter plate was held vertically, that is, the surface of the plate wall was perpendicular to the floor and 10 μl of microbial suspension, approximately 1 x 10^6 cells in BHI, was placed on the tested materials (group A). The plate remained in the vertical position and was incubated for 1 h at 37°C. Evaporation of the suspension liquid, after 1 h at 37°C incubation, ensured direct contact between the bacteria and the tested materials. The plate was then positioned horizontally, and 235 μl of BHI was added to each well and the plate was gently mixed for 2 min. Then, 15 μl of BHI was transferred from each one of the eight coated wells (group A) to a set of parallel eight uncoated wells containing 205 μl of BHI (group B). When no growth was observed in group A wells and bacterial growth was observed in group B wells, it can be concluded as a bacteriostatic effect of the tested material on the microorganisms. In case there is no documented growth in the group A and group B wells, one can conclude that the tested material is bactericidal. A set of three wells served as positive control, that is, identical bacterial inoculum was placed on the sidewall of uncoated wells and processed as the experimental wells. The negative control consisted of a set of 4 wells coated with the tested materials, as in the experimental wells, which contained an equal amount of uninoculated growth medium. The plate was then incubated at 37°C in the Versamax microplate reader, and the optical density in each well at 650 nm was monitored for the next 24 h.

Calibration experiments were conducted in each plate to establish bacterial outgrowth under the experiment conditions. The calibration growth curve enables estimation of the number of viable bacteria at the end of the direct contact incubation period.

For this purpose, 10 μl of bacterial suspension was placed on each sidewall in 3 wells of a 96-well microtiter plate in the experimental setup. Then, 275 μl of fresh growth medium was added, and the plate was gently mixed for 2 min. From each well, 55 μl was transferred to an adjacent set of wells that contained 220 μl of fresh medium. This was repeated seven consecutive times.

1) The slope of the linear portion of the growth curve expressed changes in bacterial growth rate. 2) The distance of the ascending portion of the growth rate from the *Y* axis that correlates with the number of viable microorganisms at time zero. The growth curves for each well were calculated, and a regression line was performed on the linear segment of each curve. Each experimental microtiter plate, processed throughout the study, contained all its controls as well as calibration set of wells.

Experiments were conducted in which the tested materials were allowed to age for 24, 48, and 72 h after the last fluoride rinse. The aging process was performed by replacing the 275 μl of PBS every 24 h. Another experiment was conducted immediately after the 30-day aging of the materials, without the following 2 weeks of fluoride rinsing.

Statistical analysis

The recorded data were plotted as semilogarithmic growth curves. The linear portion of the curve, which correlated with the bacterial growth rate, was transferred and expressed as a linear mathematical formula. One-way ANOVA, two-way ANOVA, and Tukey's multiple comparison procedures were applied to the slopes of these linear formulas. The level of confidence was set at 0.05.

Results

To maintain the quantitative nature of the DCT, a calibration growth curve was performed for each experiment. For this purpose, bacteria were diluted by a factor of five; each point on the curve was the average of three wells measured at the same time (not shown). The calibration growth curve allows the estimation of the number of viable bacteria at the end of the incubation period. The gradual decrease in viable bacteria due to serial dilution at time zero consistently affected the constant in the linear portion of the curve and had no effect on the bacterial growth rate (slope) or the final density of bacteria at the stationary phase. The calibration curves served as a scale to measure the relative number of bacteria in a quantitative and reproducible manner. Based on this finding, it is possible to express the material–bacteria interaction measured by the DCT, as changes in bacterial growth rate and/or in the number of viable bacteria.

Direct contact test was performed on eight specimens of each of the materials tested. A regression line was fitted to the linear segment of the curve in the group A wells and group B wells, which represented the logarithmic phase of growth. The R^2 value of all of the growth curves ranged between 0.92 and 0.99. The two-way ANOVA, performed on all of the experiments, indicated a significant difference in the bacterial growth rate (slope) between the microorganisms as a function of time and material ($p < 0.001$). None of the 30-day-aged materials showed any antibacterial properties.

Twenty-four hours after the last fluoride rinse, the resin-modified glass-ionomer-based GC Fuji Ortho LC and the glass-ionomer-based CX Plus in the group A wells both showed potent antibacterial properties, whereas the other composite-based materials showed no such properties. Transbond XT was shown to encourage *S. mutans* growth, while Transbond Plus exhibited a minimal growth inhibition (Fig. 1 and Table 1). In group B wells, no growth was documented for the resin-modified glass-ionomer-based GC Fuji Ortho LC and the glass-ionomer-based CX Plus. Transbond Plus showed similar growth to the control, whereas Transbond XT encouraged growth (not shown).

Forty-eight hours after the last fluoride rinse, the resin-modified glass-ionomer-based GC Fuji Ortho LC and the glass-ionomer-based CX Plus showed potent bacteriostatic properties, whereas the composite-based materials – Transbond XT, encouraged bacterial growth at the same time point. Transbond Plus was similar to the control. (Table 1). In group B wells, growth was documented for the resin-modified glass-ionomer-based GC Fuji Ortho LC and the glass-ionomer-based CX Plus only after a lag time of 14 h. Transbond Plus showed similar growth to the control, whereas Transbond XT exhibited some growth encouragement (Fig. 2).

Seventy-two hours after the last fluoride rinse, only the GC Fuji Ortho LC maintained its bacteriostatic properties, while the glass-ionomer-based CX Plus exhibited a slight bacteriostatic effect. Transbond Plus was similar to the control, and Transbond XT continued encouraging bacterial growth (Fig. 3 and Table 1).

Discussion

The ability of a dental material to act as a fluoride reservoir is mainly dependent on the type of the material, its matrix, its setting mechanism, and the fluoride content (3, 11, 12). Previous *in vitro* and *in situ* studies have shown that various methods of aging, periods of aging, and methods of fluoride recharge may also act as contributory factors (12, 13, 15, 21, 22). In the current study,

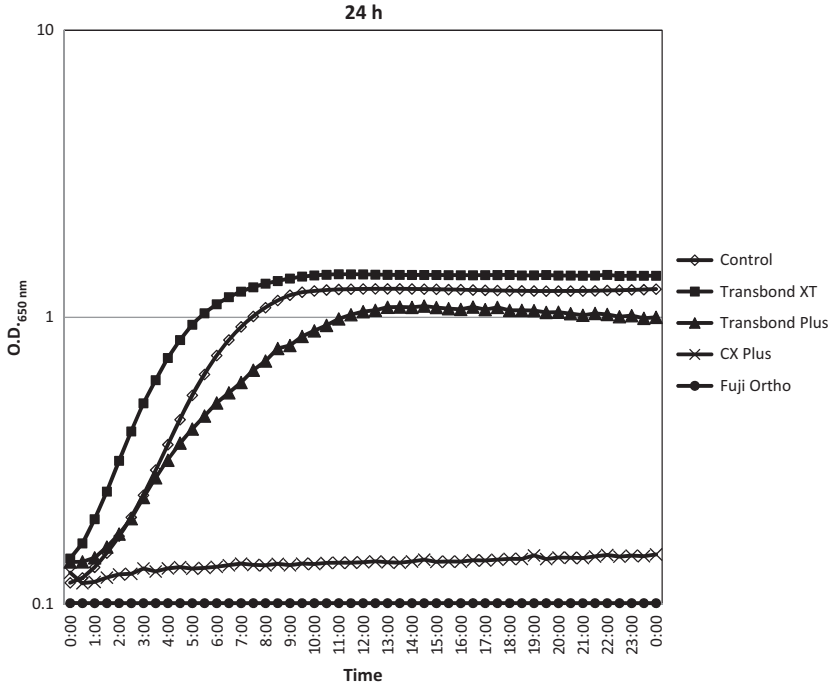

Fig. 1. Group A wells bacterial outgrowth on the surfaces of the orthodontic cement samples 24 h after a period of 14 days during which the samples were rinsed twice a day with a fluoride mouth rinse, as measured by changes in the optical density. Each point is the average of the optical densities (OD) measured in eight wells at the same time.

Table 1. **Bacterial growth rates in group A wells according to the slopes of the linear portions of their growth curves**

Material / Time	24 h	48 h	72 h
Transbond XT	96.4 ± 6.2	96.4 ± 5.2	100 ± 9.7
Transbond Plus	44.4 ± 2.5	72.1 ± 8.5◆	80.4 ± 3.4◆
CX Plus	0.4 ± 0.03♦	0.01 ± 0.001♦	58.1 ± 0.3
Fuji Ortho LC	0.01 ± 0.001♦	0.01 ± 0.001♦	7.9 ± 0.3
Control	72.3 ± 2.5	72.5 ± 3.54◆	78 ± 3.6◆
Significance	$p < 0.001$	$p < 0.001$	$p < 0.001$

Each number in the table is the average [($\times 10^{-3}$) ± standard deviation ($\times 10^{-3}$)] of the slope of bacterial growth in eight separate wells in the same microtiter plate. Similar symbols indicate values which do not differ significantly (Tukey's comparison).

we aimed to eliminate all antibacterial potential by aging the materials for 1 month (16). The potential to regain an antibacterial effect on *S. mutans* by a clinical protocol of exposure to a fluoride rinse was then assessed. A previous study showed that among all of these cements, only the RMGI cement (GC Fuji ORTHO LC) exhibited potent antibacterial activity on *S. mutans*, which lasted 1 week only and diminished over the next 3 weeks of aging in PBS (16). In the present study, similar materials were allowed to age for 1 month to unsure evacuation of all potential fluoride release from the tested cements, after which the materials were treated via a short exposure to NaF solution (0.05%) for 30 s' twice a day in a 12-h interval for 2 weeks to allow fluoride recharge. The antibacterial activities over the next 3 days were tested using the direct contact test.

RMGI cement (GC Fuji ORTHO LC) and conventional GIC (CX Plus) showed complete inhibition of bacterial growth when tested 1 day after the succession of fluoride rinses in group A wells as well as in group B wells indicating bactericidal ability at that specific experimental time point. As such, these materials successfully regained their initial antibacterial activity. After 48 h, in the group B wells, both cements CX Plus and GC Fuji ORTHO LC allowed bacterial growth after a long lag time indicating the bacteriostatic

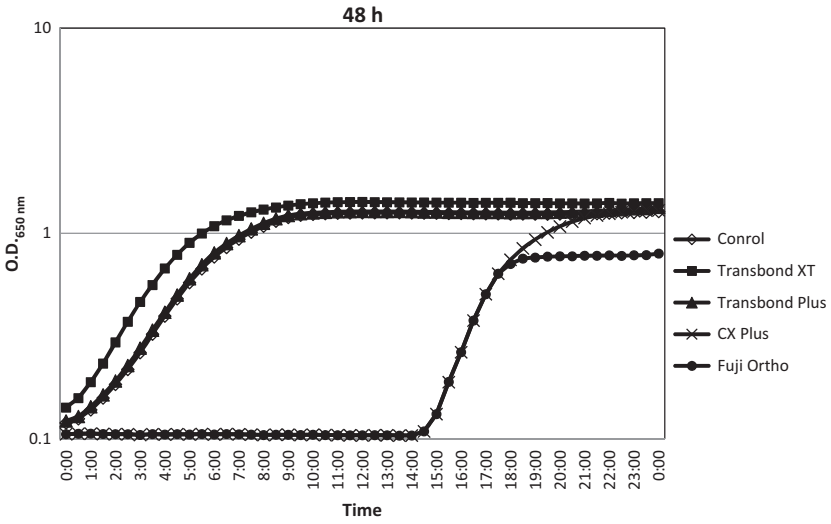

Fig. 2. Group B wells bacterial outgrowth without direct contact to the tested material. The bacterial inoculum was transferred from corresponding group A wells in which the bacterium was allowed to establish direct contact to the surfaces of the orthodontic cement samples 48 h after a period of 14 days during which the samples were rinsed twice a day with a fluoride mouth rinse. The growth was measured by changes in the optical density. Each point is the average of the optical densities (OD) measured in eight wells at the same time.

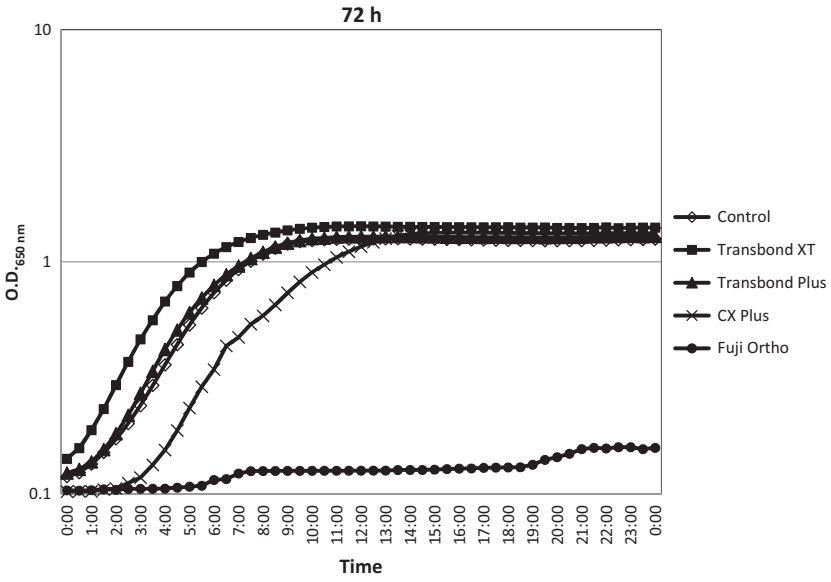

Fig. 3. Group A wells bacterial outgrowth on the surfaces of the orthodontic cement samples 72 h after a period of 14 days during which samples were rinsed twice a day with a fluoride mouth rinse, as measured by changes in the optical density. Each point is the average of the optical densities (OD) measured in eight wells at the same time.

nature of direct contact with the tested bacterium. While CX Plus showed a decrease in this regained activity over time, GC Fuji ORTHO LC maintained its maximal inhibiting effect on the bacteria for at least 3 days. A previous study showed that a low-viscosity glass-ionomer-based sealant maintained potent antibacterial properties 2 days after the last fluoride rinse (23). The differences in duration between the regained antibacterial activities may be explained by the differences between the materials in terms of their fluoride recharge and release capabilities as well as their correlation with initial fluoride release (11, 22, 24). The RMGI has a titer bound and a lower permeability than GIC; this may result in a prolonged fluoride release, therefore explaining the more potent antibacterial activity of RMGI in comparison with conventional GIC after 72 h in this study.

Transbond Plus had little antibacterial capabilities for at least 24 h after the last fluoride rinse; this may be explained by the incorporation of fluoride in a small amount only into material outer surface layer. The other tested resin composite cement, Transbond XT, even showed a slight stimulatory effect on bacterial growth. These results are not surprising because most studies have demonstrated that cured resin composites do not release any antibacterial components and fail to show any antibacterial effects

against oral bacteria (18, 19). Moreover, polymerized resin monomers demonstrated bacterial growth enhancement (19, 25, 26). Additionally, glass ionomers are often found to have a significantly better capability to act as a fluoride reservoir than composite resin-based materials (24). This result can be explained by the loosely bound water in the glass ionomer and by its high permeability, which may lead to the absorption of fluoride ions deep into its bulk, whereas a relatively impermeable resin-based material can only absorb fluoride into the immediate subsurface (3, 11).

Although fluoride-releasing materials may act as a fluoride reservoir and may increase fluoride levels in the saliva, plaque and hard dental tissues, clinical studies have exhibited conflicting data as to whether or not these materials significantly prevent or inhibit secondary caries (3). However, there is evidence to suggest that glass ionomer cement is more effective than composite resin at preventing white spot formation, even though the evidence is weak (27). White spot lesions are a major clinical problem around cemented brackets and are a potential liability. Although GIC may lead to increased bracket bonding failures, the use of RMGI could satisfy the clinician's need for reasonable bond and at the same time superior longitudinal fluoride release and antibacterial effect. The constant fluoride-rich environment around the cemented bracket probably will not be achieved by fluoride rinse only.

The results of this *in vitro* study do not guarantee that the release of fluoride from the recharged RMGI and GIC will prevent the establishment of caries inducing biofilm and white spot lesions.

Conclusion

The current study suggests that the use of resin-modified glass-ionomer-based orthodontic cements, which can be recharged by daily rinses with fluoride-containing oral hygiene products, may be beneficial as a preventive strategy during long-term orthodontic treatments although it does not guarantee total elimination of white spot lesions unless a meticulous regiment of oral hygiene is kept to which this phenomena is an added bonus.

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