Original Article

Virulence modulation of *Streptococcus mutans* biofilms by metal ions released from orthodontic appliances

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ABSTRACT

Objectives: To evaluate the impact of metal ions commonly shed from orthodontic appliances on the virulence of *Streptococcus mutans* ATCC®25175™ biofilms.

Materials and Methods: Biofilms were grown in the presence of Ni^{2+}, Fe^{3+}, Cr^{3+}, Co^{2+}, and a metal ion pool at concentrations similar to those released in saliva of orthodontic patients for 72 hours. Once mature, biofilms were treated for up to 12 hours with 5% glucose.

Results: Ions interfered with the growth of *S mutans* by reducing its biomass (Ni^{2+}, Fe^{3+}, Cr^{3+}), raising its rates of sugar metabolism (Ni^{2+}, Fe^{3+}, Cr^{3+}), and raising its secretion of lactate (Ni^{2+}, Fe^{3+}, Cr^{3+}, pool).

Conclusion: The laboratory data presented here point to the possibility of virulence increase of *S mutans* by metal ions commonly released during orthodontic therapy. (*Angle Orthod.* 2013;83:987–993.)

KEY WORDS: *Streptococcus mutans*; Biofilm; Orthodontic apparatus; Metal ions; Virulence

INTRODUCTION

Stainless-steel, cobalt-chromium-nickel (CoCrNi), nickel-titanium (NiTi), alloy of β-titanium (β-Ti), and pure titanium are used in orthodontics as a result of their mechanical properties.[1] Once in the mouth, they become prone to corrosion due to dynamic stress,[2] fatigue,[3] and acids from the diet or from lactic fermentation by adjacent microbiota. This degradation leads to an increase in the salivary concentration of metal ions,[4,5] and continued exposure of oral microorganisms to these metal ions likely induces some degree of physiological change in these organisms.

Streptococcus mutans is a bacterium, the physiology of which is known to be affected by metal ions such as Zn^{2+},[6] Al^{3+},[7] Mn^{2+},[8] and Fe^{2+}.[9] Moreover, *Streptococcus mutans* produces a glucan-rich extracellular polymeric matrix that retains metal ions to considerably higher concentrations within the plaque than in the surrounding environment.[10]

In drinking water, the ability of metal ions to interfere with the growth of *S mutans* has previously been investigated.[11] However, little is known about changes in the physiology of this species induced by ions from orthodontic appliances. Out of the few studies performed investigating this question, two stand out: an investigation of the inhibitory effect intrinsic to orthodontic appliances[12] and a quantification of Ni^{2+} in the dental plaque of orthodontic patients.[13]

Although it is known that metal ions are continuously released into the oral cavity of patients undergoing orthodontic therapy, the virulence-modulating ability of these metal ions with regard to the cariogenic microbiota is unknown. To test the null hypothesis that metal ions commonly released from orthodontic appliances have no effect on the virulence of *S mutans* ATCC®25175™ biofilms we conducted a series of laboratory tests.

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MATERIALS AND METHODS

Collection of Stimulated Saliva

Stimulated saliva (10-mL aliquots) was collected from three healthy volunteer donors, aged 20–45 years, who were not on drug therapy, were not undergoing orthodontic treatment, and did not have amalgam fillings and/or metal prostheses. At the moment of saliva collection, their salivary mutans streptococci counts were inferior to 2.38×10^3 colony-forming units/mL, their decayed, missing, filled teeth (DMFT) were between 0 and 2, and no active caries were present. Donor participation was conditional upon provision of written informed consent, in accordance with the Ethics Committee on Human Research (Protocol 5410; Approval term 3588/09) at the Pontifical Catholic University of Paraná.

These donors were instructed not to eat or brush their teeth for at least 2 hours before the collection period. Collections were performed by patients chewing a sterile silicone tube. Saliva aliquots were mixed in order to ensure formation of pellicles with more receptors.[14] Immediately after collection samples were cooled and centrifuged (10,000 g, 4°C, 20 minutes).

Microorganism Growth

Planktonic cells of *S mutans* ATCC®25175™ were grown in 50 mL of brain heart infusion (BHI) at 37°C, at 100 rpm and 10% pCO_2. After 24 hours, cells were washed with sterile 145 mM NaCl. Cells were resuspended in sterile 145 mM NaCl to an optical density at 600 nm (OD_{660}) of 1.00.

Preparation of Metal-Containing Broths

BHI containing different metal ions was prepared using metal ion at the same concentrations found in saliva from orthodontic patients.[4,5] The ions and their concentrations were 10 ng/mL (170 nM) nickel (Ni^{2+}), 6.77 ng/mL (121 nM) iron (Fe^{3+}), 4.5 ng/mL (86.5 nM) chromium (Cr^{3+}), and 0.44 ng/mL (7.46 nM) cobalt (Co^{2+}). The bacterium was also treated with a pool (Mix) dissolved in BHI at the combined concentrations above. Because of their solubility, high-purity metal nitrates were used (Merck Chemicals Co, Rio de Janeiro, Brazil). Negative control was grown in culture broth without any added metal ions. The solvent used for broths and challenge glucose solutions (CGS) was Type II reagent water with specific resistivity above 2 Mohm/cm.[15,16]

Two hundred microliters of standardized suspension of *S mutans* ATCC®25175™ was added to the broth (1 mL) and incubated at 37°C, at 100 rpm and 10% pCO_2. After 24 hours, cells were washed with sterile 145 mM NaCl. The cells were resuspended in 145 mM NaCl to an OD_{660} of 1.00.

Biofilm Growth

Biofilms were grown in 24-well polystyrene plates.[17] One hundred–microliter aliquots of clarified saliva were transferred to wells and incubated for 2 hours at 37°C and 100 rpm. Wells were washed twice with sterile water.

One milliliter of standardized suspension (OD_{660} 1.00) was added to wells and incubated at 37°C, at 100 rpm and 10% pCO_2. After 2 hours, culture was removed and wells were washed twice with sterile 145 mM NaCl to remove weakly adhering and planktonic cells. Control wells received 1 mL of BHI, and experimental groups received 1 mL of BHI+ions. The plates were incubated at 37°C and 10% pCO_2 for 72 hours, and media were replaced with fresh broth with or without metal ions every 24 hours.

For each experimental group, six replicates of biofilms were grown in four separate moments; therefore, for each subsequent experiment 24 repetitions were analyzed.

Quantification of Glycolytic Rate

The rate of glucose uptake was determined using cells from intact biofilms. After 72 hours of growth, each well was gently washed twice with sterile 145 mM NaCl. One milliliter of CGS containing 27.77 mmol/L glucose with/without ions was then added to each well, and cells were incubated at 37°C with 10% pCO_2 and 90 rpm. At 6 hours and 12 hours, 10-μL aliquots were collected and mixed with 1 mL of enzyme reagent containing glucose oxidase.[18] Subsequent analytical steps followed the manufacturer's recommendations (Laborlab Inc, São Paulo, Brazil). Measured values were subtracted from 27.77 mmol/L. The values obtained were divided by time (minutes) and subsequently by biomass (quantified by absorbance) to obtain metabolic rates expressed as (μm/L)/min/biomass.

Quantification of Lactate Production

Two microliters of CGS was mixed with 200 μL of enzyme reagent containing lactate oxidase.[19] Subsequent analytical steps followed the manufacturer's recommendations (Biotecnica Ltd, Varginha, Brazil). Values were expressed in mmol/L and were divided by time (minutes). Subsequently, values were divided by biomass (quantified by absorbance) to obtain lactate secretion rates expressed as (μmol/L)/min/biomass.

Biomass Estimation by Crystal Violet

Biofilms grown for 72 hours and challenged by 6 hours or 12 hours with CGS were washed twice with sterile 145 mM NaCl, and the cells were fixed in 99% methanol for 15 minutes. After rapid methanol evaporation, cells were incubated in 1 mL of 0.02% crystal

Table 1. Biomasses Achieved by 72-Hour-Old Biofilms Grown in Presence/Absence of Metallic Cations Followed by 6-Hour/12-Hour Challenges with 5% Glucose

	Biomass [CV Retention (ΔOD_{540nm})] ± Standard Deviation					
Incubation Time	Control	Nickel[a]	Iron	Chromium[a]	Cobalt[a]	Mixture[a]
6 h	1.488 ± 0.295 [ac]	1.414 ± 0.298 [a]	1.819 ± 0.766 [b]	1.756 ± 0.374 [c]	1.540 ± 0.456 [ac]	1.420 ± 0.380 [a]
12 h	1.685 ± 0.326 [A]	1.048 ± 0.326 [B]	1.414 ± 0.470 [C]	1.302 ± 0.494 [C]	1.774 ± 0.355 [A]	1.676 ± 0.339 [A]

[a] Differences of results between 6 hours and 12 hours of incubation with 5% glucose (Games-Howell; $P < .05$). Different uppercase letters denote statistically significance differences (Games-Howell; $P < .05$) among different treatments for the same incubation time. CV indicates crystal violet.

violet (CV) for 20 minutes. After four washings with distilled water, incorporated CV was released for 10 minutes in 1 mL of 33% acetic acid, and the OD_{540} was then determined.[20]

Statistics

All data were assessed for homogeneity of variance using the Levene index and were analyzed by the one-way analysis of variance test with a Games-Howell multiple comparison test for heterogeneous variances using SPSS 18.0 (SPSS Inc, Chicago, Ill). A *P* value of $<.05$ was considered statistically significant.

RESULTS

Quantification of Biofilm Biomass

After 6 hours of treatment, increases in biomass were achieved following the addition of Fe^{3+} ($P \leq .049$) (Table 1). Biomasses obtained with Ni^{2+}, Cr^{3+}, Co^{2+}, and ion pool had means with slight fluctuations but were similar to the control ($P \geq .506$).

When cultures were continued for 12 hours, the average biomass of the controls had an absorbance of 1.685 ± 0.326. Reductions ($P \leq .015$) in biomass were obtained for biofilms grown in the presence of Ni^{2+} (1.048 ± 0.326), Fe^{3+} (1.414 ± 0.470), and Cr^{3+} (1.302 ± 0.494). The ion pool (1.676 ± 0.339) and Co^{2+} (1.774 ± 0.355) produced biofilms with biomasses similar to that of the control ($P \geq .419$).

The biomasses of groups grown with Ni^{2+}, Fe^{3+}, and Cr^{3+} were reduced by glucose treatment for 12 hours compared to their values following treatment for 6 hours. Possible methodological errors were not taken in account, and we concluded that the most plausible hypothesis is that the sharp drop in pH over 12 hours caused the ions to become toxic, resulting in a reduction in cell viability.

Quantification of Glycolytic Rate

At the 6-hour time point, none of the experimental groups were different from controls ($P \geq .657$), indicating that metal ions did not produce metabolic changes (Table 2). However, after 12 hours of glucose challenge, we observed increases in the metabolic rate of biofilms grown in the presence of Ni^{2+}, Fe^{3+}, and Cr^{3+} ($P \leq .033$).

Quantifying Lactate Production Rate

Although there were substantial variations among the results of different ion-treated groups (eg, Fe^{3+} ions vs other ions) in 6-hour cultures, lactate production results were not significantly different from those of the control ($P \geq .065$). The secretion of lactate after 12 hours of glucose treatment was increased in the presence of Ni^{2+}, Fe^{3+}, Cr^{3+}, and ion pool ($P \leq .046$; Table 3).

Stoichiometry

A scatter plot (Figure 1) was built to demonstrate the influence of metal ions on the relationship between the use of glucose and the consequent secretion of acid. However, the distribution of means did not generate clusters for a given ion or for the duration of treatment (6 hours and 12 hours).

Correlation analysis using Pearson's coefficient (r_P) revealed that after 6 hours of glucose treatment, the controls showed a weak nonsignificant negative

Table 2. Glucose Metabolic Rates of 72-Hour-Old Biofilms Grown in Presence/Absence of Metallic Cations Followed by 6-Hour/12-Hour Challenges with 5% Glucose

Incubation Time	Glucose Metabolic Rate {[(μmol/L)/min]/biomass} ± Standard Deviation					
	Control	Nickel[a]	Iron	Chromium[a]	Cobalt	Mixture
6 h	42.123 ± 3.811 [ab]	43.396 ± 7.315 [a]	34.293 ± 12.481 [b]	35.587 ± 10.223 [b]	40.933 ± 9.564 [ab]	43.116 ± 9.086 [ab]
12 h	36.891 ± 5.604 [A]	57.962 ± 17.735 [B]	44.081 ± 12.431 [C]	46.114 ± 13.756 [BC]	34.429 ± 4.868 [A]	36.429 ± 5.467 [A]

[a] Differences of results between 6 hours and 12 hours of incubation with 5% glucose (Games-Howell; $P < .05$). Different uppercase letters denote statistically significance differences (Games-Howell; $P < .05$) among different treatments for the same incubation time.

Table 3. Lactate Secretion Rates of 72-Hour-Old Biofilms Grown in Presence/Absence of Metallic Cations Followed by 6-Hour/12-Hour Challenges with 5% Glucose

	Lactate Secretion Rate {[(μmol/L)/min]/biomass} ± Standard Deviation					
Incubation Time	Control[a]	Nickel	Iron[a]	Chromium	Cobalt	Mixture[a]
6 h	9.991 ± 3.627 [ab]	4.636 ± 1.022 [a]	15.354 ± 5.838 [b]	2.935 ± 0.507 [a]	3.553 ± 2.917 [a]	7.299 ± 2.814 [a]
12 h	0.351 ± 0.069 A	6.139 ± 2.880 B	4.015 ± 1.473 C	2.761 ± 0.454 C	0.308 ± 0.052 A	1.602 ± 0.194 D

[a] Differences of results between 6 hours and 12 hours of incubation with 5% glucose (Games-Howell; $P < .05$). Different uppercase letters denote statistically significance differences (Games-Howell; $P < .05$) among different treatments for the same incubation time.

correlation between glucose consumption and lactate secretion ($r_P = -0.157$, $P > .05$). The ion pool also resulted in a weak negative correlation ($r_P = -0.024$, $P > .05$). Strong positive correlations were obtained for Ni^{2+} ($r_P = 0.777$, $P < .05$) and Fe^{3+} ($r_P = 0.742$, $P < .05$). After 12 hours of treatment, correlation coefficients become positive for all groups except for the ion pool ($r_P = -0.111$, $P > .05$). Results for Ni^{2+} were particularly noteworthy, demonstrating a strong positive correlation ($r_P = 0.833$, $P < .05$).

To determine the influence of metal ions on the efficiency of the conversion of glucose to lactate, a simplified homolactic fermentation equation (1 glucose → 2 lactate) with no metabolic deviation (eg, heterolactic fermentation) was considered. Lactate concentration values (in mmol/L) were divided by two to obtain the amount of glucose converted to lactate. The ratios obtained were multiplied by 100 and divided by the total concentration of glucose consumed. These calculated ratios were again multiplied by 100 and divided by the average values of controls for each time point. Thus, the values of controls were arbitrarily considered as 100%. At this threshold, the percentages of conversion of glucose to lactate by biofilms were recalculated (Figure 2). The results for the 6-hour treatment showed that only Fe^{3+} was able to increase the conversion rate above the mean control value (32.83% increase). The remaining ions reduced the conversion rate by about 52.69% (Ni^{2+}), 66.85% (Cr^{3+}), 66.77% (Co^{2+}), and 26.20% (pool). In contrast, glucose treatment for 12 hours produced conversion rates in the ion-treated groups that were much higher than that of the control, with increases ranging from 351.47% (mix) to 1064.01% (Ni^{2+}). The exception was the Co^{2+}-treated group, which showed a reduction of 3.35%.

DISCUSSION

In terms of methodology, our treatment of biofilms with glucose for up to 12 hours could be regarded as being too long and thus not physiologically relevant. However, we opted to include this treatment time because patients (and, in certain instances, their parents) commonly report the consumption of sugar throughout the waking period, even in multiple doses per day. Nevertheless, the choice to assess these biofilms over 72 hours is based on the clinical

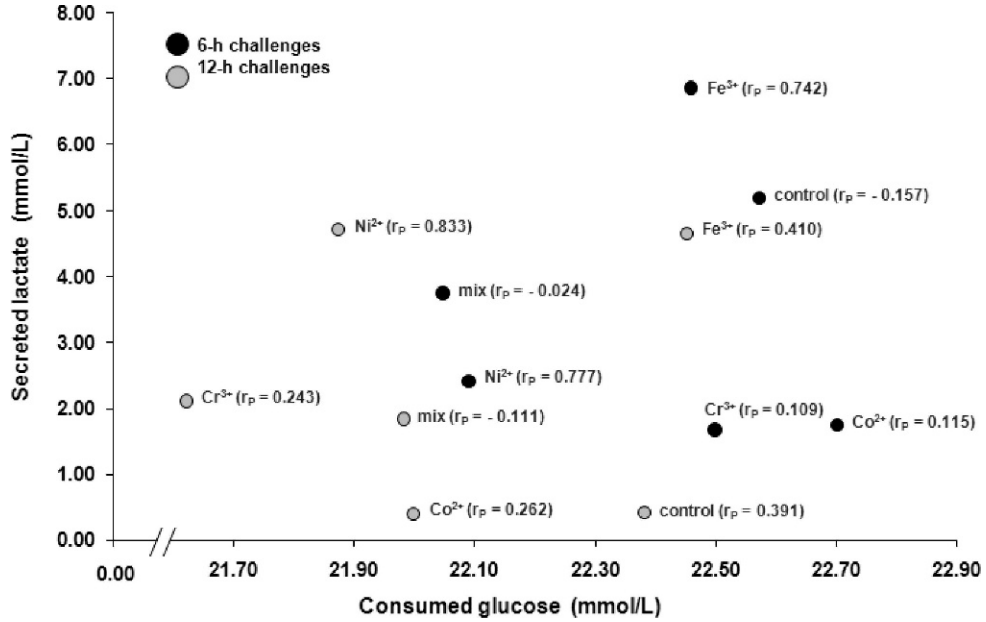

Figure 1. Cartesian combinations of nominal secreted lactate values (mmol/L) by consumed glucose (mmol/L). Each point represents the arithmetic average of 24 repetitions. r_P values represent the Pearson correlation coefficients for lactate vs glucose combinations.

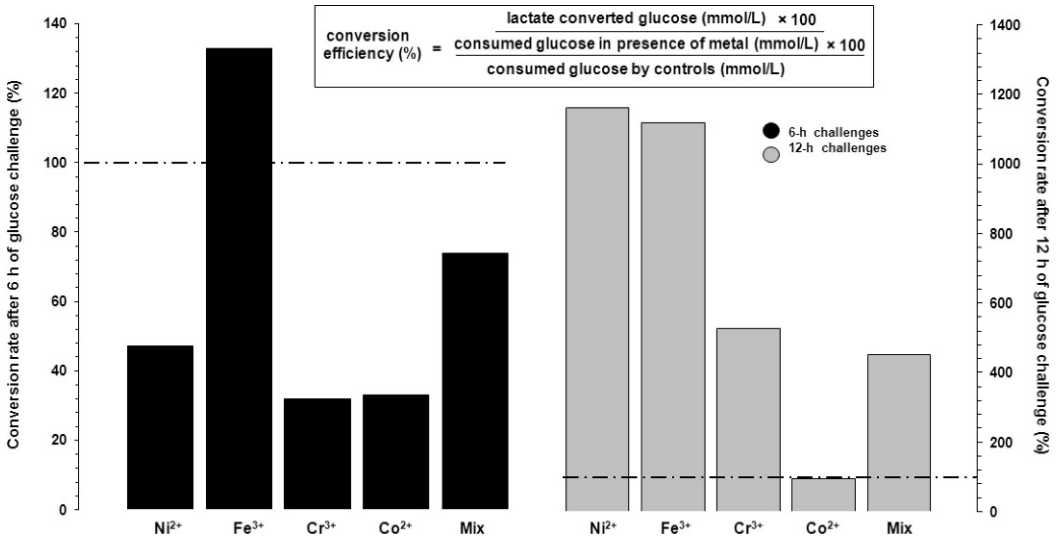

Figure 2. Efficiency (%) of glucose to lactate conversion modulated by metal ions. Dashed lines perpendicular to the y axis represent controls arbitrarily considered as being 100%.

observation that some patients are not collaborative and conduct oral hygiene far below the desirable level.

Of note, some metal ions, particularly Fe^{3+} and Cr^{3+}, increased the biomass of biofilms during the early hours of treatment with glucose compared to the control. However, these same ions did not maintain this effect when the treatment was extended. It is possible that these ions acted during the lag phase of biofilm growth over 72 hours caused by the addition of glucose and promoted its shortening. However, because *S mutans* lacks catalase with which to detoxify reactive oxygen species (ROS), it is also possible that trivalent ions were reduced to Fe^{2+} and Cr^{2+} as a result of the acidity of the biofilm and the low availability of molecular oxygen.[21] Once internalized by the bacterial cells, these reduced ions might have begun to catalyze the Fenton reaction, with subsequent production of hydroxyl radicals, which are strong oxidizing agents that affect cellular functions. At the level of microbial populations, the presence of ROS would have killed cells of outer layers of biofilm, as well as internal layer cells, to a lesser extent, resulting in lower biomass after 12 hours of treatment.

Based on the above hypothesis, it would be expected that the ion pool would also induce an increase in biomass in the first 6 hours, followed by a reduction when the treatment is extended to 12 hours. In fact, the mixture of ions induced an increase in biomass after 12 hours. Although Ni^{2+}, Cr^{3+}, and Fe^{3+} caused biomass reductions, Co^{2+}, which promoted increases between the sixth and 12th hours in glucose treatments, may has counteracted the cell loss, resulting in the maintenance of biofilm biomass exposed to ion pool.

Some ions were able to influence glucose metabolism by increasing consumption when the treatment was prolonged to 12 hours. However, the stoichiometry of glucose consumption and lactate production indicated that the hexose was not fully digested for energy purposes, as might be thought. Three possible scenarios, which may have occurred simultaneously, could explain the pattern of glucose expenditure. First, some of the glucose may have been converted to pyruvate and used for homolactic and/or heterolactic fermentation. Second, because *S mutans* is deficient in α-ketoglutarate dehydrogenase, its Krebs cycle is incomplete.[22] Thus, some of the pyruvate produced may have been converted into amino acids for biomass formation. Third, bacterium may have secreted extracellular glycosyltransferases to transform the surrounding glucose into an extrapolymeric matrix for biofilm formation.

Previous studies have found that *S mutans* internalizes only ferrous iron (Fe^{2+}),[9] which can be formed in the anaerobic and reducing environment of the biofilm interior as a result of either partial oxygen[23,24] or the action of reducing substances from glucose metabolism, possibly via flavoprotein-dehydrogenase.[9] Once Fe^{2+} enters the membrane, it can be internalized into cells. In *Lactobacillus casei*, it has been shown[25] that Fe^{2+} and Ni^{2+} can activate lactate dehydrogenase, increasing the conversion of pyruvate to lactate by 1.78 and 2.34 times, respectively. Our results are in agreement with that finding, which may at least partially explain the observed increases in the lactate secretion rate induced by ions (Figure 2). However, this effect is likely concentration dependent because bacterial exposure to nanomolar ion concentrations

stimulates microbial growth and metabolic activity,[26] in agreement with our results. Moreover, micromolar or greater ion concentrations lead to decreases in these.[11,27]

Two possible problems arose and must be referenced. First, we did not carry out any pH measurement during glucose metabolism. It may have interfered negatively with the biofilm formed for 12 hours, as depicted in Table 1. Second, amounts of glucose and lactate were determined enzymatically, and metal ions may have exerted some influence on the assay system. The extension of such interference is unknown. We consider that they could not determine punctual variations, once groups were under the same paired conditions and, therefore, with the same amount of susceptibility.

Notwithstanding that the significant impact of metal surface on oral biofilm formation has been demonstrated,[28] to our knowledge,[29] this is the first study that prospected the action of released metal ions over bacterial cariogenic potential. However, the compiled data obtained in our study, although interesting, must be considered with some reservation. Laboratory conditions may have increased some biofilm behavior, and no conclusive bench-to-bed inferences can be drawn. The results presented here must be validated by in vivo experiments, as well as in situ ones, involving multispecies biofilms and real intraoral conditions. The main contribution of this study is its demonstratation that released metal ions, similar to those released from orthodontic devices, may exert some interference on mutans physiology and virulence.

CONCLUSION

- We concluded that metal ions, in concentrations similar to those obtained from patients undergoing orthodontic intervention, may interfere with the physiology of *S mutans,* changing its virulence behavior.

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