

# Microbiological or Chemical Models of Enamel Secondary Caries Compared by Polarized-Light Microscopy and Energy Dispersive X-ray Spectroscopy

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**Abstract:** Different secondary caries models may present different results. The purpose of this study was to compare different *in vitro* secondary caries models, evaluating the obtained results by polarized-light microscopy (PLM), scanning electron microscopy (SEM), and energy dispersive X-ray spectroscopy (EDS). Standardized human enamel specimens ( $n = 12$ ) restored with different materials (Z250 conventional composite resin–CRZ, Freedom polyacid-modified composite resin–CRF, Vitremer resin-modified glass-ionomer–GIV, and Fuji IX conventional glass-ionomer cement–GIF) were submitted to microbiological (MM) or chemical caries models (CM). The control group was not submitted to any caries model. For MM, specimens were immersed firstly in sucrose broth inoculated with *Streptococcus mutans* ATCC 35688, incubated at 37°C/5% CO<sub>2</sub> for 14 days and then in remineralizing solution for 14 days. For CM, specimens were submitted to chemical pH-cycling. Specimens were ground, submitted to PLM and then were dehydrated, gold-sputtered and submitted to SEM and EDS. Results were statistically analyzed by Kruskal-Wallis and Student-Newman-Keuls tests ( $\alpha = 0.05$ ). No differences between *in vitro* caries models were found. Morphological differences in enamel demineralization were found between composite resin and polyacid-modified composite resin (CRZ and CRF) and between the resin-modified glass-ionomer and the glass-ionomer cement (GIF and GIV). GIF showed higher calcium concentration and less demineralization, differing from the other materials. In conclusion, the glass-ionomer cement showed less caries formation under both *in vitro* caries models evaluated. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 90B: 635–640, 2009

**Keywords:** secondary caries; caries model; fluoride; *Streptococcus mutans*

## INTRODUCTION

Levels of primary caries have declined in the past decades worldwide; however secondary caries remain an unresolved issue in modern restorative dentistry.<sup>1–3</sup> Secondary caries is defined by the *Federation Dentaire Internationale* as a positively diagnosed carious lesion which occurs at the margins of an existing restoration. The lesion usually consists of two carious regions: an outer lesion formed in the enamel or cementum of the tooth surface, similar in histology to a primary lesion, which can be caused by trapped biofilm in the restoration's margin; and a wall lesion, which is a narrower defect in the enamel or dentin along the cavity wall restoration interface. These lesions are difficult to detect clinically, especially if they are incipient.<sup>2</sup>

Laboratory models for studying secondary caries have become frequent in the literature, because studies on caries formation and caries development are vital for planning prevention and treatment.<sup>1,4</sup>

The cariostatic effects of several restorative materials have been studied by *in vitro* caries models, which simulate high caries challenge and aim to develop artificial lesions comparable to those that take place *in vivo*.<sup>4</sup> Chemical models focus on physicochemical aspects of dental caries and might be static (acid solutions, for instance) or dynamic (pH-cycling). Microbiological models use caries-related microorganisms, such as *Streptococcus mutans* and *Lactobacillus* spp.<sup>4,5</sup>

Sá et al.<sup>6</sup> compared different restorative materials by *in vitro* pH-cycling and microbiological caries models, stating that glass-ionomer cements under pH-cycling showed significant anticariogenic properties, which were not verified by the microbiological model. Although the behavior of fluoride-releasing restorative materials under *in vitro* cario-

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genic challenge has been investigated and confirmed by several authors, the properties of these materials under different caries models needs to be further investigated and compared, because of the numerous open questions regarding the pathogenesis and progression of secondary caries.<sup>3,7</sup>

Caries lesions can be evaluated by different methodologies, such as scanning electron microscopy (SEM) and polarized-light microscopy (PLM). SEM enables the study of enamel micromorphology, whereas PLM allows the visualization of areas with different porosities and birefringences. On the other hand, energy dispersive X-ray analysis (EDS) provides a specific method to determine the concentration of chemical elements on substratum surfaces, being largely used in Engineering and Chemistry, but in few studies in Dentistry.<sup>8–11</sup> Thus, a study of fluoride-releasing materials by *in vitro* microbiological or chemical secondary caries models, evaluated under different methodologies has not been performed yet.

The purpose of this study was to evaluate different fluoride-releasing and non-fluoride releasing restorative materials submitted to microbiological or chemical secondary caries models, evaluating the caries lesions under PLM, SEM and EDS.

## MATERIALS AND METHODS

This study was approved by the São Paulo State University Local Ethics Committee (protocol # 069/2005-PHCEP).

### Specimen Preparation

Sixty sound embedded freshly-extracted non-impacted human third molars, indicated for extraction were used, due to orthodontic reasons. After the extraction, the roots were hand scaled and teeth were cleaned with aqueous slurry of pumice and stored in saline solution (0.85% NaCl) before use. The crowns were removed and the roots discarded.

The crowns received a standard Class V preparation on the middle third of the buccal and lingual surface, using a diamond bur (KG Sorensen # 2294, Brazil) resulting in a 2 mm-diameter × 1.5 mm-deep enamel preparation. The crowns were cut into 4 × 4 × 2 mm<sup>3</sup> enamel slabs, using a high-speed diamond saw, under air/water cooling. The enamel area around the restoration was standardized using a digital pachymeter (Mycal Absolute, Brazil), so that all specimens would have the same area (16 mm<sup>2</sup>). Each crown resulted in two specimens. Specimens were randomly restored with one of the following restorative materials according to group: CRZ: conventional composite resin (Prime and Bond 2.1, Dentsply, Brazil adhesive system + Z250 composite resin, 3M ESPE, St. Paul, MN); CRF: polyacid-modified resin composite (Prime and Bond 2.1 adhesive system + Freedom, SDI North America, Bensenville, IL); GIV: resin-modified glass-ionomer (Vitremmer, 3M ESPE, St. Paul, MN) and GIF: conventional glass-ionomer cement (Fuji IX + Fuji IX GP Varnish, GC Corpora-

tion, Tokyo, Japan). The restorative materials were handled according to the manufacturer's instructions. All the specimens were stored at 37°C and 100% relative humidity for 24 h, before being finished with two grades (fine and extra-fine) of Sof-lex disks (15 s each disc)<sup>12</sup> at low speed and water cooling. Specimens received two coats of acid-resistant varnish (Colorama, São Paulo, Brazil) in all surfaces, except the surface with the restoration, to isolate the enamel area (16 mm<sup>2</sup>) which would be submitted to the different caries models.<sup>1,4,12</sup> After this procedure, the specimens were sterilized using gamma radiation (20 kGy).<sup>13</sup> Specimens were randomly assigned to either microbiological caries model, MM (*n* = 12); chemical caries model, CM (*n* = 12); or control group (*n* = 6).

### Microbiological Caries Model

Microbiological model was performed under aseptic conditions (laminar air flow chamber). *Streptococcus mutans* ATCC 35688 standardized suspension containing 10<sup>6</sup> cells/ml was obtained in sterile saline solution (0.85% NaCl) by spectrophotometry (Shimadzu UV-1203, Kyoto, Japan), adopting the wavelength of 398 nm and optical density of 0.620.

Specimens were put in 24-well-plates for cell culture (Zellkultur Testplatte 24, Switzerland). In each well, 1.5 mL of sucrose broth, described by Gibbons and Nygaard<sup>14</sup> (20 g Trypticase, 2 g NaCl, 3 g K<sub>2</sub>HPO<sub>4</sub>, 2 g KH<sub>2</sub>PO<sub>4</sub>, 1 g K<sub>2</sub>CO<sub>3</sub>, 120 mg MgSO<sub>4</sub>, 0.015 g MnSO<sub>4</sub>, and 50 g of sucrose in 1000 mL of distilled water and sterilized) and 0.1 mL of *S. mutans* standardized suspension were added to each well. The plates were incubated at 37°C/5% CO<sub>2</sub>. Every 2 days, the sucrose broth was exchanged for fresh broth and Gram stained samples were obtained to confirm absence of contamination. The presence of bacterial growth was evaluated by plating 0.1 mL of the replaced broth in Brain Heart Infusion Agar at 37°C/5% CO<sub>2</sub> every 48 h. After 14 days,<sup>15</sup> specimens were immersed in a mineral solution to simulate the remineralizing effect of saliva, as described by Seeman et al.<sup>16</sup>: (0.33 g KH<sub>2</sub>PO<sub>4</sub>, 0.34 g Na<sub>2</sub>HPO<sub>4</sub>, 1.27 g KCl, 0.16 g NaSCN, 0.58 g NaCl, 0.17 g CaCl<sub>2</sub> • H<sub>2</sub>O, 0.16 g NH<sub>4</sub>Cl, 0.2 g urea, 0.03 g glucose, 0.002 g vitamin C, 2.7 g mucin (M-1778 porcine mucin, Sigma-Aldrich Brazil.) in 1000 mL of distilled water).

### Chemical Caries Model

For this model, pH-cycling model described by Featherstone and Rodgers<sup>17</sup> and modified by Serra and Cury<sup>18</sup> was used. Specimens for each group were cycled with a demineralizing solution (154.13 mg Ca(OH)<sub>2</sub>; 3 mL CH<sub>3</sub>COOH; Na<sub>2</sub>C<sub>2</sub>H<sub>3</sub>O<sub>2</sub> • 3H<sub>2</sub>O; 0.13 mL H<sub>3</sub>PO<sub>4</sub> in 1000 mL of distilled water) pH = 4.0 for 6 h and a remineralizing solution (115.59 mg Ca(OH)<sub>2</sub>; 1 mL HCl; 11.18 g KCl; 0.06 mL H<sub>3</sub>PO<sub>4</sub>; 2.42 g NH<sub>2</sub>C(CH<sub>2</sub>OH)<sub>3</sub>; 2 mL HCl in 1000 mL of distilled water) pH = 7.0 for 18 h during 9 days.

**TABLE I. Polarized-Light Microscopy Results of Specimens Submitted to Microbiological Caries Model and Chemical Caries Model**

Group	Microbiological Caries Model		Chemical Caries Model	
	Mean Area (mm <sup>2</sup> ) ± SD	Min.–Max. Values	Mean Area (mm <sup>2</sup> ) ± SD	Min.–Max. Values
CRZ	10.98 ± 2.42	6.66–13.49	10.09 ± 1.27	7.78–12.43
CRF	9.86 ± 1.70	7.40–12.80	9.60 ± 1.18	7.74–11.40
GIV	7.31 ± 1.49	4.26–9.27	7.05 ± 2.18	3.34–11.05
GIF	3.62 ± 1.10	2.05–4.79	3.37 ± 2.71	0.90–11.03

Legend: SD, standard deviation; CRZ, composite resin Z250; CRF, polyacid-modified composite resin Freedom; GIV, resin-modified glass-ionomer Vitremer; GIF, conventional glass-ionomer cement.

Control group had specimens that were not submitted to any caries model, being kept in 100% relative humidity at 37°C during the experimental period.

#### Polarized Light Microscopy Analysis

Specimens were cut into 1 mm-thick sections using a high-speed diamond saw, under air/water cooling.<sup>1,4</sup> Then, each section was ground to a thickness of 100 μm, measured with a digital pachymeter (Mycal Absolute, Brazil). Each section was soaked in distilled water and mounted for examination under polarizing light microscope (Axiophot 2–Zeiss–Germany). Digital images were taken and the caries-lesion area (outer lesions) was selected by a single examiner (dentistry professor, blind to the treatments) and calculated by Image J software (National Institute of Health, USA). When wall lesions were present, they were measured as the largest distance between the restoration and the inner border of lesion.<sup>1</sup>

#### Scanning Electron Microscopic and Energy dispersive X-ray Analysis (EDS)

All sections were dehydrated using crescent alcohol concentration solutions (70% for 15 min, 80% for 15 min, 90% for 15 min and 100% for 30 min),<sup>19</sup> were placed on metal stubs, gold-sputtered in the Ions Sputter (Fisions Instruments, USA) and examined through SEM with the Link Isis Oxford Instrument (Leica) at 20 kV. These procedures were developed at the Laboratory for Integration and Testing at the National Institute for Space Research (LIT-INPE, São José dos Campos, São Paulo, Brazil). For each section, two photomicrographs were taken, one with 50× magnification, to verify the formation of caries-like lesion and the other with magnifications varying from 4000 to 10,000× to examine the enamel morphology in the outer lesion. The photomicrographs were blindly analyzed by a single examiner. EDS measurements were calibrated before each stub, by a certified engineer, using standard samples of Cr<sub>2</sub>O<sub>3</sub>, titanium, silica and CaSiO<sub>3</sub>.<sup>20</sup> Element content in weight percent (%wt) of calcium (Ca) was measured with

S-UTW detector. Three windows of 1 × 1mm area were used, separated by 1 mm in the outer lesion. Link Isis Oxford software extracted the %wt of Ca. The count rate was between 1800 and 2000 counts/s with a dead time of 25%. Measuring time was 25 s (live seconds). The measurements for each specimen were averaged to provide a single mean for each specimen.

#### Statistical Analysis

The measurements were submitted to Kolmogorov-Smirnov test at 5% level of significance to assess normality of distribution. Because data had non-parametric distribution, Kruskal-Wallis and Student-Newman-Keuls statistical tests ( $\alpha = 0.05$ ) were used, using BioEstat 4.0 software (Ayres, Brazil). The correspondence between the PLM results and EDS results was analyzed by Spearman correspondence analysis ( $\alpha = 0.05$ ).

## RESULTS

PLM results for specimens submitted to MM are described in Table I. Statistical analysis showed that all groups were significantly different among them, with the exception of groups CRZ-MM and CRF-MM, with no significant statistical difference between them ( $p = 0.4932$ ). CRZ-MM showed the highest mean value and GIF-MM, the lowest.

PLM results of specimens submitted to CM are also described in Table I. Statistical analysis showed no differences between the composite resin and the polyacid-modified composite resin (CRZ-CM and CRF-CM,  $p = 0.5403$ ). In addition, the resin-modified glass-ionomer and the glass-ionomer cement showed no difference between them (GIV-CM and GIF-CM,  $p = 0.0543$ ). However, all the other statistical combinations showed significant statistical differences between the study groups: CRZ-CM and GIV-CM ( $p = 0.0070$ ); CRZ-CM and GIF-CM ( $p < 0.0001$ ); CRF-CM and GIV-CM ( $p = 0.0371$ ); CRF-CM and GIF-CM ( $p = 0.0001$ ). When MM results were compared with CM results, for each restorative material, regardless the caries model, no statistical differences were found between the caries models.

EDS mean results for control group are described in Table II. EDS mean results of the specimens submitted to MM are also described in Table II. SEM showed inhibition halos in groups GIV-MM and GIF-MM. In addition, differences in the pattern of demineralization were found between the study groups. CRZ-MM and CRF-MM showed defined pattern of demineralization. GIF-MM did not present a defined demineralization pattern, with shallow demineralization, presenting lack of exposing enamel prisms. Mixed areas, with demineralization sites mixed with areas of normal enamel were common in group GIV-MM.

Statistical analysis showed significant difference between the control group and groups CRZ-MM ( $p = 0.0015$ ), CRF-MM ( $p = 0.0277$ ) and GIV-MM ( $p =$

**TABLE II. Energy Dispersion X-ray Analysis Mean Results in Control Group, Microbiological Caries Model, and Chemical Caries Model in %wt**

Group	Control Group Ca%wt $\pm$ SD	Microbiological Caries Model Ca%wt $\pm$ SD	Chemical Caries Model %wt $\pm$ SD
CRZ	37.31 $\pm$ 2.11	23.05 $\pm$ 7.19	24.32 $\pm$ 6.09
CRF	34.83 $\pm$ 3.20	28.73 $\pm$ 5.41	25.52 $\pm$ 5.95
GIV	37.39 $\pm$ 2.62	29.69 $\pm$ 3.65	28.21 $\pm$ 6.56
GIF	37.34 $\pm$ 1.66	33.09 $\pm$ 5.17	34.09 $\pm$ 2.40

Legend: SD, standard deviation; CRZ, composite resin Z250; CRF, polyacid-modified composite resin Freedom; GIV, resin-modified glass-ionomer Vitremer; GIF, conventional glass-ionomer cement; Ca, calcium; %wt, weight percent.

0.0020) regarding Ca%wt. These results were consistent with SEM analysis, because GIF-MM specimens did not show visible signs of demineralization.

When the study groups were statistically analyzed, there were significant differences between CRZ-MM and GIV-MM ( $p = 0.0377$ ); CRZ-MM and GIF-MM ( $p = 0.0009$ ), regarding Ca%wt.

EDS mean results for specimens submitted to CM are described in Table II. SEM showed the presence of inhibition halos in groups GIV-CM and GIF-CM. No pattern of demineralization was found in any of the groups. Group CRZ-CM presented signs of more pronounced demineralization, when compared with groups CRZ-MM and CRF-MM, especially CRZ-CM. When compared with the control group, all study groups submitted to the chemical caries model showed significant statistical differences. Regarding Ca%wt, there were significant statistical differences between groups CRZ-CM and GIF-CM ( $p < 0.0001$ ); CRF-CM and GIF-CM ( $p = 0.0004$ ); GIV-CM and GIF-CM ( $p = 0.0070$ ).

When PLM results were compared with the Ca%wt in all study groups, no correspondence was found.

## DISCUSSION

The present study applied different *in vitro* caries models to verify caries lesion area, enamel morphology and enamel chemical composition evaluated by PLM, SEM and EDS. Human molars were used to avoid the effect of the variation of fluoride concentration in different tooth types.<sup>21,22</sup>

In the literature, many studies using different caries-inducing models are found.<sup>3,4,6,16-18</sup> According to Kielbassa et al.,<sup>23</sup> SEM can be considered as gold standard for qualitative information on enamel defects, but not for subsurface demineralization. In addition, when evaluating a caries-affected surface, a simple visual examination of the surface is not considered appropriate to detect the slightest surface breakdown of caries lesion even under laboratory conditions.<sup>23</sup> SEM is probably a more sensitive model to visualize small enamel changes than light microscopy and microradiography.<sup>24</sup> PLM is a methodology that allows the visualization of areas with different porosities. On the other

hand, EDS provides a semi-quantitative technique for analyzing caries-affected surface and should be added to PLM, SEM, and microradiography analysis, because EDS is a sensitive method, which provides additional surface information, allowing to monitor chemical surface variation.<sup>1</sup>

Under SEM, CRZ-MM and CRF-MM showed characteristic patterns of enamel demineralization, when compared with groups GIV-MM and GIF-MM, which presented hollow enamel cores, as described by Wang et al.<sup>25</sup> These results suggest that the mixture of fluoride and composite resin may have influenced enamel demineralization by *S. mutans*. The deepest demineralization observed by SEM was found in CRZ-CM, corroborating the results found by Rodrigues et al.,<sup>26</sup> who related that Z250 conventional composite resin showed high degree of adjacent enamel demineralization in Class V restorations submitted to a chemical caries model, when analyzed by visual examination. It is important to mention that although no significant statistical difference was found between the PLM and EDS results when the findings of both caries models were compared, there was a marked morphology difference under SEM. This difference regarding the restorative materials (CRZ and CRF, compared with GIV and GIF) might be explained by the fluoride release. Because fluoride release is greater on the first day, followed by a progressive and gradual decrease in release rate,<sup>27</sup> the interaction between fluoride release (or lack of it) and cause of enamel demineralization (non-bacterial acids or bacterial acids) may have influenced the morphological results.

Regarding the morphology of the specimens observed by SEM, the results may also be explained by the fact that the observation of simultaneous remineralization and demineralization within outer and inner regions of enamel, respectively, is consistent with the suggestion that the effective composition of the experimental solution changes as ions diffuse and precipitate within the partially demineralized outer enamel region.<sup>28</sup> As a result of remineralization of the outer enamel region, the remineralization potential of the resultant solution that penetrates the inner enamel pore volume is reduced, due to the loss of constituent ions. This implies that the rates of simultaneous demineralization and remineralization are controlled by local variations in relevant ion products<sup>28</sup> and because in the present study, different restorative materials were used, fluoride release by the restorative materials may have affected the results.

Yamazaki et al.<sup>28</sup> stated that kinetics of enamel demineralization is controlled by surface reactions. The caries models used in the present study have different kinetics: microbiological caries model uses the acid-production by *S. mutans* as the demineralizing substance, whereas chemical caries model uses a production of eroded-like lesions, which were specially pronounced in CRZ-CM. It is suggested that specific interactions of acid species with enamel mineral may modify the rate of enamel demineralization<sup>29</sup> and enamel morphology, as shown in the present study.

The inhibition halo produced by GIV and GIF (both under caries model) has been described by González et al.<sup>30</sup> and it can be assumed that this is due to the fluoride release from these materials, known as demineralization inhibition effect. This can be explained by the fact that fluoride ion inhibits enamel demineralization by enhancing the deposition of less soluble fluoridated hydroxyapatite phases within the enamel surface. When the rate of this deposition exceeds the rate of demineralization, the sections appears as if there is no net loss of enamel mineral, which could explain the SEM morphology of group GIF under both caries models, showing less visible signs of demineralization. In addition, fluoride may affect the adherence of bacteria (*S. mutans*, in the present study), altering their metabolism and the caries process and many questions still arises on how exactly fluoride influences the development of secondary caries.<sup>5-7,27</sup>

The results of EDS in the present study are in agreement with the results of Moshonov et al.,<sup>31</sup> in which the Ca%wt in human enamel varied from 33.8 ( $\pm 2.3$ ) to 35 ( $\pm 4.3$ ). However, the mean results of the present study regarding Ca%wt were lower than Aires et al.,<sup>32</sup> in which the mean Ca%wt was 39.4 ( $\pm 17.2$ ). These differences can be explained not only by the usage of different caries models but also by the different procedures for EDS calibration in addition to different number of measurements taken for each specimen. In the present study, three EDS measurements were taken before the mean result was averaged. In addition, different count rate, dead time, measurement time and measurement windows could explain the findings of the present study when comparing it to other studies in the literature.

In the present study, it was able to conclude that no significant differences were found between the two caries models. GIF presented less caries area in both caries models, and a higher Ca%wt.

In the present study, two *in vitro* caries models were used and although *in vitro* caries models present limitations, such as the lack of interaction among saliva, bacterial species and immunological response, these models are less expensive, less time-consuming, easily reproducible and has fewer ethics issues than *in situ* or *in vivo* models and these are important characteristics when studying caries formation and development.

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