

# Subtraction radiography of dentinal caries-like lesions induced in vitro by cariogenic bacteria

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### Abstract

*Purpose:* The objective of this project was to develop an in vitro model system for investigations of dentinal caries.

*Methods:* Five extracted primary molar teeth with circular cavity preparations to the depth of the dentinoenamel junction were mounted individually in cold-cure acrylic bases constructed to fit a positioning jig in a Schick-Technologies™ digital radiographic imaging apparatus. The mounted teeth (MT) were incubated with pure cultures of *Streptococcus mutans* for 7 days and *Lactobacillus casei* for 38 days. At baseline and on day 23 and 45, four digital radiographs of each MT were made. Subtraction radiography was performed and analyzed using custom interactive software.

*Results:* Lesions progressed roughly halfway through the dentin in all teeth at 23 days and approximated the pulp chambers at 45 days of incubation. Images resulting from subtraction procedures clearly revealed incremental caries progression which could be quantitated.

*Conclusion:* The model may be useful for screening cariostatic dental materials or treatments and investigating microbial mechanisms in dentinal caries. (*Pediatr Dent* 20:5 345–349, 1998)

Dentinal caries-like decalcification has been produced in extracted human teeth in vitro with pure cultures of lactobacilli,<sup>1–3</sup> mutans streptococci,<sup>2–7</sup> *Actinomyces viscosus*,<sup>2, 3, 5</sup> and *Streptococcus salivarius*.<sup>3</sup> Nagoka<sup>3</sup> recently found that certain mixtures of bacterial species increased dentinal decalcification compared to single species. Other investigators achieved in vitro caries-like effects with acidic solutions.<sup>7–10</sup> Progression of in vitro decalcification has been evaluated and quantitated by microradiography,<sup>2, 9, 11</sup> polarized light microscopy,<sup>5–8</sup> light microscopy using stain reagents,<sup>3</sup> microhardness measurements,<sup>3</sup> and subtraction radiography.<sup>12–14</sup>

With digital subtraction radiography (DSR) teeth do not need to be sectioned or probed to determine lesion progression.<sup>12–14</sup> Instead, radiographs are taken with the same exposure geometry and conditions on succeeding occasions. These images are then superimposed in a computer and a subtraction is performed. If the superposition of the two images is accurate and

the exposure factors are the same, the subtracted image will show any changes that occurred in the time interval between the exposures. By convention, the earlier radiograph is subtracted from the later radiograph producing an image where negative values signify demineralization and positive values signify remineralization. DSR has been employed in periodontal<sup>15–23</sup> and caries studies,<sup>12–14</sup> but not in in vitro caries investigations using pure cultures.

The objective of this investigation was to construct an in-vitro model which closely simulated natural dentinal caries and could be assayed longitudinally by quantitative methods. This would allow quantitative assessment of incremental caries progression without the necessity of employing invasive histological techniques and should be useful in evaluations of materials or treatments which inhibit dentinal caries.

### Methods

Five intact primary molars (one mandibular, four maxillary) extracted from children for orthodontic reasons were mounted in acrylic by the following procedures. An alginate impression (Jeltrate™, Dentsply, Milford, DL) was made of a hexagonal aluminum template constructed to fit the positioning jig of a digital radiographic imaging apparatus (see subtraction radiography, below). Cold-cure acrylic (Halther Acrylics™, Unitek Corp., Monrovia, CA) was poured into the impression and a tooth inserted. After setting, the acrylic was trimmed to expose the crown, which was coated with light-cure sealant (Concise™, 3M Dental Products Division, St. Paul, MN). A circular preparation, 1.5 mm in diameter and extending to the dentinoenamel junction, was made in the occlusal surface of the tooth.

### Culture procedures

The mounted teeth (MT) were disinfected by immersion in 5.25% sodium hypochlorite solution (Chlorox™, Chlorox Co. Oakland, CA) for 2 h followed by soaking in six changes of sterile distilled water over a 48-h period, and then placed in sterile 50-mL glass jars containing 25 mL of Brain Heart Infusion

broth (Difco, Detroit, MI) made 5.0% (wt/vol) with respect to sucrose (BHI-S). The broth was inoculated with 100  $\mu$ L of an overnight culture of *Streptococcus mutans* (GEM, a biotype I clinical isolate), cultivated in BHI-S. The jars were incubated at 37°C in air containing 10% carbon dioxide. MT were transferred daily into jars containing fresh BHI-S. After 7 days the medium was changed to MRS broth (Difco), a nonselective medium for cultivation of lactobacilli, made 5.0% (wt/vol) with respect to sucrose (MRS-S). This was inoculated with 100  $\mu$ L of an overnight culture of *Lactobacillus casei* (ATCC 11578) cultivated in BHI-S. MT were transferred daily into a fresh medium for an additional 38 days. The MRS-S was alternated daily with an 0.85% saline solution containing 5.0% sucrose (NaCl-S). Incubation conditions remained the same. Plaque from MT was evaluated periodically for presence and purity of the test microorganisms, by streaking wire-loop specimens on MM10 Sucrose Blood agar<sup>24</sup> and Mitis Salivarius Bacitracin agar (MSBS),<sup>25</sup> selective for *S. mutans*, or Rogosa SL agar (Difco), selective for *L. casei*.

### Subtraction radiography

DSR was used instead of more traditional sectioning and subsequent histological examination to measure sequential decay progression. Sectioning in this type of investigation would require a larger starting number of teeth as teeth would be destroyed after each caries measurement. A positioning apparatus ensured exact repositioning of the tooth samples, the x-ray tube, and the electronic sensor from one exposure to the next. All radiographs were taken with a Schick Technologies CDR (Computed Dental Radiography) size-2 sensor using a Kevex™ PXS7-730 EA x-ray machine with 50- $\mu$ m focal spot at 70 kVCP. An Al step wedge with steps of 1-, 2-, 4-, 8-, and 16-mm thickness was attached over an unused portion of the sensor to measure any changes in the exposures of the teeth from one radiograph to the next. All samples were radiographed individually, prior to culturing in the bacterial solution, and on days 23 and 45. To reduce quantum x-ray noise, four radiographs were taken and averaged together for every sample at each time period.

Even with rigid control of the exposure geometry, it was found necessary to perform subpixel translations and small rotations of the images before they were subtracted. These were performed using custom, interactive software which transposed the two images, translated one image with respect to the other, and then performed a subtraction in real time. The positioning was considered optimal when the edges of the crowns were maximally canceled.

The decalcification resulting from mineral loss was computed by drawing a region of interest (ROI) with the computer's mouse around the decayed area on the

subtracted image. The area of the region and the average gray-scale value of the region were then computed. A DSR value was defined as the area of the ROI multiplied by the average gray-scale value of the ROI. This value corresponds to the computer-assisted densitometric image analysis (CADIA) value mentioned in the literature which has previously been shown to correspond directly to mineral loss.<sup>19</sup>

The average gray value was measured under each step of the step wedge. These values were then compared on all radiographs. All the average values were within  $\pm 2$  gray levels out of a possible 4096. This showed that the exposures were identical for all radiographs. As a further test, the accuracy of the DSR measurement was also checked by taking the DSR value on regions of the subtracted images where no decay occurred. In this instance, the DSR values were between  $\pm 2$ .

Two ROIs were chosen for each subtraction. In the first ROI, care was taken to carefully circumscribe the lesion. In the second ROI, a wide area around each lesion was chosen. This ROI was made as large as possible without including neighboring carious lesions or tooth/air boundaries. The average of the two DSR values was used as the DSR value for the subtraction. The difference between the two DSR values was taken as an upper bound on the measurement inaccuracy of each subtraction.

### Results

After exposure to *S. mutans* for 7 days, flocculent plaques were present on the acrylic bases and the sealant covered crowns of MT. *S. mutans* was recovered on MM10 and MSBS during this period. While plaques remained after exposure to *L. casei*, only *L. casei* could be recovered from them 7 days after incubation in MRS-S and NaCl-S.

Radiographs of all teeth showed obvious dentinal caries-like lesions at 23 days of incubation which progressed roughly halfway through the dentin (Fig 1). During the next 22 days progression of lesions appeared to accelerate and approached the pulp chambers (Fig 1). DSR revealed extensive decalcification of the teeth at both time periods which appeared to extend beyond the radiographic profile of the lesions.

Physical examination of the crowns of MT showed marked decay on many cusp tips not associated with the sites of the original cavity preparation. This occurred because the pit and fissure sealant was not thick enough at the cusp tips to form more than an air inhibited layer which quickly washed off during the incubation period.

Decay was detected in the prepared sites and was easily followed using DSR. The results of the DSR measurements are shown in the Table.

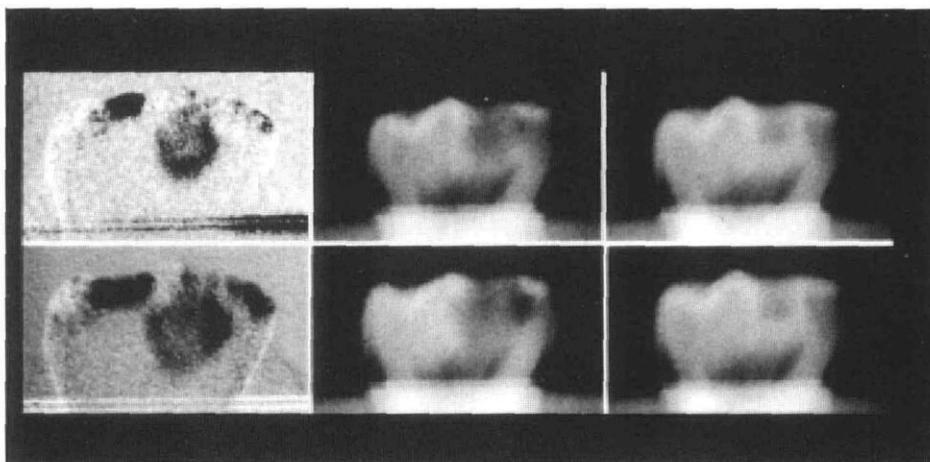


Fig 1. Radiographic and subtraction images. Top, left to right: subtraction image, 23-day radiograph, baseline radiograph. Bottom, left to right: subtraction image, 45-day radiograph, baseline radiograph.

**TABLE. DIGITAL SUBTRACTION RADIOGRAPHY (DSR) MEASUREMENTS OF PROGRESSION OF DECAY IN EXPERIMENTAL LESIONS**

Day	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
0	0	0	0	0	0
23	-264 ± 3*	-265 ± 2	-191 ± 7	-240 ± 3	-197 ± 7
45	-736 ± 29	-601 ± 18	-657 ± 49	-549 ± 65	-640 ± 31

\*DSR values (Area of Radiolucency x Gray-Scale Value). The errors are differences between two measurements.

## Discussion

This model system demonstrated that dentinal caries-like lesions could be induced by pure cultures within a relatively short time period and that longitudinal progression could be accurately measured. Nonvitality of the experimental teeth may have contributed to the rapid expansion of the lesions, as caries rates have been found to be slower in vital teeth.<sup>26</sup> The present model did not, however, duplicate the microbial profile of natural dentinal lesions which harbor complex arrays of bacterial species.<sup>27, 28</sup> In preliminary studies we attempted unsuccessfully to stimulate progression of natural lesions in extracted teeth by incubation in various artificial media. Opportunistic species in the carious material or contaminants became enriched and readily overgrew the indigenous inhabitants. Successful induction of dentinal caries progression in extracted teeth with natural lesions has been reported, but the teeth were not cultured.<sup>13</sup>

We attempted to simulate environmental and mi-

crobiological conditions of natural lesions by introducing *S. mutans* as an initial colonizer and cariogen and then *L. casei* after 7 days. *S. mutans* has been associated with dental caries in enamel and root surfaces and is an early colonizer of the teeth.<sup>29-32</sup> Lactobacilli, while frequently present at dental sites, numerically increase once cavitation occurs and eventually predominate the microflora of a

dentinal lesion.<sup>29-31, 33, 34</sup> Both the glucan-rich plaques, which may enhance colonization,<sup>35</sup> and an acidic environment in the vicinity of *S. mutans* microcolonies within plaque theoretically encourage and stimulate succession of lactobacilli.<sup>36</sup> A possible cooperative effect by both species was demonstrated in vitro<sup>3</sup> whereby a mixture of *S. mutans* and lactobacilli penetrated to a greater depth in dentin than did either species alone. The proportion of lactobacilli in carious dentin varies widely in the literature when inhabitation according to depth of the lesion was not accounted for.<sup>28, 31, 33, 34, 37, 38</sup> When dentinal lesions were cultured at four depths from enamel to pulp, lactobacilli comprised 92% of the viable microflora in the deepest portion.<sup>28</sup>

Fluctuations of high and low nutrient availability were imposed on the cultures in an effort to partially simulate nutrient variations which occur in the oral cavity. Bacteria in supragingival dental plaque, particularly in sites distant from crevicular regions have shown cellular characteristics associated with a nutrient environment deficient in nitrogen and high in carbon.<sup>39</sup> Although our media regimen did not perfectly duplicate the oral nutrient environment, daily alternation of MRS-S with NaCl-S was meant to introduce periods of high and low nutrient availability.

Using the same culture regimen, our preliminary studies showed that dentinal lesions could also be induced in extracted permanent molars, but the required length of incubation time was 8 to 10 weeks, as opposed to 6 to 7 weeks for primary teeth. Slower in-vitro dentin-decalcification rates of permanent versus primary teeth have been reported.<sup>40</sup>

While this model system involved previously used

techniques,<sup>1, 3, 4, 6, 13, 14</sup> our particular combination of methods differs from other reports. In the referenced studies that did not use DSR,<sup>1, 3, 4, 6</sup> decalcified teeth were evaluated by either histological or radiographic methods. Histological determinations would preclude sequential measurements of caries progression within the same lesion. Radiographic assessments, conversely, would not alter the experimental lesion, but could not reveal small incremental changes as effectively as DSR. A study to determine caries progression under a restoration placed over an experimental lesion, for example, could be most effectively assessed by DSR. An idealistic in-vitro caries model was described by Maggio et al.,<sup>13</sup> whereby incremental caries progression was evaluated by DSR in extracted teeth with natural carious lesions. Variability of specimen microflora and lesion characteristics, however, would limit effectiveness of this system when multiple experimental specimens were required. Use of pure cultures to induce experimental lesions in our model, while not ideal from the standpoint of artificiality of the microbial content and variability among dental specimens, would permit implementation of controlled preliminary studies prior to in-vivo trials.

The precision and high reproducibility of the DSR measurements was due to the nature of DSR and also to the control that can be obtained through in vitro experiments. Precise control of the positioning of the specimens during the radiographic process is easily achieved when patients are not involved, as is the luxury of averaging four radiographs together to reduce x-ray noise. Errors in positioning are exceptionally noticeable in caries detection measurements because of their location on the exterior surfaces of the enamel. Because of the sharp boundaries between the air and enamel, errors in registration of the two images cause very large difference artifacts as a white enamel edge in one radiograph is superimposed over the black air gap of its paired image. This can totally obscure small changes in mineralization occurring in the misregistered area.

In this study, after minor rotations and translations, misregistrations were not observed and the resulting subtracted images were highly diagnostic. It became obvious during the first subtractions on day 23 that DSR could have detected changes in calcification much earlier (see Fig 1). In subsequent experiments, radiographs will be spaced at shorter intervals to more closely monitor the decay process. Future studies will also address progression and microbial viability of carious lesions under restorations, and cariogenicity of dentinal inhabitants.

## Conclusions

1. The cultural system employed resulted in extensive dentinal caries-like lesions in extracted primary teeth within a short time period relative to

natural human caries progression.

2. Determination of extent and quantitation of caries progression were accomplished by a DSR technique.
3. The model system may be useful for in vitro studies of sequential caries progression within individual teeth.

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