A MicroCT Study on Mineral Change over Time Associated with Demineralisation in Primary Teeth

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Objective: *To evaluate the change in demineralisation depth (DD)and mineral density (MD) over time in primary teeth exposed to a demineralisation protocol with microcomputed tomography (microCT).*

Methods: *Caries lesions were artificially induced on the labial surfaces of 9 primary incisors by way of a demineralisation protocol using 0.1 M lactic acid with 10% methylcellulose gel for 7 and 14 days. The specimens were scanned with microCT and CTAn software (Bruker, Billerica, MA, USA) was used to analyse the changes in DD and MD. Statistical analyses were performed using SPSS software (IBM, Armonk, NY, USA). Repeated analysis of variance (ANOVA) test and Pearson bivariate correlation were used and the level of significance was set at $P < 0.05$.*

Results: *The DD ranged from 0.00 to 0.99 μm (mean ± standard deviation [SD] 0.70 ± 0.43 μm) at baseline, 11.18 to 29.5 μm (18.15 ± 5.23 μm) at 7 days and 18.00 to 55.30 μm (34.20 ± 8.70 μm) at 14 days. The MD for all specimens (n = 9) ranged from 1.48 to 1.76 g/cm^3 (1.65 ± 0.08 g/cm^3) at baseline, from 1.47 to 1.74 g/cm^3 (1.62 ± 0.08 g/cm^3) at 7 days demineralisation and 1.33 to 1.72 g/cm^3 (1.54 ± 0.13 g/cm^3) at 14 days. There were statistically significant differences in DD ($P < 0.001$) and MD ($P = 0.016$) between different durations of demineralisation.*

Conclusion: *DD and MD change with time after being exposed to demineralising solution. MicroCT is a nondestructive method that allows repeated MD evaluations of the same sample.*

Key words: *demineralisation, lesion depth, microcomputed tomography, mineral density, primary teeth*

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It is well established that acid attack of tooth enamel can cause a reduction in mineralisation and mechanical strength. In the oral environment, demineralisation of the enamel surface is reversible through ions in saliva and the effect of fluoride[1]; however, there is a gap in knowledge regarding the remineralisation potential of different remineralisation agents relative to density change and demineralisation depth (DD). Thus, a laboratory model using microcomputed tomography (microCT) to assess changes in mineral density (MD) and DD may improve understanding of initial caries progression in primary teeth and how to treat them in a minimally invasive manner.

There is strong evidence that caries in the primary dentition is predictive of later caries experience[2]; however, primary teeth may be more susceptible to initial caries progression compared to permanent teeth for several reasons. First, primary teeth have much thinner enamel. The mean thickness of the buccal enamel on the maxillary central incisors is 0.787 mm for primary

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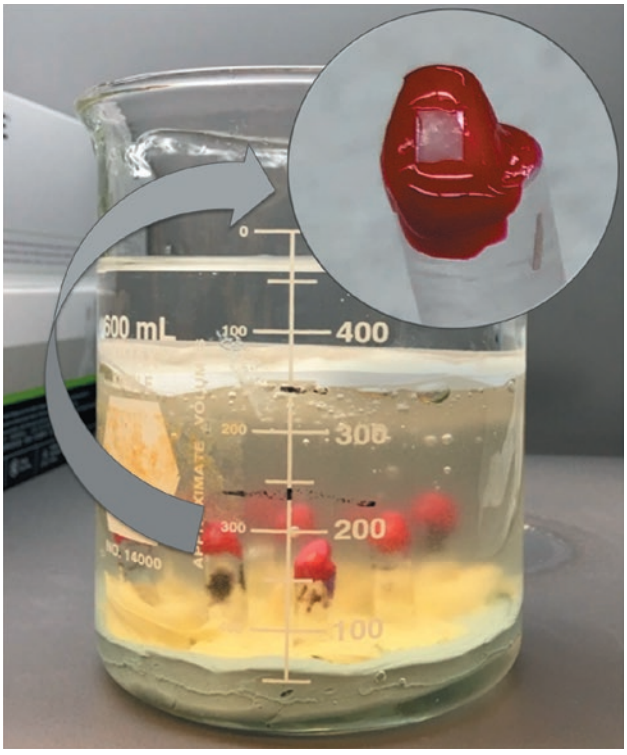

Fig 1 Experimental design. Mounted specimen in 10% methylcellulose gel and 0.1 M lactic acid on filter paper; insert: specimen mounted on acrylic rod with a 2 × 2 mm^2 exposure window.

teeth[3] compared to 0.945 mm in permanent teeth[4]. Second, primary teeth have a thicker aprismatic layer that exhibits low porosity[5]. Third, primary teeth exhibit far greater porosity overall, making them more prone to caries-like attacks[6]. Last, the interprismatic fraction and prism-junction density are also greater in primary teeth[6]. However, only one study has have evaluated enamel density changes after acid attacks related to early caries progression in primary teeth[7].

Laboratory demineralisation methods create standardised and reproducible caries lesions. Artificial caries have been created on primary teeth mainly using two demineralisation protocols. The most common methodof creating artificial caries lesions is using a demineralisation solution containing 2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 0.05 M acetic acid with the pH adjusted to 4.4 to 5.0[8]. The direct immersion of primary teeth samples in the solution for 4 days yields an artificial lesion of 60 to 200 µm in depth[9,10]. Another method involves using 8% methylcellulose gel and 0.1 M lactic acid. After 3 to 14 days, lesion depth of 110 µm was noted[11]. Both methods were proven to be reliable in other studies[12,13].

Despite the widespread use of light microscopy to measure demineralisation and remineralisation, its main limitations include the need to section the samples and the fact that the two-dimensional view does not enable measurement of changes in density[6]. However, several methods have also been used to measure caries depth, including microCT[10] and scanning electron microscopy (SEM)[14]. MicroCT is a modified version of medical computed tomography (CT) and is used to assess the mineral concentration at micron levels, thus differentiating between sound and carious dentine. The new, cutting-edge technique enables nondestructive visualisation of dental structures in 3D and provides an easy way to monitor the progression of caries lesions without destroying the samples[15].

The present study aimed to evaluate the change in DD and mineral density (MD) over time in primary teeth exposed to a demineralisation protocol as measured using microCT. The null hypotheses tested were:

1. There would be no difference in DD depending on the amount of time exposed to the demineralisation agent.
2. There would be no difference in MD depending on the amount of time exposed to the demineralisation agent.
3. There would be no correlation between MD and DD.

Materials and methods

This study was approved by the Institutional Review Board of Loma Linda University, CA, USA (IRB#5180417).

Specimen preparations

Extracted/exfoliated primary incisors (N = 9) without restorations or caries were collected and stored in 0.1% sodium azide solution. Enamel-dentine blocks with an approximate size of 4 × 4 × 3 mm^3 were cut using a water-cooled diamond-edged blade and mounted on acrylic rods with the labial surface facing upwards. The tooth surfaces were then painted with acid-resistant nail varnish (Maybelline, New York, NY, USA) to leave a 2 × 2 mm^2 window to be exposed to the demineralisation agent (Fig 1).

Demineralisation protocol

A well-established demineralisation protocol using 0.1 M lactic acid with 10% methylcellulose gel was used[11]. The tooth specimens mounted on rods were stabilised in a 600 ml beaker with polyvinylsiloxane. 10%

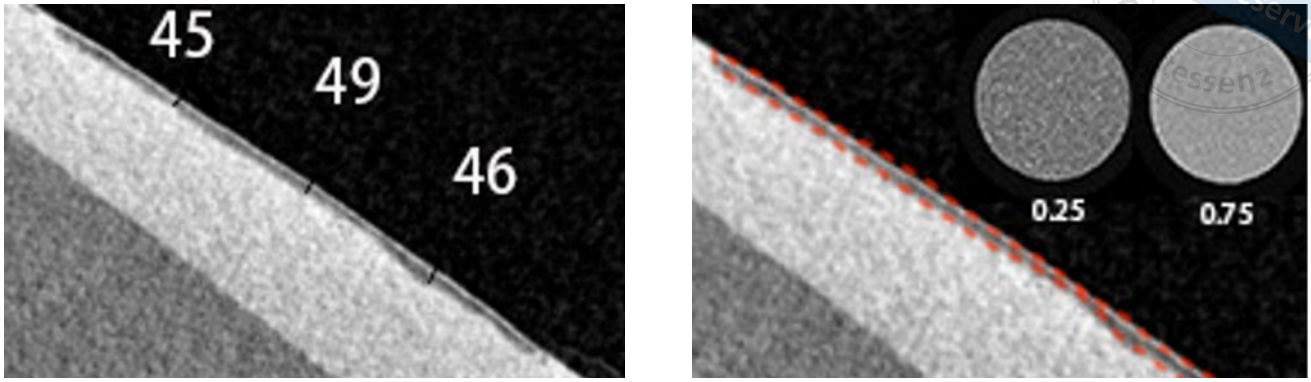

Fig 2 MicroCT images. **(a)** Lesion depth after 14 days of demineralisation; **(b)** region of interest set up (dotted line) for mineral density measurement and reconstructed images of phantoms at 0.25; 0.75 g/cm^3.

methylcellulose gel was poured onto the rods so that the specimens were covered with gel. The beaker was kept in the refrigerator for 4 hours for the gel to solidify. A filter paper was placed on top of the gel and 200 ml of 0.1 M lactic acid adjusted to pH 4.6 was poured over it (Fig 1). The specimens were kept for 7 days at 37°C then removed, rinsed with deionised water, kept in artificial saliva and scanned with microCT. This procedure was repeated for the 14-day measurements.

MicroCT parameters

A desktop radiograph microCT system SkyScan1272 scanner (Bruker, Billerica, MA, USA) was used to evaluate DD (μm) and enamel MD (g/cm^3) compared to the baseline measurements. Phase-contrast enhancement allowed object details as small as 4.0 μm to be detected. The enamel-dentine blocks mounted on acrylic rods were scanned with X-rays generated by a sealed microfocus X-ray tube (tungsten anode) at 85 keV and 100 mA with an integration time of 60 minutes. The samples were rotated over 180 degrees at rotation steps of 0.4 degrees. A 1.0-mm-thick aluminum filter was placed in front of the detector to remove low-energy X-rays. Scanning was performed under 100% humidity to avoid dehydration of the specimens, which were placed in a small container with wet gauze on top of them. A positioning jig was used for repeated measurements and precise superimposing of images.

Image analysis

Each specimen was reviewed and the parameters showing clearly defined margins and best contrast between enamel and dentine were recorded. When reduced noise and artificial defects in the images were observed, the critical value was chosen by one operator, then the mean of the values was calculated and the same parameters were used to reconstruct each image. Reconstruction of 800 to 900 images per specimen per scan was performed using NRecon software (Bruker). All images were reconstructed with a beam-hardening correction of 40%, smoothing of 2 and a ring artefact correction of 4. DataViewer software (Bruker), which provides tools for intensity-based image registration for both two and three dimensions, was used for visualisation of the image. The evaluated specimens served as their own controls for the assessments. The sagittal views of baseline, 7-day and 14-day images were superimposed using the 3D registration function in DataViewer. CTAn software (Bruker) was used to analyse the mineral concentration. Phantoms with different densities (0.25; 0.75 g/cm^3) were scanned with the same parameters and were used to calibrate the enamel density (Fig 2b). A linear calibration curve relationship between greyscale (linear attenuation coefficient, μ) and MD could then be generated from these phantoms. The calibration standards needed to fulfil basic requirements were that the X-ray attenuation must reflect the absorbance of the interested object and cover a representative range of MDs, and the calibration phantoms must be homogeneous at the spatial resolution of the scanner. After calibration, the images were analysed between baseline and after treatment to measure DD, and changes in MD were determined (Fig 2). The demineralisation area was separated from sound enamel and other tissue using the region of interest function (Fig 2b). DD was measured in the section from the middle of the nail varnish window. From that section, DD was determined by calculating the mean of the measurement from the one-third, one-half and

Table 1 Descriptive summary of DD and MD at different time points (mean ± SD).

Variable	Baseline	7 days	14 days	*P* value
DD, µm	0.70 ± 0.43[a]	18.15 ± 5.23[b]	34.2 ± 8.7[c]	< 0.001*
MD, g/cm^3	1.65 ± 0.08[a]	1.62 ± 0.08[ab]	1.54 ± 0.13[b]	0.016*

*Different superscript letters within rows indicate a significant difference determined by repeated measures ANOVA, $P < 0.05$.

two-thirds points of the window. MD was determined by the mean value of fifty sections from the middle of the nail varnish window.

Statistical analysis

Descriptive and inferential statistics were performed using SPSS Statistics 25 software (IBM, Armonk, NY, USA). Repeated analysis of variance (ANOVA) tests were used to evaluate the differences in DD and MD over time. A Pearson bivariate correlation was run to determine the relationship between MD at different times and DD and MD. The level of significance was set at $P < 0.05$.

Results

DD by exposure time to demineralisation agent

DD measured at different time points is summarised in Table 1. It ranged from 0.00 to 0.99 µm (mean 0.70 µm), 11.18 to 29.50 µm (mean 18.15 µm) and 18.00 to 55.30 µm (mean 34.20 µm) at baseline, 7 days and 14 days, respectively. There was a statistically significant difference between DD at the different time points as determined by repeated measures ANOVA (Pillai trace $P < 0.001$). The range and distribution of DD by time point are illustrated in Fig 3.

MD by exposure time to demineralisation agent

MD measured at different time points is summarised in Table 1. It ranged from 1.48 to 1.76 g/cm^3 (mean 1.65 g/cm^3), 1.47 to 1.74 g/cm^3 (mean 1.62 g/cm^3) and 1.33 to 1.72 g/cm^3 (mean 1.54 g/cm^3) at baseline, 7 days and 14 days, respectively. There was statistically significant difference in MD between the different time points, as determined by repeated measures ANOVA (Pillai trace $P = 0.016$). After multiple pairwise comparisons, there was a statistically significant difference between MD at baseline and 14 days ($P = 0.035$), whereas there was no difference between baseline and 7 days ($P = 0.654$) and 7 days and 14 days ($P = 0.089$).

Correlation between MD and DD

A Pearson bivariate correlation was run to determine the relationship between MD and DD. There was a strong negative correlation between them, which was statistically significant ($r = -0.406$, $P = 0.035$).

Discussion

The present study explored the applicability of microCT to evaluate changes in DD and MD in primary teeth after exposure to a specific demineralisation protocol. To the best of the authors' knowledge, this is the first study to use microCT and evaluate changes over time on the same primary teeth. Based on the results, we rejected our null hypotheses as there were differences in DD and MD depending on the length of time exposed to demineralisation agents. This supports that microCT methods can be used to detect changes in DD and MD over time in the same specimens, and can therefore be a valid and reliable method for future research into primary teeth.

For primary teeth, artificial caries depth ranged from 26 µm created by lactic acid with pH 4.5 by 7 days to 350 µm created by acetic acid with pH 3.9 and a soaking time of 10 days[16,17]. The main advantage of using a gel method is the indirect contact of the demineralisation solution facilitated by the gel that may simulate the oral environment that presents a pellicle or biofilm on top of the tooth structure. Compared to a previous study that recorded a mean DD of 36.1 ± 8.2 µm measured by transverse microradiography (TMR) and microscopy after 7 days' exposure to lactic acid and 6% hydroxyethyl cellulose with pH 4.5[16], the present results showed less lesion depth, namely 19.11 ± 5.49 µm after 7 days of demineralisation. This may be attributed to factors such as the use of a denser concentration of methylcellulose gel (10%) and immersion in a demineralising solution with a slightly higher pH (pH 4.6) in the present study. DD varied from previous studies due to different demineralisation protocols, length of soaking time and type of teeth used[16,17].

The MD of sound enamel of primary incisors in the present study (mean 1.65 ± 0.08 g/cm^3) was similar to a previous study measuring mandibular primary anter-

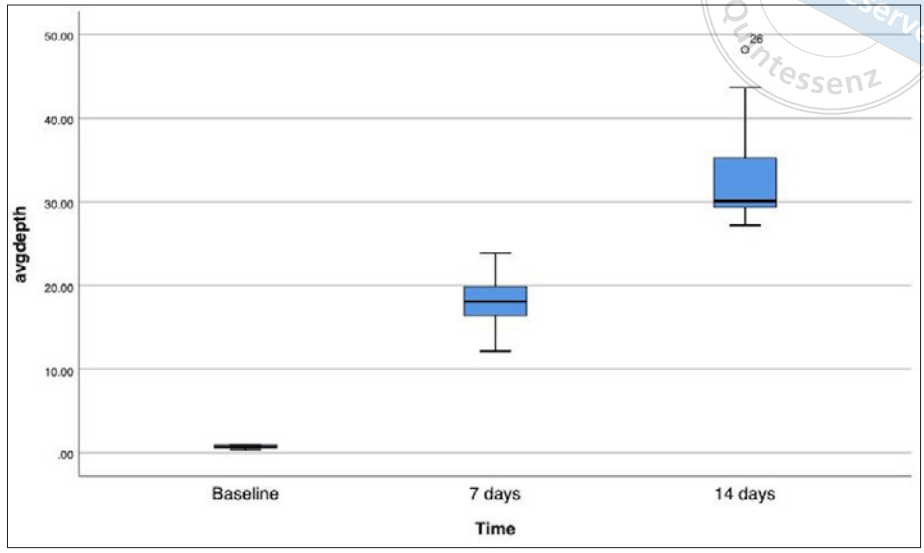

Fig 3 Changes in DD over time.

ior teeth using microCT (mean 1.68 g/cm^3)[18]. Kecuk et al[19] measured the MD of permanent premolars using microCT and reported a mean of 2.32 g/cm^3. This result was similar to other microCT studies measuring permanent premolars (2.43 g/cm^3)[20] and third molars (2.74 ± 0.19 g/cm^3)[21]. It is reasonable to expect higher MD for permanent teeth compared to primary teeth due to the increased porosity of primary tooth enamel[6]. Loss of MD of 1.85 g/cm^3 was measured using microCT from permanent premolars after 21 days' demineralisation with pH 4.5 lactic acid solution, containing 6% methylcellulose and 500 mg/l hydroxyapatite[20]. With a different demineralisation protocol with 2.2 mM $CaCl_2$, 2.2 mM KH_2PO_4 and 0.05 M acetic acid with pH 4.4, mineral loss of 1.38 ± 0.21 g/cm^3 was noted with permanent third molars after 96 hours of exposure[21]. The present results showed less MD loss (0.11 g/cm^3 after 14 days of demineralisation) compared to previous studies; this may be due to the demineralisation protocol involving use of methylcellulose gel for milder artificial caries progression.

It is noteworthy that previous studies only measured either DD or MD and none compared the two parameters. The present authors found a negative correlation between DD and MD. DD became deeper as caries progressed and the MD of the whole demineralised area decreased. As a result, progression of DD may predict loss of MD; however, the upper limit of DD in our study was 55.3 μm, so the correlation of DD and MD is not generalisable to depths beyond our upper limit.

Light microscopy is used widely to measure caries depth in many diagnostic studies of caries lesions[6,8]; however, it analyses 3D alterations using a two-dimensional method. As such, some characteristics can be lost during sample preparation, which can be particularly critical for analysis of caries depth[15]. TMR is considered the gold standard for determining the mineral content and MD change of dental enamel[22]. It is a reliable technique, but the cross-sectional sample preparation is difficult and makes it impossible to take repetitive scans of the same specimen[23].

MicroCT provides 3D images with no sample preparation required, and repeated scanning is possible, with the enamel surface of the lesion not being damaged during scanning[24,25]. From the 3D images provided by microCT, changes in depth, surface area, volume and density of the tooth structure can be calculated. The disadvantages of microCT are the long amounts of time needed to form a 3D model of each sample and the high cost of the process. Different settings of microCT scanners from different studies may affect the results. This is therefore a technique-sensitive method with a steep learning curve for image processing.

The present study has inherent limitations that warrant interpretation. Because no similar studies on primary teeth had been conducted previously, to the best of the authors' knowledge, the sample size was determined based on a pilot study. During the pilot study, specimen fracture was noted after prolonged microCT scanning; this may have been due to the dryness during the scanning time. Modification was done by placing a wet gauze over the specimens while scanning to maintain 100% humidity. Future studies should be conducted with a longer demineralisation time and with deeper

artificial caries lesions being analysed in both sagittal and horizontal directions; this would enable a 3D map showing caries progression over time to be created.

Conclusion

Within the limitations of this study, we concluded that the microCT method makes it possible to evaluate the MD of the same sample repeatedly and in a way that is comparable to the traditional method. DD increased with exposure time and MD changed over time after exposure to a demineralisation agent.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Ke Chung WU contributed to the conception and design of the study and drafted the manuscript; Dr Jung Wei CHEN contributed to the acquisition, analysis and interpretation of data; Dr So Ran KWON contributed to the conception and design of the study; Drs Jung Wei CHEN and So Ran KWON critically revised the manuscript for important intellectual content. All the authors gave their final approval of the manuscript for publication.

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