A Newly Designed Automatic pH-cycling System to Simulate Daily pH Fluctuations

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The aim of this study was to develop and evaluate an automatic pH-cycling system, which was intended for performing time-lapse analyses of dental caries. Two single-section specimens prepared from each of the five extracted human incisors were studied under a condition of 3- or 9-cycles/day for seven weeks. Increase of mineral loss ($\Delta\Delta Z$) and increase of Ld (ΔLd) were examined and compared at the end of every week during the study. In the 3-cycles/day condition, $\Delta\Delta Z$ and ΔLd significantly increased only at Week 7. In the 9-cycles/day condition, $\Delta\Delta Z$ increased significantly from Week 5. ΔLd increased from Week 2, and then there were no further increases for the subsequent five weeks. Based on the results of this study, progression pattern was found to be significantly different between the two conditions. We therefore concluded that the automatic pH-cycling system which we have developed qualified as a model system that simulated the human oral cavity. As such, it could be beneficially employed to clarify the roles of many factors that cause or prevent caries development in human teeth.

Key words : Automatic pH-cycle, Demineralization and remineralization, Time-lapse analysis

INTRODUCTION

Although many intrinsic and extrinsic factors are involved in the etiology and pathogenesis of dental caries, pH in the oral cavity is known to be one of the main factors. In particular, fluctuations in dental plaque pH after carbohydrate consumption exacerbate caries occurrence and severity. As shown by Stephan[1], the pH value following sucrose intake drops rapidly to its minimum level (which is below pH 5.0), and then over a period of 30 minutes to several hours, the pH value slowly returns to its original value (which is above pH 7.0). As a result, pH in the oral cavity is subjected to successive fluctuations between pH below 5 and then above 7 with each carbohydrate intake. Such a pH fluctuation occurs because metabolism of fermentable substrates by the plaque flora leads to acid production[2]; following which, acid dilution and/or neutralization occurs according to the flow rate of saliva and its buffering capacity[3].

It should also be highlighted that these pH fluctuations play a key role in de- and remineralization of teeth. This is because enamel dissolution rate is reduced when pH value is increased[4], and when pH is sustained above 5.5 both hydroxyapatite (HAP) and fluorapatite (FAP) are synthesized. Moreover, when pH is between 4.5 and 5.5, HAP is dissolved, but FAP synthesis is still in progress if fluoride exists[5].

Caries formation is a dynamic process that involves alternating periods of de- and remineralization of the teeth with pH changes. When the rate of remineralization exceeds the rate of ion transport out of the tooth surface, the surface layer can be retained with the continuous renewal. However, if demineralization is the predominant process over a defined period, or that the rate of demineralization exceeds the rate of remineralization, the net result will be a gradational loss of tooth minerals leading to irreversible cavity formation. Thus, the outbreak occurrence and progression of a caries lesion are associated with an imbalance between de- and remineralization. In various researches[6−9], protocols have been designed to investigate caries progression using a pH cycle in which the teeth were immersed alternately and periodically into two different pH solutions. Those studies indicated that the rate of demineralization process was highly dependent on the low pH and ion concentration of such ions as Ca^{2+}, PO_4^{3-}, and F^- of demineralization solutions[10,11]. Among the ions mentioned above, fluoride is known to have anticariogenic effect. As such, there are many studies concerning fluoride release from fluoride-containing materials[12−15].

However, most of the studies performed did not indicate the influence of demineralization-remineralization imbalance on the teeth in human oral conditions, as shown in Stephan's curve under changing pH. Therefore, in the clinical setting, a direct correlation between pH changes simulating oral cavity conditions and caries formation is still a matter of discussion.

For the purpose of observing caries occurrence

and progression, we have designed an automatic pH-cycling system. It could simulate the daily pH changes in the oral cavity, as in a Stephan's curve. The aim of this study, therefore, was to evaluate the newly designed automatic pH-cycling system, which was intended for performing time-lapse analyses of dental caries.

MATERIALS AND METHODS

Specimen preparation
Five extracted non-carious human incisors from different patients were used for the present study. The present study was approved by the Ethical Committee of the Hokkaido University Graduate School of Dental Medicine. Two longitudinal sections of 300-μm thickness were generated from each of the five teeth by means of a low-speed saw (Isomet, Buehler, USA) under water coolant. All sections were reduced to a thickness of approximately 100 μm by the use of ♯1000 and ♯2000 whetstones. After rinsing with distilled water, all surfaces of the tooth specimen, except for the original surface, were coated with Sticky Wax (Kerr, USA) dissolved in xylene (single-section specimen). Two single-section specimens from each tooth were divided into two experimental conditions: 3-cycles/day and 9-cycles/day. Therefore, each of these two groups had five specimens from the same five patients.

Automatic pH-cycling system
Fig. 1 shows our automatic pH-cycling system. This system was designed such that the fluid in a specimen-containing beaker could achieve the minimum pH 4.5 for demineralization and the maximum pH 6.8 for remineralization, using a method modified from Almqvist *et al.*[16]. Table 1 shows the components of the de- and remineralizing solutions used in this study.

These two experimental solutions were injected alternately into a small plastic beaker (10 ml) by two pump systems, resulting in a spontaneous outflow of the solution. The demineralizing solution flowed at 1000 ml/hour for two minutes followed by the remineralizing solution at 100 ml/hour for the next 60 minutes. During the whole experimental period, a magnetic stirrer was used to mix the fluid in the beaker to keep it at the desired pH value uniformly throughout the beaker. The pH value was monitored continuously every three minutes by a pH meter (ORION A290, ORION Research Inc., USA),

and recorded on a computer as text data using a data import software (ORION Data Collect for Windows, ORION Research Inc., USA). As for the infusion speeds of the two pumps, they were controlled by a programmable electronic timer with four channels. Duration period of each cycle was 62 minutes, and between cycles the specimens were left in the remineralizing solution. Fig. 2 shows the pH fluctuations under the two different experimental conditions within a day.
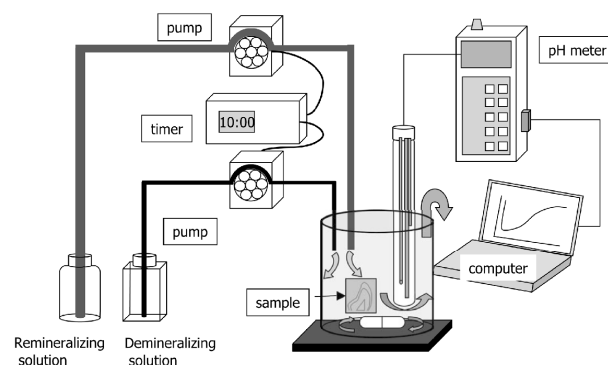

Fig. 1 Schema of the experimental instrument used for our pH-cycling system. Demineralizing and remineralizing solutions were pumped into a plastic beaker (10 ml) with two variable delivery pumps controlled by a programmable timer. The pH value of immersion fluid in the beaker was measured by a pH electrode connected to a pH meter and recorded by a personal computer at every 3-minute interval.
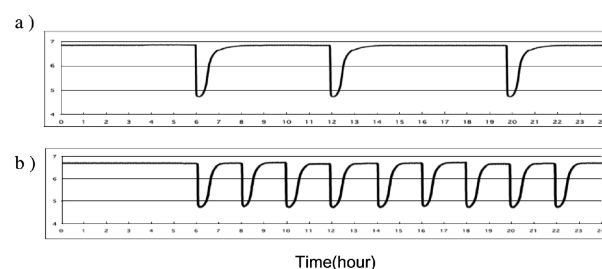

Fig. 2 24-hour pH fluctuation patterns of: (a) 3-cycles/day (at 6:00, 12:00, and 20:00 hours); and (b) 9-cycles/day (from 6:00 to 22:00 hours at one-hour interval period). Duration of each cycle was 62 min, and between cycles the specimens were left in the remineralizing solution.

Table 1 Components of demineralizing and remineralizing solutions used in this study

Solution	Composition	pH
Demineralizing solution	0.2 M Lactic acid, 3.0 mM $CaCl_2$, 1.8 mM KH_2PO_4	4.5
Remineralizing solution	0.02 M HEPES, 3.0 mM $CaCl_2$, 1.8 mM KH_2PO_4	6.8

Assessment of experimental system and evaluation of caries progression

To produce an artificial caries lesion, the single-section specimens were incubated in the system under a 3- or 9-cycles/day condition for seven weeks. For both groups, the transverse microradiography (TMR) image of each specimen was examined for caries progression. For each specimen, a series of microradiographs was made: before and after lesion formation at one-week intervals. After the radiography was performed, all specimens were placed again into the same beaker. Micrographs with aluminum step wedge using high-resolution films (type 1A, oc, Kodak) were obtained by means of a Softex X-ray system (CSM-2, Softex). Exposure time was 30 minutes at 10 kV, 3 mA, and with a focus-specimen distance of 44 mm. Microdensitometric image analysis of the developed X-ray films was then done with a system comprising a film scanner (DIMAGE san elite 5400, MINOLUTA, Japan) and a personal computer (Power Macintosh G5, Apple, USA).

For quantitative measurement of the same area in a series of microradiograph images of each specimen, enamel just adjacent to the EDJ (enamel-dentin junction) was superimposed as baseline for each image using Adobe Photoshop 7.0 (Adobe Systems Incorporated, USA) (Fig. 3). At three given areas of each sample, the average density of each 50-μm width at every 4.7-μm depth increment was obtained by NIH image. Mineral content profile was then calculated using the formula of Angmar[17]:

$$\text{Vol}\% = 50.48 \times t_a/t_s$$

where t_a is the thickness of the corresponding aluminum step wedge and t_s the thickness of enamel section. ΔZ (Vol%·μm) and Ld (μm) were calculated from the mineral content profile of each specimen before experiment commenced and at each experimental stage. $\Delta \Delta Z$ (Vol%·μm) and ΔLd (μm) then indicated the differences for ΔZ and Ld before and after pH-cycling, as shown in Fig. 4.
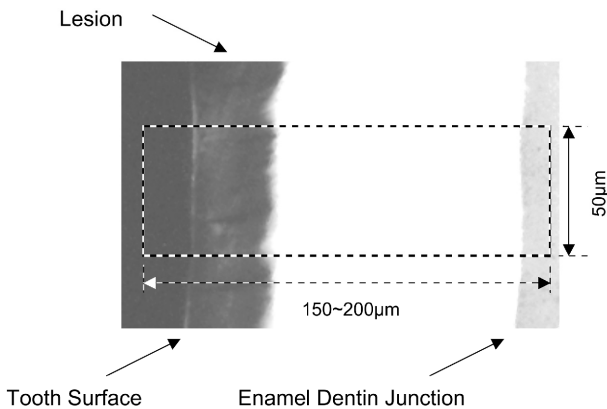
Statistical analysis

All statistical analyses were performed using SPSS Ver. 10.0. Increase patterns of ΔZ and ΔLd between each group were compared using a repeated-measures ANOVA (ANOVA). Differences in ΔZ and ΔLd between null stage and stipulated experimental stages during the study were analyzed using Dunnett's post-hoc test (Dunnet). For the same week in 3- and 9-cycles/day groups, ΔZ and ΔLd comparisons were also made using a paired t-test.

RESULTS

Automatic pH-cycling system

Fig. 5 shows the means and standard deviations of pH values in a specimen-containing beaker at the given times using our pH-cycling system. In this system, the demineralizing solution was infused at 1000 ml/hour for the first two minutes followed by remineralizing solution at 100 ml/hour for the next 60 minutes. The pH level dropped rapidly from 6.8
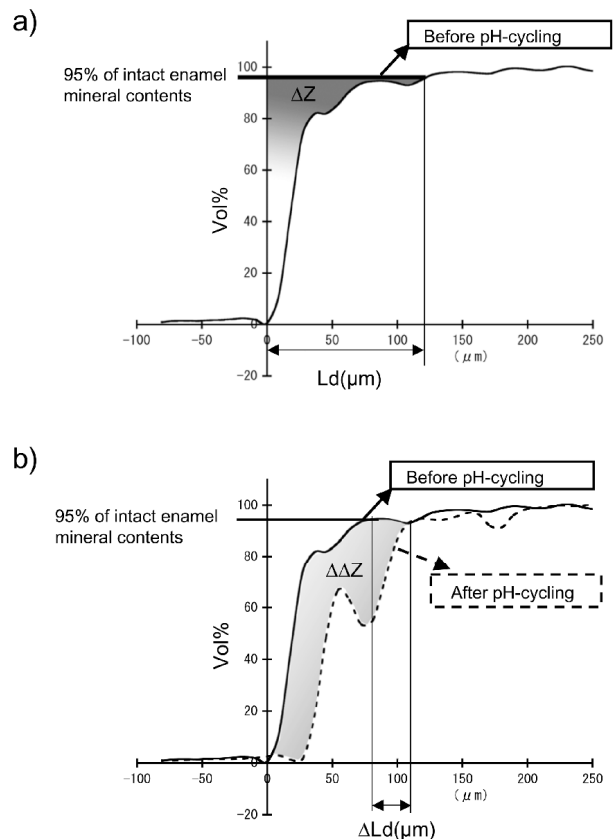
Fig. 4 (a) Schematic drawing of an average mineral content profile showing total mineral loss in the lesion (ΔZ) and the lesion depth (Ld); (b) Increase of ΔZ ($\Delta \Delta Z$) and increase of Ld (ΔLd), versus the data obtained before pH-cycling, were calculated at end of each week.

Fig. 3 Analysis area of a digitized radiographic image of enamel specimen containing a lesion and enamel-dentin junction.

to 4.5 during the first 4.5 minutes (SD: 1.2) and gradually returned to 6.8 at 67 minutes (SD: 2.5) after the start of infusion. As shown in Fig. 2, this pH changing pattern was similar in both groups regardless of cycling number — although pH fluctuations were observed to occur more frequently in 9-cycles/day group.

Caries progression pattern
All specimens were examined by the method described previously (Figs. 3 and 4). In the 3-cycles/day condition, subsurface lesion was observed at Week 7 in all samples. In the 9-cycles/day condition, surface-softened lesion and subsurface lesion were observed respectively during Weeks 5 to 6 and Weeks 6 to 7 in examined samples. From the increase pat-

terns of ΔZ and ΔLd, the caries progression pattern was found to be significantly different between the two conditions (ANOVA: $p < 0.001$) (Fig. 6).

In the 3-cycles/day condition, there were no significant increases in ΔZ and ΔLd for six weeks. Significant increases of ΔZ (0 to 3000 Vol%・μm) and ΔLd (0 to 40 μm) appeared only at seven weeks after the start of lesion formation (Dunnet: $p < 0.05$). In the 9-cycles/day condition, ΔZ increased significantly from Week 5 (Dunnet: $p < 0.05$) and increased further (Dunnet: $p < 0.05$) at Week 7. For ΔLd, a significant increase appeared at Week 2 (Dunnet: $p < 0.05$), and there were no further increases even with the experimental period extended by two more weeks. (For ΔLd, the experimental period was extended for two more weeks when a significant increase appeared. Nonetheless, there were no significant increases even with the extension of the experimental period.)

When the two conditions were compared, the values of ΔZ and ΔLd for 9-cycles/day condition were higher than those of 3-cycles/day condition. In particular, there were significant differences in both ΔZ and ΔLd between both conditions and between experimental periods at Weeks 2, 5, and 7 after the start of lesion formation (paired t-test: $p < 0.05$).

DISCUSSION

pH fluctuation after carbohydrate consumption is known to be one of the contributing factors to the etiology and pathogenesis of dental caries. Against this background, we designed an automatic pH-cycling system which could simulate the 24-hour pH changes in the oral cavity, such as in Stephan's curve,
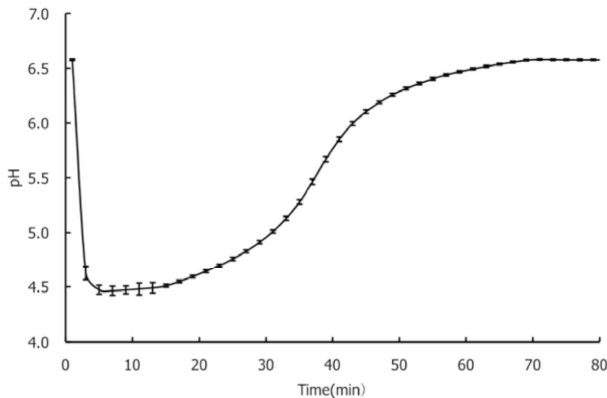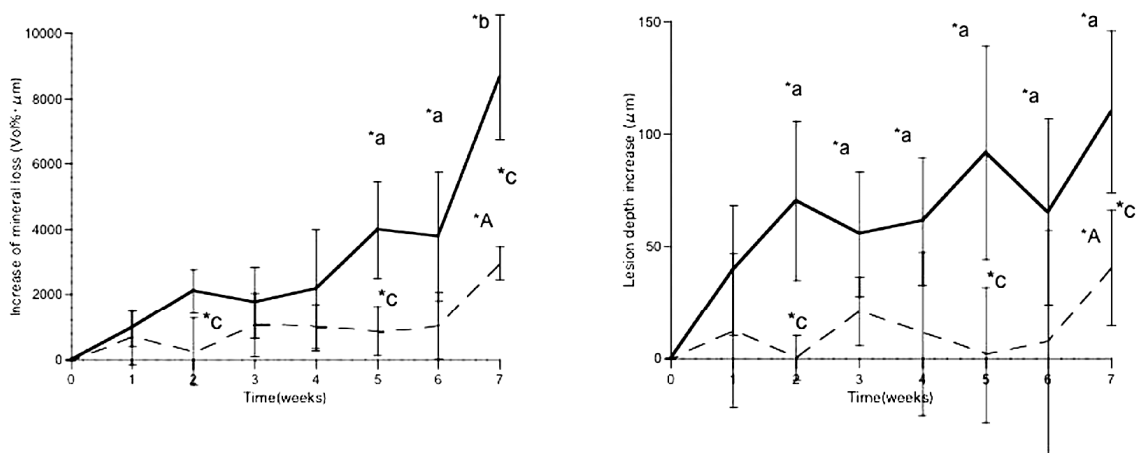

Fig. 5 pH variation-versus-time curve obtained at every 3-minute interval in our automatic pH-cycling model. Vertical bar represents standard deviation (n=5).

Fig. 6 Mineral loss increase ($\Delta \Delta Z$) and lesion depth increase (ΔLd) of two 3-cycles/day and 9-cycles/day groups. Means and standard deviations (vertical lines) are shown. Note the following:
- Dotted lines are for 3-cycles/day and solid lines are for 9-cycles/day.
- *a, *A: statistically different ($p < 0.05$) from the original stage (time 0).
- *b: statistically different ($p < 0.05$) from 5-6 week stage.
- *c, *C: statistically different ($p < 0.05$) between 3- and 9-cycles/day conditions at the same week.

for the purpose of observing caries initiation and progression.

In any ordinary lifestyle, the acid-attacking period after carbohydrate intake seems to be limited. This is because for the most part of a day, teeth are bathed in saliva of near-neutral pH such that minerals may be redeposited. In this connection, the influences of the duration and frequency of pH fluctuations should be considered. Almqvist et al.[18] reported on an automatic pH-cycling system simulating Stephan's curve, in which pH cycles were repeated 18 times per day. We have thus modified the pH cycle to that in the oral cavity to simulate pH changes that occurs in a daily lifestyle. In our study, we established two different situations to evaluate caries progression pattern in our system. With the 3-cycles/day condition, we intended to simulate an ordinary meal supply in a daily life. With the 9-cycles/day condition, we expected to see the effect of a more severe condition on tooth demineralization, although this would be an unusual condition in daily life.

In the 3-cycles/day condition, there were no significant increases in ΔZ and ΔLd for six weeks. This suggested that de- and remineralization would be in equilibrium under conditions of an ordinary daily lifestyle. However, at Week 7, significant increases appeared. This meant that when no protection against demineralization was available, demineralization would start eventually[19]. These results indicated that it was possible to maintain the equilibrium of de- and remineralization of teeth through an ordinary lifestyle with additional protection. In the 9-cycles/day condition, significant increases of ΔZ and ΔLd appeared at Week 5 and Week 2 respectively. It should also be highlighted that the values of ΔZ and ΔLd for 9-cycles/day condition were higher than those of 3-cycles/day condition throughout the experimental period. It is very understandable that in the 9-cycles/day group, a severe caries progression pattern and an aggressive demineralization process were observed. As a matter of fact, differences in caries progression between the two groups, thus reflecting the different lifestyles, were detected and captured by the automatic pH-cycling system.

Results of this study supported the notion that three variables are involved in caries initiation and progression. They are namely — frequency, duration, and reaction rate of pH variation at tooth-plaque interface in the oral cavity[20]. Thus, it would be useful to use our system to investigate the effect of a caries preventive strategy under a severe condition.

In our study, ΔZ and ΔLd of the single-section specimens were analyzed by TMR[21]. The key advantages of the single-section specimen procedure for analytical evaluation of tooth de- and remineralization were that the same specimen was employed continu-

ously, and that the lesion was confirmed by a visual approach.

In previous studies, samples were immersed in de- and remineralizing solutions at a given condition. Therefore, it would be difficult to compare their results with ours directly. In a study by Argenta et al.[22], they immersed the samples in a demineralizing solution for 3 hr/day for five days and observed a more significant mineral loss than ours. This was simply due to longer immersion periods in an acid solution. As for Damato et al.[23], they immersed the samples for 16 hr/day for five days. In their case, the mineral loss was less than that of Argenta et al.[22] and similar to ours. The difference between the two cases, Argenta et al.[22] and Damato et al.[23], could be attributed to the different Ca^{2+} and PO_4^{3-} concentrations of the immersion fluids. Kielbassa et al.[24] performed an in vivo study using human teeth which remained in the oral cavity for six weeks. Their obtained data of mineral loss (1337 ± 1546 vol%·μm) was similar to ours obtained at 3-cycles/day for six weeks (1046 ± 1023 vol%·μm). Furthermore, regarding the subsurface lesion and surface-softened lesion observed in our study, they were likewise observed in an in vitro study by Dijkman et al.[25].

In conclusion, although there are many intrinsic and extrinsic factors that lead to caries formation, this experimental system could evaluate the influences of these various factors. These factors included the frequency, duration, and reaction rate of pH variation on caries formation, as well as the various compositions of de- and remineralizing solutions. With the latter factor, issues such as saturation of calcium, phosphate, and fluoride ions on de- and remineralization of dental hard tissue will be examined in the future. Based on the results obtained in this study, this system qualified as a model system which simulated the oral conditions. As such, it could be beneficially employed to clarify the roles of many factors that cause or prevent caries formation in human teeth.

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