An *in vitro* study to assess the efficacy of OH^- ion superadded alkali-ion water in inhibiting caries and secondary caries

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Purpose: This study assessed the caries and secondary caries prevention efficacy of OH^- ion superadded alkali-ion water (AW) using an *in vitro* model.
Materials and Methods: Class 1 type cavities were prepared on bovine teeth blocks and they were immediately filled with Clearfil AP-X composite with application of an adhesive system. Biofilms were formed on the specimens using three species of cariogenic bacteria inside an oral biofilm reactor for 20 hours and specimens were further incubated separately for 7 days. During the incubation period the specimens were lightly rinsed with three pH levels of AW (AW-H: 11.5, AW-M: 10.5, and AW-L: 9.5) as the experimenting functional waters and control solutions (tap water; TW: 7.5 and heart infusion broth; HI: 7.3) dividing into five groups. The progression of the developed caries and secondary caries lesions were examined using a fluorescence microscope. Sizes of the lesions were measured by using an image analyzing software. Data were statistically analyzed by one-way ANOVA and Turkey's HSD methods.
Results: The caries and secondary caries were visible mainly in enamel at the edge of resin restorations in all specimens. Photomicrographs and data on image analysis clearly showed that the lesion size was smaller in AW samples compared to control samples, which was statistically significant when compared between AW-H and TW ($p<0.05$).
Conclusion: It was suggested that AW can be used to inhibit the progression of caries and secondary caries by adjusting the pH levels as required. **(Asian Pac J Dent 2011; 11: 1-8.)**
Key Words: alkali-ion water, glucan, secondary caries, *Streptococcus mutans*

Introduction

Biofilm-dependent oral diseases that include dental caries and periodontal diseases involve number of oral pathogens.[1,2] Among many types of bacteria that participate in the formation of cariogenic biofilms mutans streptococci, namely *Streptococcus mutans* and *Streptococcus sobrinus*, are acidogenic and considered to be the primary organisms that are responsible for dental caries.[1-4]

In recent years, resin composite restorative materials are becoming the most common indwelling medical devices used to restore decayed functional tooth structures and tooth restoration is a widely accepted dental clinical procedure.[5,6] Especially in minimally invasive restorations, direct resin composites have been widely used in clinical practice.[7] These types of resin composites also play a major role in operative, aesthetic, as well as prosthodontic treatments. However, one of the most frequently occurring reasons for replacement of direct resin composite restorations is secondary caries.[8-11] Secondary caries around restorations which require replacement of the restoration is a major concern in dentistry.[12] One of the major causative factors for the development of secondary caries is bacterial biofilm formation on the tooth structures and resin composite surfaces followed by invasion along the interface by damaging the tooth structures.[13] That may also cause as a result of wear or distortion of the restorations.

An artificial biofilm-induced secondary caries model has been established recently[14,15] by using an oral biofilm reactor (OBR)[9,16,17] which has been used to study oral biofilm formation on tooth structures and different dental materials *in vitro* by simulating the human oral environment.[18] The study model was established to provide a better understanding of the secondary caries development around resin composite restorations and to

assess the efficacy of various antibacterial agents on caries or secondary caries inhibition. One of those studies reported that the model secondary caries developed using the OBR were clearly visible by 7-days time.[14] Therefore, preclinical information can be obtained using this study model within very limited period of time.

A functional-water, commonly known as "alkaline water/ alkali-ion water (AW)", being used as drinking water contains a high concentration of OH^- ion and it was reported that AW dissolve cariogenic bacteria induced water insoluble glucans (WIG).[19]

At present, there is almost no available research report that evidently demonstrates the efficacy of AW in preventing caries or secondary caries. With a view to obtain more information on these issues, AW and secondary caries model were used in this study. Therefore, the purpose of this study was to assess the efficacy of AW in inhibiting the progression of artificial caries and secondary caries in bovine teeth using an OBR.

Materials and Methods

Solutions

Three different pH levels of AW: AW-H (pH11.5), AW-M (pH10.5) and AW-L (pH9.5) were produced using a water electrolysis device (TK7705, Panasonic Electric Works Co., Ltd., Osaka, Japan). Tap water (TW) and a solution of Heart Infusion broth (HI, Becton Dickinson, Sparks, MD, USA) with sucrose (1% final concentrations) were used as a control solution. The pH level of TW was 7.5 and that of HI was 7.3 on average.

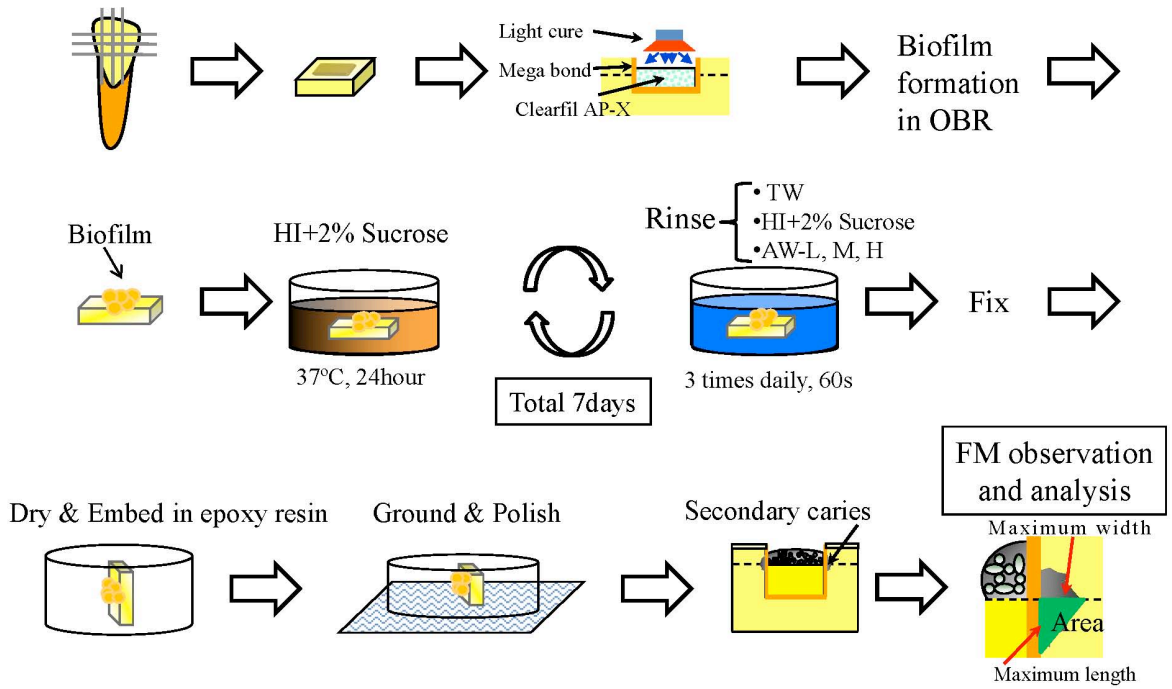

Fig. 1. Schematic diagrams sequentially show the specimen preparation, secondary caries induction and observation using a fluorescence microscope (FM). Class-1 type cavity prepared, immediately restored with resin composite followed by mutans streptococci biofilm formation inside an OBR and then rinsed 3 times daily with the solutions during final 7-day incubation period. Zone of measuring the maximum width, the maximum length and area of a lesion is indicated.

Tooth block preparation

Bovine lower incisor teeth were used to prepare square shaped dentino-enamel blocks in this study. Tooth blocks (approximately 5×4×4 mm^3) were prepared from mid-labial parts by cutting with a low speed diamond

saw (Isomet, Buehler, Lake Bluff, IL, USA) under running water as coolant. The convex enamel surfaces on the outermost buccal slices were reduced up to 0.5 mm by gently polishing on an 800-grit silicone carbide paper under running water to prepare a flat enamel surface and then polished with water based diamond paste of 0.25 μm. Finally, the blocks were cleaned with deionized water (Milli-Q water, Japan Millipore Corp., Tokyo, Japan) ultrasonically cleaned for 5 minutes to remove the remaining grit left during polishing. The blocks were rinsed with Milli-Q water three times for two minutes each dried accordingly and enamel surfaces were coated with nail varnish (Fig. 1).

Cavity preparation and composite restoration

Class 1 cavities (3 mm long, 2 mm wide, 2 mm deep) were prepared in specimens using a diameter 0.8-1.0 mm regular-grit tapered bur (ISO #170, Shofu, Kyoto, Japan) mounted in a milling machine (Cendres & Metaux SA CH-2501, Biel-Bienne, Switzerland) and cavity surfaces were finished with a straight bur (ISO #109, Shofu) in fixed in the milling machine under water coolant. Cavities of the specimens were filled with a two-step self-etching primer/adhesive system and a-light-cured hybrid composite (Clearfil AP-X). The cavities were not filled completely, keeping about 0.8 mm deep unfilled space for biofilm accumulation at the top, and the adhesive was light-cured for 10 s using a conventional halogen light curing unit (XL3000, 3M-ESPE, Minneapolis, MN, USA). The cured adhesive surface was then carefully filled with a hybrid restorative (Clearfil AP-X, shade A3) and light-cured for 40 s (Fig. 1).

Restorative materials

A two-step self-etching adhesive materials (Clearfil SE Bond) and a hybrid composite (Clearfil AP-X) were used in this study. The lot numbers and chemical compositions of the materials are listed in Table 1 according to the information provided by the manufacturer (Kuraray Medical, Tokyo, Japan).

Table 1. Materials used in the present study

Material	Lot number	Composition	Procedure	Manufacturer
Clearfil Mega Bond	00927A	Primer: HEMA, MDP, Hydrophilic dimethacrylates, Photo initiator, Water	Apply 20 s, Gentle air-blow	Kuraray Medical Inc., Tokyo, Japan
	01371A	Bond: Bis-GMA, MDP, HEMA, Photoinitiator, Filler	Apply 15 s, Mild air-blow, and light-cured 10 s	
Clearfil AP-X	01213A	Bis-GMA, TEGDMA, Photoinitiator, Barium glass, Silanated colloidal silica (filler content 85.0 wt%)	Light-cured 40 s	Kuraray Medical Inc.

HEMA, 2-hydoroxyethyl methacrylate; MDP, 10-methacryloyloxydecyl dihydrogen phosphate; Bis-GMA, bisphenol-A diglycidyl methacrylate; TEGDMA, triethylene glycol dimethacrylate

Preparation of bacterial suspensions

Suspensions of *Streptococcus mutans MT8148* (*S. mutans*), *Streptococcus sobrinus 6715* (*S. sobrinus*), and *Streptococcus gordonii ATCC10558* (*S. gordonii*) in phosphate buffered saline (PBS) at an optical density 500 nm (OD_{500}) of 2.5 were prepared from a 16-hour fresh cultures in brain heart infusion broth (Becton Dickinson, Sparks, MD, USA) after washing three times with PBS. The suspensions were stored at 4˚C with continuous gentle stirring until use.

Growing biofilm on the specimens inside OBR

Artificial *S. mutans*, *S. sobrinus*, and *S. gordonii* biofilms were grown on each of the Class-1 cavity filled with resin composite material. Four specimens from each group were placed on a Teflon holder around a flat bulb pH electrode of the OBR by using red utility wax (GC, Tokyo, Japan) keeping the top of each cavity open for biofilm accumulation. The Teflon holder bearing the specimens set through the bottom opening of the chamber

by a silicon plug. Pooled sterile saliva was then poured on the specimen surfaces and incubated for 30 minutes to obtain a coating of salivary pellicle. The chamber encircled by a water jacket was sealed with another silicon plug fitted with five stainless steel tubes (21gauge) so that the chamber itself served as an incubator with a 37ºC inner temperature. The other ends of the five stainless steel tubes were connected to silicon tubes passing through peristatic pumps regulated by a computer operated controller (EYALA EPC-2000, Tokyo Rika, Tokyo, Japan). One of them was used to collect the mixed suspension of *S. mutans, S. sobrinus*, and *S. gordonii*, two to collect sucrose added HI and the other two to collect PBS from the prepared stock as described above. All of these liquids form water domes which are mixed by the force of gravity exerted from the falling liquid drops on the holder and are diffusely distributed over all of the specimens. After 20 hours incubation of the biofilm in the OBR chamber, each specimen with artificial biofilms was removed from the Teflon holder in the OBR. The specimens with the undisturbed biofilms were inoculated for further 7 days to observe the effects of AWs in inhibiting the progression of the secondary caries lesions. A 24-well culture plate (Corning Inc., New York, NY, USA) was used to keep all the specimens in separate wells to incubate at 37ºC and sucrose added HI was supplemented by changing on every alternate day.

Rinsing with the solutions

During the 7-day incubation period in HI, specimens were separately rinsed with AW-H, AW-M, AW-L, TW and HI (n=4 in each group) for 20 s three times daily. In this study, a Water Bath Shaker (Taitec, Personal-11, Tokyo, Japan) was used with a moderate stroke of 10 Hz for 20 s three times with an interval of 1 minute each to simulate the gargling condition of an adult.

Fluorescence microscopy (FM)

Morphology of the lesions developed at resin-enamel interface was investigated by using a fluorescence microscope (FM) followed by image analysis of the lesions. In order to detect and measurement of the secondary caries lesions, after the incubation period, the samples were rinsed with PBS buffer and fixed in 4% paraformaldehyde with 1% glutaraldehyde in PBS for 1 hour. The samples were rinsed with PBS three times for 2 minutes each, and finally rinsed with Milli-Q water three times for 20 minutes each and dried. Following these procedures, the samples were embedded in a self-curing epoxy resin (Epon 815, Nissin EM, Tokyo, Japan), subsequently ground to expose in the longitudinal direction with 800-grit silicon carbide paper under running water and finished with diamond pastes down to 0.25 μm particle sizes. The samples were cleaned with Milli-Q water applying mild ultrasonication in between each step for 10 minutes and finally dried. Secondary caries lesions at the interfaces were evaluated with an inverted fluorescent microscope (Olympus CKX41, Olympus Imaging Corp., Tokyo, Japan) and imaged using a DP70 system with the aid of DP Manager Software (Olympus). In order to observe blue fluorescent BP460-490C filter was used and that produced green micrographic pictures on the PC. Images were taken at ×40 and ×100 magnifications after setting a scale bar each time. Maximum length (from the edge of the restoration to deepest point of the lesion) and maximum width (from the edge of the restoration to widest point of the lesion) were directly measured using the DP70 system and Manager. Later, area of the lesions was measured using ImageJ V.1.34 software[20] utilizing the scale bar on each image as reference for calibration. Only this part of a lesion that remained adjoined with resin considered as the secondary caries lesion (Fig. 1).

Statistical analysis

All numerical data was analyzed using the Statistical Package for the Medical Science (SPSS Ver.11 for

Windows, SPSS Inc., Chicago, IL, USA) for statistical procedures. The number of specimens was four in each group (n=4) and the experiments were repeated three times under the same conditions to ensure reproducibility. The data for maximum width, maximum length and area of secondary caries lesions were analyzed by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test with a confidence level of 95%.

Results

FM images clearly displayed development of caries and secondary caries like lesions in all groups with remarkable morphological patterns loosing green fluorescence and appearing brighter (Fig. 2). Lesions entirely were remarkably large in TW and HI specimens although the incubation was only for 7 days. On individual observation of each of the FM image followed by group-wise comparison and data on image analysis (Fig. 3) it appeared that the secondary caries like lesion size was smaller in AW-H and AW-M treated specimens compared to AW-L, TW, HI treated specimens. AW-H displayed statistically significant differences ($p<0.05$) with TW and HI specimens (Fig. 3a and 3b).

Demineralization along the cavity wall extended most (length of the lesion) in TW specimens, and that extension was shortest in AW-H specimens (Fig. 3b). However, there were no statistical significant differences when compared between the groups. The width of the demineralized enamel lesion extended most in HI specimens compared to all other specimens; only in AW-H and AW-M specimens showed significantly smaller lesion size ($p < 0.05$) compared to TW and HI specimens (Fig. 3b).

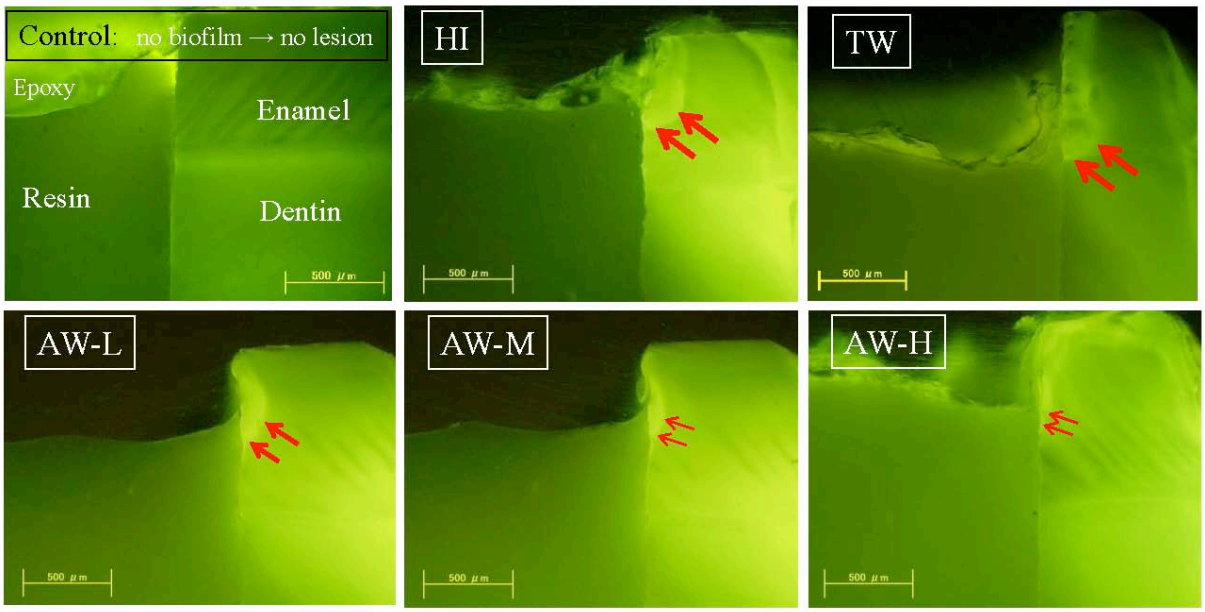

Fig. 2. Fluorescent photomicrographs represent typical secondary caries lesions from each group. A 'Control' specimen without any lesions and the experimental specimens; HI and TW (upper panel), AW-L, AW-M and AW-H (lower panel) are seen. Large-sized lesions (indicated by large red arrows) are seen in HI and TW specimens. In AW-L, AW-M and AW-H specimens the size of the lesions appeared to be reducing with increase of pH level (indicated by small red arrows).

Discussion

Currently, a good number of electrolyzed waters including AW are either being used or being investigated for medical and dental purposes.[21] AWs that were produced by electrolyzing tap water in the present study

contained high concentrations of OH^- ion which showed resemblances with NaOH;[22] specifically in disintegrating the WIGs of cariogenic biofilms as reported previously.[19] In this study, the direct effects of three pH levels of AW after application on a secondary caries model were investigated. Both morphologically and numerically AW displayed the efficacy of inhibiting the progression of enamel demineralization and that was more effective with the increase of pH level. It is unlikely to achieve remineralization of enamel lesions once induced by the acid attack from the biofilms on application of AW according the experimental conditions in this study. In contrary, there would be inhibitory factors that either prevented or slowed down the progression of demineralization of the enamel on first place.

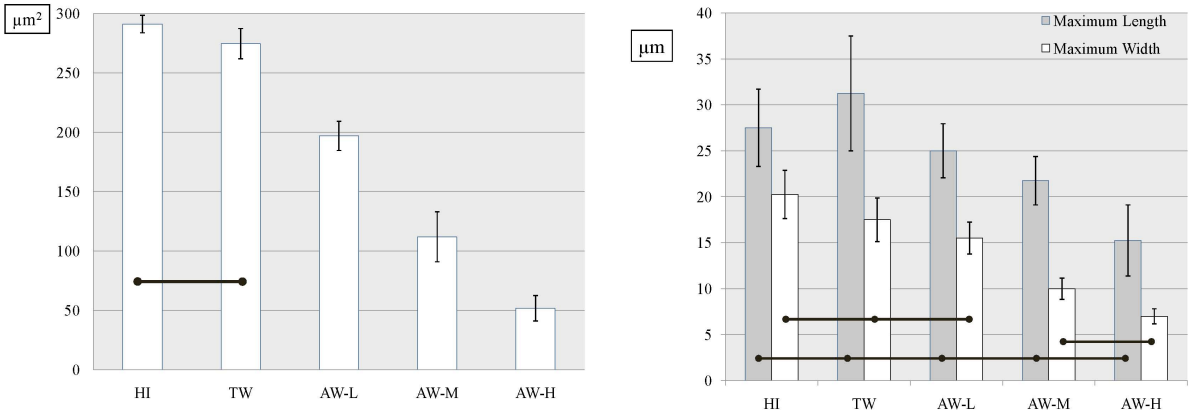

Fig. 3. **a** **b**
Graphs represent the size of the secondary caries lesions; a) area and b) maximum length and maximum width. Horizontal lines indicate no significant differences, otherwise found significantly different according to one-way ANOVA and Tukey's HSD test ($p<0.05$), n=4 in each group. Horizontal lines indicate no significant differences.

S. mutans and *Streptococcus sobrinus* (*S. sobrinus*) are the micro-organisms most strongly associated with dental caries.[4] Their cariogenicity is largely determined by their ability to adhere to tooth surfaces and the production of water insoluble glucan (WIG) resulting in subsequent formation of dental plaque or biofilm.[23,24] These two species of mutans streptococci produce at least seven extracellular glucosyltransferases (GTFs) that convert sucrose into glucan.[25] *S. mutans* also produce acid that may cause demineralization. *Streptococcus gordonii* (*S. gordonii*) is another species that also plays some role in the synthesis of WIG.[26]

Mutans streptococci promote tooth decay through the ability to adhere, production of extra-cellular matrix (biofilms) and production of large amounts of acids. Biofilm formation occurs through the synthesis of water-soluble and water-insoluble glucans via the catalyzing activity of the glucosyltransferase enzymes (GTF).[23,25] Glucan is believed to be the major factor contributing to the ability of mutans streptococci to adhere to the tooth surface and for aggregation of the bacterial cells within a biofilm. The ability of mutans streptococcal GTF to synthesize WIG from sucrose is an important virulence factor in initial caries development, potentially by increasing the colonization ability of infecting cariogenic bacteria and by influencing the diffusion characteristics of dental plaque.[24] Synthesis of WIG is known to be one of the most important virulence properties of cariogenic bacteria that contributes to the development of mature biofilm and the potency of AW on disintegrating WIG were understood in a previous study.[19] Therefore, the 20-hr biofilms formed on the restored specimens in the present study might have been disintegrated similarly on AW application, so that the existing bacteria were not capable enough to produce acids. Also, AW itself may have contributed in changing the acidic atmosphere to a non-acidic one by delivering the available OH^- ions.

AW is being used as drinking water that contains a high concentration of OH^- ion usually produced by the electrolysis of ordinary tap water. The Japanese Ministry of Health and Welfare has certified that alkaline electrolyzed water assists in the alleviation of gastrointestinal disorders, acidosis, chronic diarrhea, and poor digestion in 1960.[27,28] Hence, the possibility of intra-oral use of AW for caries and secondary caries prevention thought to be wide open and efficacy of OH^- ion superadded AW was evaluated in this study.

Since the prevalence of primary caries is on the decline worldwide, secondary caries remains an unresolved problem in restorative dentistry. It was expressed that extensive experimental and clinical researches are needed to develop suitable prevention approaches for secondary caries expression and to know the complex interaction among the restoration, the oral environment and the tooth surface.[12,13] The development of an OBR made it possible to create an in vitro secondary caries model induced by cariogenic streptococci on the enamel wall at composite resin interface. In the present study, similar types of carious lesion formation on the exposed tooth surfaces around the composite resin restorations were observed as reported previously.[14] The caries lesions were clearly visible at the interface of restorations by FM in all specimens due to the loss of green fluorescence in both enamel and dentin. For instance, no detectable lesion was visible in a control specimen without biofilm formation (Fig. 2). In order to permit accurate quantitative analyses of the secondary caries size, we measured maximum width and maximum length of the outer lesions using the FM images at low magnification (×40).

In vitro caries model allows the simultaneous production of primary and secondary caries-like enamel lesions in a considerable number of specimens, and facilitates the possibility to manipulate and transfer plaque and tooth without necessarily terminating the experiment.[29] In all groups the length of the lesion was larger than the width indicating easier acid penetration along the enamel-resin interface rather than widely spreading into the enamel structure. Expectedly, the sizes of secondary caries lesions in AW-H and AW-M groups were smaller than that of AW-L, TW and HI groups. That might also be because OH^- ion can interfere the adhesion of biofilm on resin composite and enamel surfaces.

In this study, the results indicated that AW with pH levels above 11.5 found to be significantly effective in inhibiting the progression of demineralization. AW with 10.5 pH level was also found to be effective. The results suggested that higher OH^- ion concentrations may have disintegrated more glucan by a mechanism similar to that of NaOH.[30] In addition, it can be considered that more pathogenic bacteria along with the disintegrated WIG may have been detached by AW than that of TW or HI resulting in less acid production and smaller sized lesion production. Therefore, it is anticipated that simple gargling with alkali-ion water may effectively prevent dental caries and secondary caries in human oral cavity. It is suggested that AW can be used to inhibit the progression of caries and secondary caries and also as prophylactic therapy by adjusting the pH levels as required.

Acknowledgment

This work was supported by the Global Center of Excellence Program; International Research Center for Molecular Science in Tooth and Bone Diseases at Tokyo Medical and Dental University, Tokyo, Japan.

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Accepted December 4, 2010.

Copyright ©2011 by the *Asian Pacific Journal of Dentistry*. Online ISSN 2185-3487, Print ISSN 2185-3479