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The in vivo Inhibition of Oral Biofilm Accumulation and *Streptococcus mutans* by Ceramic Water

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Keywords
Biofilms · Ceramic water · Dental caries · Dental plaque · *Streptococcus mutans*

Abstract
Combustion-synthesized titanium carbide ceramics uniformly disperse silver, producing silver ions and hydroxyl radicals in water. This generates antimicrobial activity against various bacteria. One such bacterium is *Streptococcus mutans*, a gram-positive anaerobic bacterium known as a major pathogen of dental caries. In this study, we analyzed the inhibition of oral biofilms and *S. mutans* by ceramic water in in vitro and human studies. *S. mutans* strains showed significantly lower antimicrobial and sucrose-dependent adhesion activity in the presence of ceramic powder compared with untreated culture medium. Confocal microscopy revealed that *S. mutans* biofilm structures with ceramic powder were thin and coarse. Twenty-seven volunteers (13 males, 14 females; 18–37 years old, mean 25.2 years) were enrolled for subsequent studies. After each meal, one group was asked to rinse with ceramic water while the other rinsed with untreated water for 1 week. After 1 week, the rinsing contents were switched between the groups and the same protocol was followed for an additional week. After rinsing with ceramic water, the average plaque score was 43.0 ± 3.7, which was significantly lower than the baseline value (74.1 ± 5.7, $p < 0.001$). However, no significant difference was observed when rinsing with untreated water. In addition, the total number of *S. mutans* in saliva was significantly reduced after rinsing with ceramic water compared with untreated water ($p < 0.05$). These results suggest that ceramic water possesses antimicrobial activity against *S. mutans* and inhibits its biofilm formation. Rinsing with ceramic water can also inhibit dental plaque formation and *S. mutans* colonization in humans.

Streptococcus mutans has been implicated as a primary causative agent of dental caries in humans [Hamada and Slade, 1980]. In the oral cavity, *S. mutans* produces acid and synthesizes water-insoluble glucan in the presence of sucrose, allowing the bacterium to decalcify and firmly

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adhere to the tooth surface [Mukasa et al., 1989]. Furthermore, adherent *S. mutans* forms biofilms, contributing to dental caries development [Hamada et al., 1984].

Several food products for reducing dental caries have been reported such as sugar substitutes and polyphenols contained in fruits and vegetables [Smith et al., 2002]. Furthermore, polyphenols from drinks such as coffee, tea, and wine have also been shown to inhibit the growth of oral bacteria [Matsumoto et al., 1999; Signoretto et al., 2010]. In addition, many antiplaque agents such as mouth rinses and toothpastes have been developed [Smith et al., 2002]. Though such food products and antiplaque agents contribute to the reduction of dental caries risk, which leads to the reduction of decayed, missing, and filled teeth, especially in developed countries, the elimination of dental caries is quite difficult. Thus, dental caries products that have a strong preventive effect and are convenient as well as safe to use must be developed.

Recently, artificial materials originally derived from engineering approaches have been applied to inhibit bacterial growth in medical and dental fields [He et al., 2015]. Combustion-synthesized porous titanium carbide ceramics (TiC), which contain titanium, carbon, and silver (Ag) in a ceramic pellet, uniformly disperse Ag. Ag ions dispersed from TiC ceramics are obtained by heating the ceramic pellet at extremely high temperatures (3,000 K), a process referred to as "combustion synthesis." With water contact, Ag-dispersed TiC ceramics generate hydroxyl and methyl radicals, Ag ions, and microbubbles, all of which exhibit antimicrobial activity against various bacteria.

In the present study, we analyzed the inhibitory effects of ceramics against *S. mutans* as a novel, simple, and safe approach for dental caries prevention. First, we analyzed the inhibitory effects of ceramics against the cariogenicity of *S. mutans* strains in vitro. Subsequently, we analyzed the effects of rinsing with ceramic water on the inhibition of dental plaque accumulation in humans.

Subjects and Methods

Ceramic Preparations

Porous TiC ceramics dispersed with Ag were produced by combustion synthesis. The starting materials, titanium, carbon, and Ag (mixing ratio of 16 wt%), were combined and press-molded into a ceramic pellet. Ignited by point heating the pellet, a combustion synthesis reaction was propagated spontaneously through the sample, and Ag-dispersed TiC ceramics were obtained within seconds (Fig. 1a). The ceramic pellet contains a porous microstructure, superior conductivity, and covalent character (Fig. 1b). A powder form was obtained by milling the ceramic pellet (Fig. 1c). Suspended water was prepared by adding "ceramic powder" into water, and ceramic water for rinsing was prepared by placing a "ceramic pellet" into water for over 8 h.

Characteristics of Ceramic Water

Combustion-synthesized nonoxide TiC ceramics were radically heat-treated (heated up to over 3,000 K and cooled down to below 1,000 K within a few microseconds). Thus, quenched TiC ceramics contained many anode and cathode spots that constituted a battery. When TiC ceramics were added to water in an SiO_2 bottle, H_2O dissociated into hydrogen and hydroxyl radicals. Ag was ionized as Ag^+ at a saturated concentration of around 100 ppb in water (Fig. 2a) when 100 mg of the ceramic powder or 1 ceramic pellet was added to 1 L of water in an SiO_2 bottle. Methyl radicals were also formed by the reaction of hydrogen radicals and carbon. Two kinds of radicals were detected by electron spin resonance spectroscopy, whereas no radicals were detected in water in the SiO_2 bottle when ceramics were not added (Fig. 2b).

S. mutans *Strains*

S. mutans strains MT8148 (serotype *c*) [Ooshima et al., 1983] and SA31 (serotype *k*) [Nakano et al., 2008] were selected from the stock culture collection in our laboratory. Strains were confirmed to be *S. mutans* based on their biochemical properties and observation of rough colony morphology on Mitis Salivarius agar (Difco Laboratories, Detroit, MI, USA) plates containing bacitracin (0.2 U/mL; Sigma-Aldrich, St. Louis, MO, USA), and 15% (w/v) sucrose (MSB agar). *S. mutans* strains were cultured in brain-heart infusion (BHI) broth (Difco Laboratories) at 37°C for 12 h and used in subsequent studies.

Antimicrobial Activity

The antimicrobial assay was performed according to methods described previously with some modification [Sasaki et al., 2004]. Cultures of *S. mutans* strains at the stationary growth phase were collected by centrifugation at 3,000 *g* for 10 min. The cultures were washed and resuspended in sterile distilled water or phosphate-buffered saline (PBS) to reach an optical density at 550 nm (OD_{550}) of 1.0. Bacterial suspensions were incubated at 37°C at different time intervals, specifically 1.5, 3, 6, and 12 h, with or without ceramic powder. The ceramic concentration was adjusted to 1, 10, and 100 ppb. Bacterial suspensions were then streaked onto Mitis Salivarius agar plates supplemented with 15% (w/v) sucrose and 0.2 U/mL of bacitracin and anaerobically cultured at 37°C for 48 h. The numbers of colonies were counted after identifying the characteristic colonial morphology of mutans streptococci. The bacterial survival rate was calculated by reading OD_{550} as follows: (OD_{550} with ceramics at each examined time point)/(OD_{550} without ceramics at each examined time point) ×100.

Sucrose-Dependent Adhesion

Sucrose-dependent adhesion to a glass surface was analyzed by culturing the tested strains in BHI broth containing 1% sucrose as previously described with some modification [Nakano et al., 2005]. Briefly, the tested strains were cultured in BHI broth or 1/10 concentration of BHI broth containing 1% sucrose at 37°C for 18 h at a 30° angle. If necessary, ceramic powder was added at a final concentration of 100 ppb. After incubation, the culture tubes were vigorously vibrated with a vortex mixer for 3 s and nonadhesive cells were transferred to fresh tubes. Cells remaining on the glass

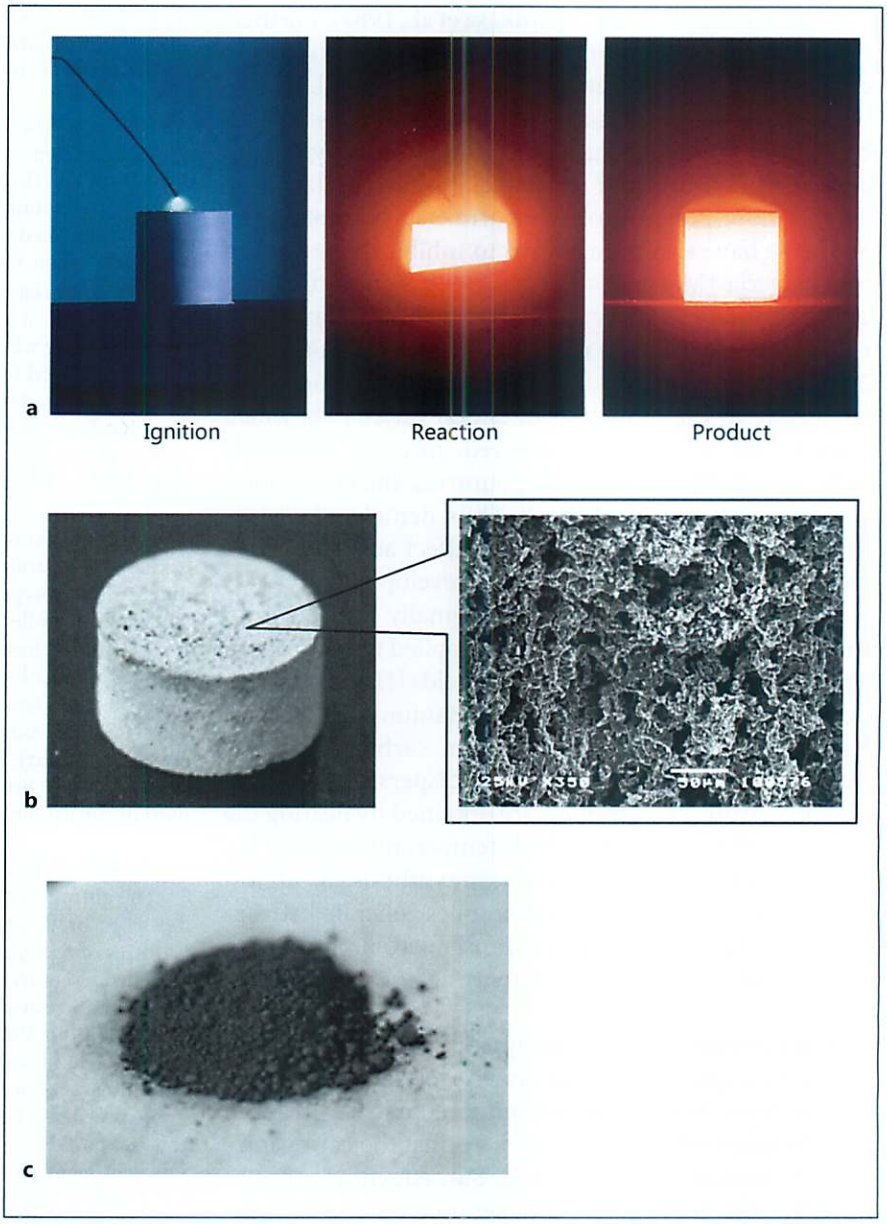

Fig. 1. Ceramics used in the present study. **a** Combustion synthesis reaction. **b** Ceramic pellet. **c** Ceramic powder.

surface (adhesive cells) were removed using a rubber scraper and suspended in 3 mL of water. Both adhesive and nonadhesive cells were dispersed by ultrasonication, and the mass was determined by densitometry at OD_{550}. Total cells were defined as OD_{550} (adhesive cells + nonadhesive cells), and the percent adherence was defined as $100 \times OD_{550}$ (adhesive cells)/OD_{550} (total cells).

Microscopic Observation of in vitro Biofilms

The effects of ceramics on the quantitative amount and biofilm structure produced by *S. mutans* were assessed using confocal laser scanning microscopy, as described previously with some modification [Kuboniwa et al., 2006]. Mutans streptococci were stained with hexidium iodide (15 μg/mL; Molecular Probes, Eugene, OR, USA), which was adjusted to 0.1 at OD_{600} in chemically defined medium, with or without 100 ppb of ceramic powder. The organisms were anaerobically cultured with rocking in saliva-coated wells of a chambered cover glass system (CultureWell™; Grace Bio-Labs, Bend, OR, USA) at 37°C for 18 h in the dark. Nonattached *S. mutans* were washed with PBS, and biofilms were observed by confocal scanning laser microscopy using a TCS-SP5 microscope (Leica Microsystems GmbH, Wetzlar, Germany) with reflected laser light at 488 nm, as well as a DMI6000 B fluorescence microscope (Leica) and a 63× oil immersion objective. Next, the obtained images were analyzed using the software package ImageJ version 1.34s (National Institutes of Health, Bethesda, MD, USA). Digitally reconstructed images of x-z sections (225 mm appropri-

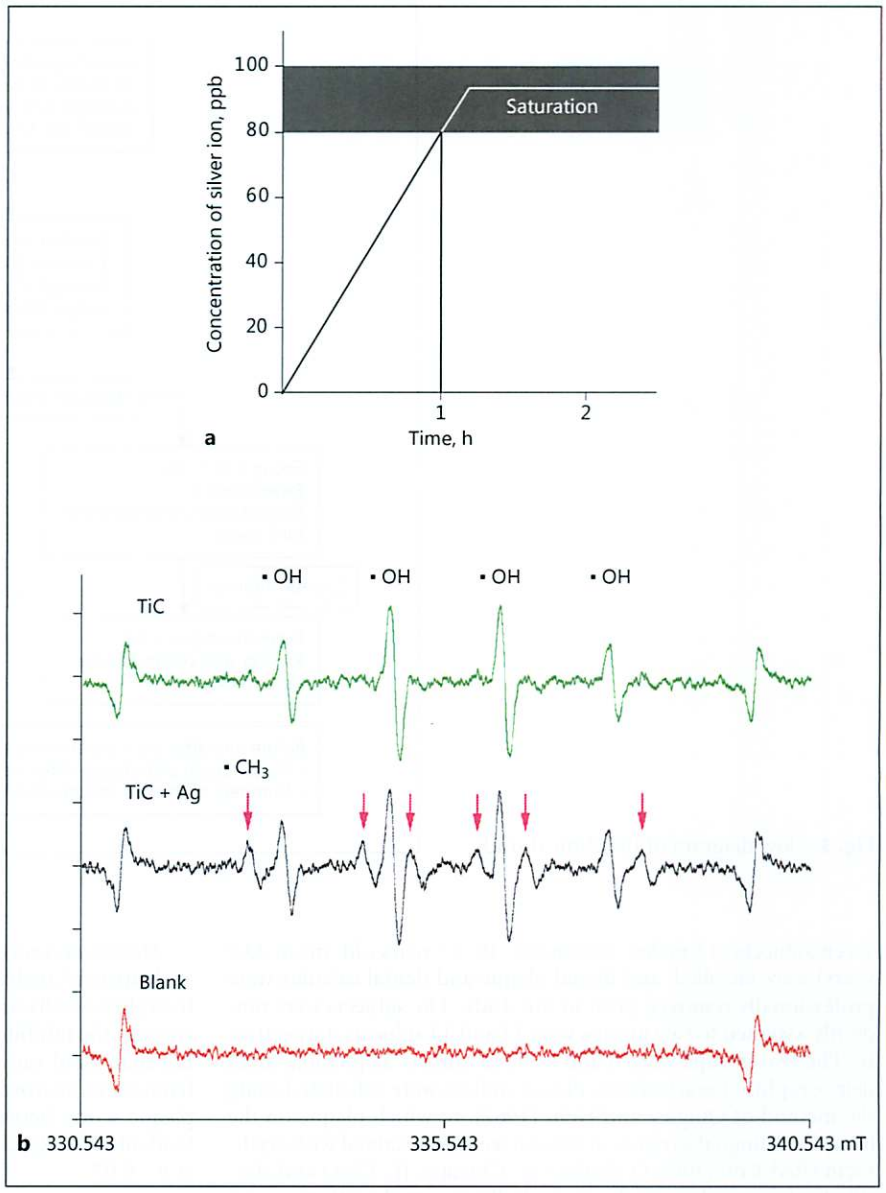

Fig. 2. Characteristics of ceramic water. **a** Concentration of silver ions in water. **b** Hydroxyl radicals appeared in water in the SiO_2 bottle when TiC was used (upper wave profile), and methyl and hydroxyl radicals appeared in water when TiC-dispersed Ag was used (middle wave profile). The radicals disappeared in water in the SiO_2 bottle when ceramics were not added (lower wave profile).

ate height) with 30 μm spaced y-series slices were created using the "Reslice" function of ImageJ. Image series of x-z sections were processed using the "Find Edges" function, and then the peak height was calculated by ImageJ. Each analysis was independently repeated 3 times for each strain in triplicate.

Subjects for Human Studies

The study protocol was approved by the Ethics Committee of Osaka University Graduate School of Dentistry (approval No. H26-E31). Prior to the collection of specimens, the subjects were informed of the study contents by the use of written forms as well as verbal explanations, and written informed consent was obtained from all subjects. Forty-one subjects (17 males, 24 females; 18–37 years old, mean 22.1 years) were initially enrolled in the present study. The selection and sampling of the study population are shown in Figure 3. Subjects were recruited in the following manner: (1) subjects were informed of the contents of the study and provided their signed consent; (2) subjects with special general or oral health conditions were excluded; and (3) subjects with *S. mutans* counts of less than 1×10^3 colony-forming units (CFU)/mL in unstimulated whole saliva were excluded. Finally, 14 subjects were excluded, and 27 subjects were evaluated in the present study.

Design for Human Studies

Human studies were performed according to methods described previously with some modification [Ooshima et al., 1994]. The schedule for the subjects is summarized in Figure 3. Twenty-

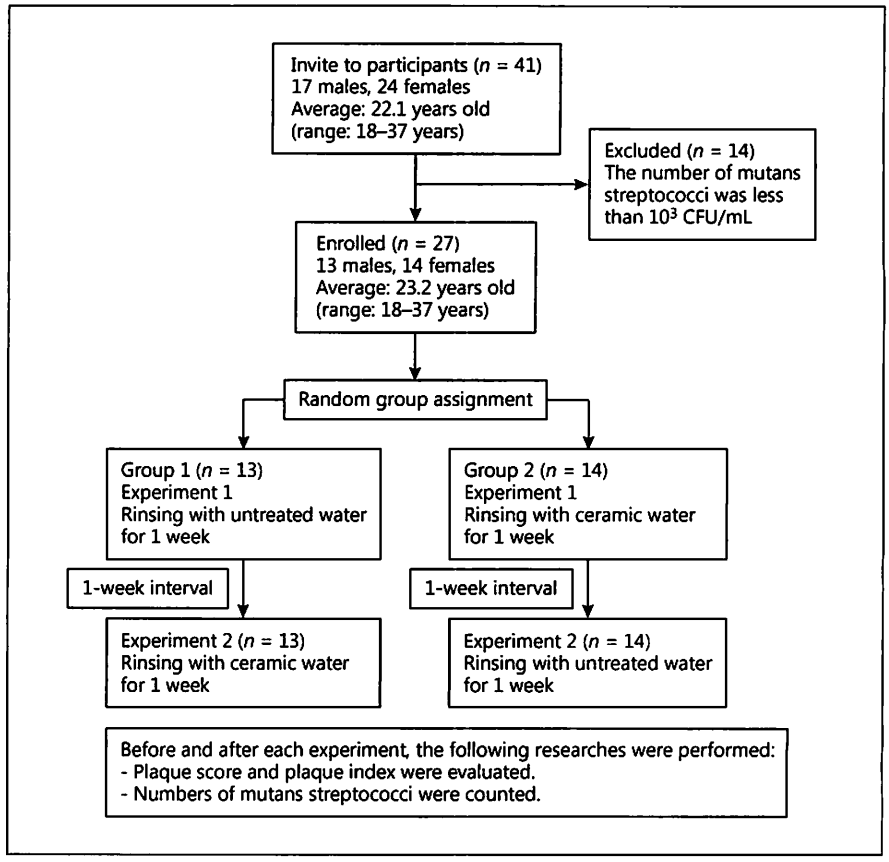

Fig. 3. Flow diagram of the clinical study.

seven subjects (13 males, 14 females; 18–37 years old, mean 23.2 years) were enrolled, and dental plaque and dental calculus were professionally removed prior to the study. The subjects were randomly assigned to two groups with 13 and 14 subjects, respectively. The two groups were made to be as similar as possible in all demographic characteristics. Plaque indices were calculated using the method of Quigley and Hein [1962], in which plaque on the buccal and lingual surfaces of natural teeth was stained with erythrocin (Red-Cote; John O. Butler Co., Chicago, IL, USA) and classified into 6 grades (grades 0–5). Fully crowned teeth were not evaluated in this study. In addition, saliva specimens were collected and streaked onto Mitis Salivarius agar plates supplemented with 15% (w/v) sucrose and 0.2 U/mL of bacitracin, then anaerobically cultured at 37°C for 48 h. The numbers of colonies were counted after identifying characteristic colony morphology of mutans streptococci. After every meal for 1 week, one group was asked to rinse with ceramic water for 10 s while the other rinsed with untreated water. Saliva specimens were collected, followed by colony counting of mutans streptococci. Next, plaque accumulation was evaluated by a single examiner. The groups were then asked to switch rinsing contents, and the same protocol was followed for 1 week. Both the subjects and the examiner were blinded to which subjects were rinsing with ceramic water or untreated water. During the experimental periods, the subjects were not asked to refrain from any oral hygiene procedures or from drinking tea, coffee, or alcohol.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA). Intergroup differences in the inhibitory effects of ceramic water were estimated using analysis of variance followed by the Bonferroni method. Differences in sucrose-dependent adhesion properties and average plaque scores between the two groups were compared using the Student *t* test. Results were considered to be significantly different at $p < 0.05$.

Results

Inhibitory Effects of Ceramics on Bacterial Survival

The bacterial survival rates of MT8148 were drastically reduced by 1.5 h after ceramic powder was added to sterile distilled water (Fig. 4a), which was also observed when the powder was added to PBS (Fig. 4b). Similar results were observed when *S. mutans* strain SA31 was used (online suppl. Fig. 1A, B; for all online suppl. material, see www.karger.com/doi/10.1159/000452343). The antimicrobial activities against MT8148 increased with increasing ceramic concentrations (Fig. 4c).

Inhibitory Effects of Ceramics on Sucrose-Dependent Adhesion

The sucrose-dependent adhesion rates of MT8148 cultured under normal BHI concentrations with or without ceramic powder-suspended water showed no significant differences (Fig. 5a). However, the sucrose-dependent adhesion rate of MT8148 with ceramic powder-suspended water was significantly greater than that without when the amount of BHI was at 1/10 concentration ($p < 0.001$) (Fig. 5b).

Inhibitory Effects of Ceramics on Biofilm Formation

Confocal scanning laser microscopy of MT8148 revealed prominent biofilm formation (Fig. 6a). By contrast, biofilm formation was drastically reduced when ceramic powder was added. Such reduction in the presence of ceramic powder was also observed in MT8148 grown at 1/10 BHI concentration, though bacterial growth was decreased compared with MT8148 grown in BHI at the original concentrations (Fig. 6b).

Human Studies

After rinsing with ceramic water, the average plaque score was 43.0 ± 3.7, which was significantly lower than the baseline value (74.1 ± 5.7, $p < 0.001$) (Fig. 7a). With untreated water rinsing, there was no significant difference in the average plaque score between the baseline value and after 1 week (Fig. 7b). Representative images of dental plaque accumulation before and after rinsing with ceramic water or untreated water are shown in Figure 7c and d, respectively. In addition, the total number of mutans streptococci was reduced after rinsing with ceramic water compared with untreated water ($p < 0.05$) (Fig. 7e). Among 27 subjects, 22 (81.5%) showed decreases in the total number of mutans streptococci when the subjects rinsed with ceramic water (there was an increase in 4 subjects, and 1 subject did not show any changes). The average number of the total mutans streptococci of these subjects after rinsing with ceramic water was 8.2×10^4 CFU, which was significantly lower than that before rinsing with ceramic water (1.6×10^5 CFU; $p < 0.05$). On the other hand, the average numbers of the total mutans streptococci before and after rinsing with untreated water were 2.0×10^5 and 2.8×10^5 CFU, respectively, with no statistically significant difference.

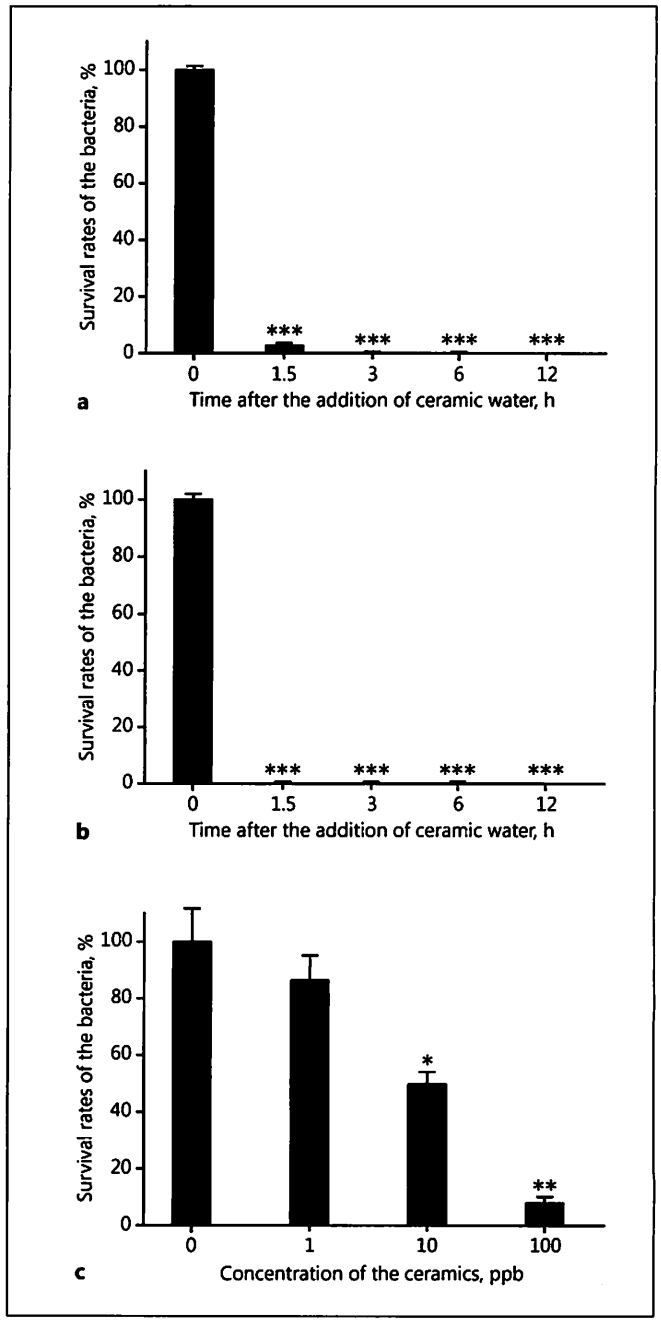

Fig. 4. Inhibitory effects of ceramic water on bacterial survival of *S. mutans* strain MT8148. The survival rates of MT8148 were calculated by determining the percentage of bacteria in ceramic water relative to that without ceramic water at each time point. MT8148 was suspended in sterile distilled water (**a**) and phosphate-buffered saline (**b**), respectively. Significant differences were determined using the Student *t* test. *** $p < 0.001$ vs. time point 0 after the addition of ceramic water. **c** MT8148 was added to varying concentrations of ceramic water. Significant differences were determined using the Student *t* test. * $p < 0.05$ and ** $p < 0.01$ vs. bacterial survival without ceramic water.

Discussion

To our knowledge, this is the first study focusing on the inhibitory effects of Ag-dispersed TiC ceramics against the cariogenicity of *S. mutans* and dental plaque

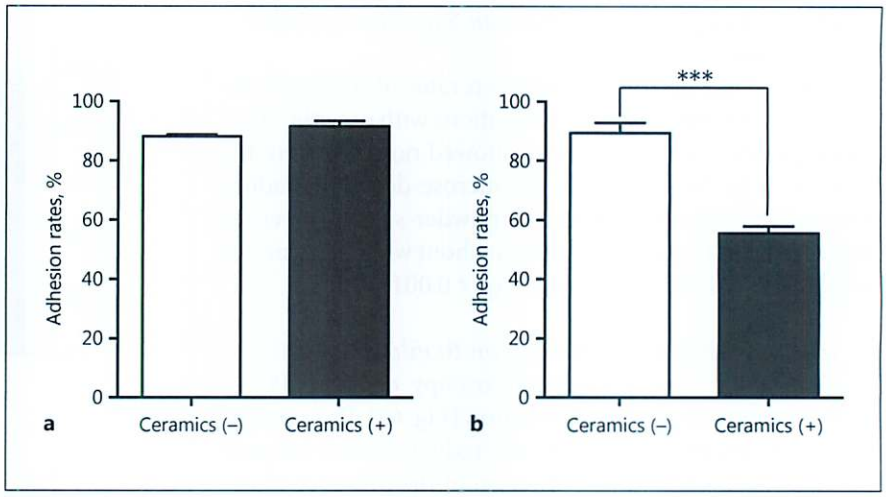

Fig. 5. Sucrose-dependent adhesion properties of *S. mutans* MT8148 in brain-heart infusion broth at normal concentration (**a**) and 1/10 concentration (**b**). Significant differences were determined using the Student *t* test. *** $p < 0.001$.

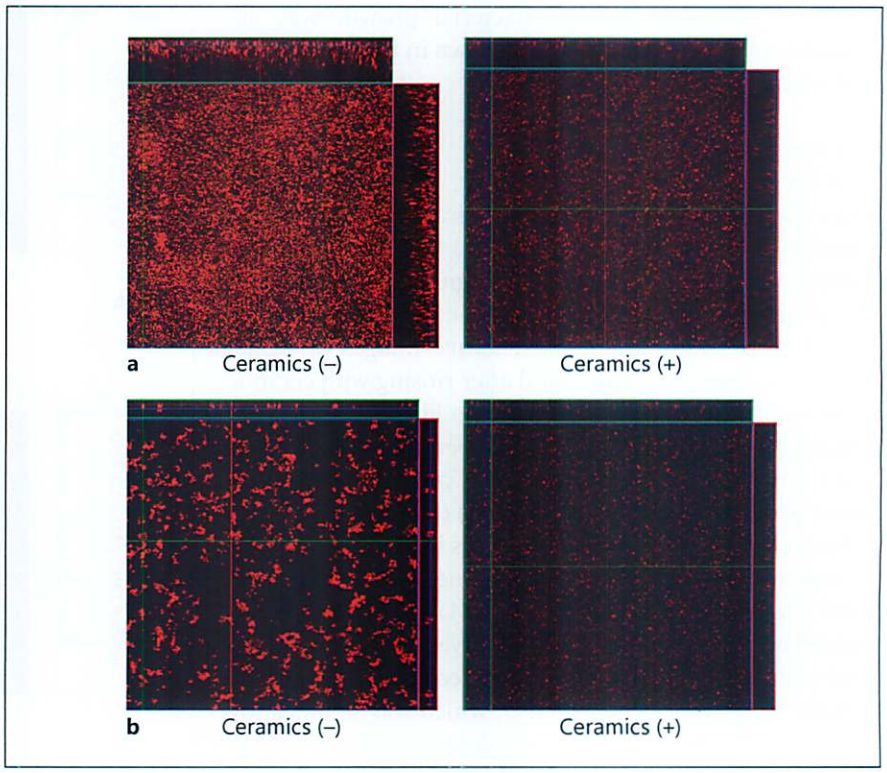

Fig. 6. Inhibitory effects of ceramic water on *S. mutans* biofilm formation. Confocal scanning laser microscopy of *S. mutans* MT8148 biofilms, which were grown in brain-heart infusion broth at normal (**a**) and 1/10 concentrations (**b**).

accumulation. There has been no attempt to produce any antiseptic solution with nonoxide ceramics heretofore. When combustion-synthesized TiC dispersed with Ag contacts water, methyl, hydroxyl radicals, and Ag ions are generated. In contrast, oxide ceramics such as TiO_2 SiO_2, which were widely used as water bottle products, produced by usual sintering, do not generate any free radicals in water, as shown in Figure 2b (lower wave profile "blank"). In the present study, we propose a simple method for preventing dental caries by rinsing with Ag-dispersed TiC ceramic water, as silver ions are known to have an inhibitory effect on bacterial growth [Burne et al., 1987].

Several food products such as sugar substitutes and polyphenols have been reported to reduce dental caries [Smith et al., 2002]. We previously evaluated the effects

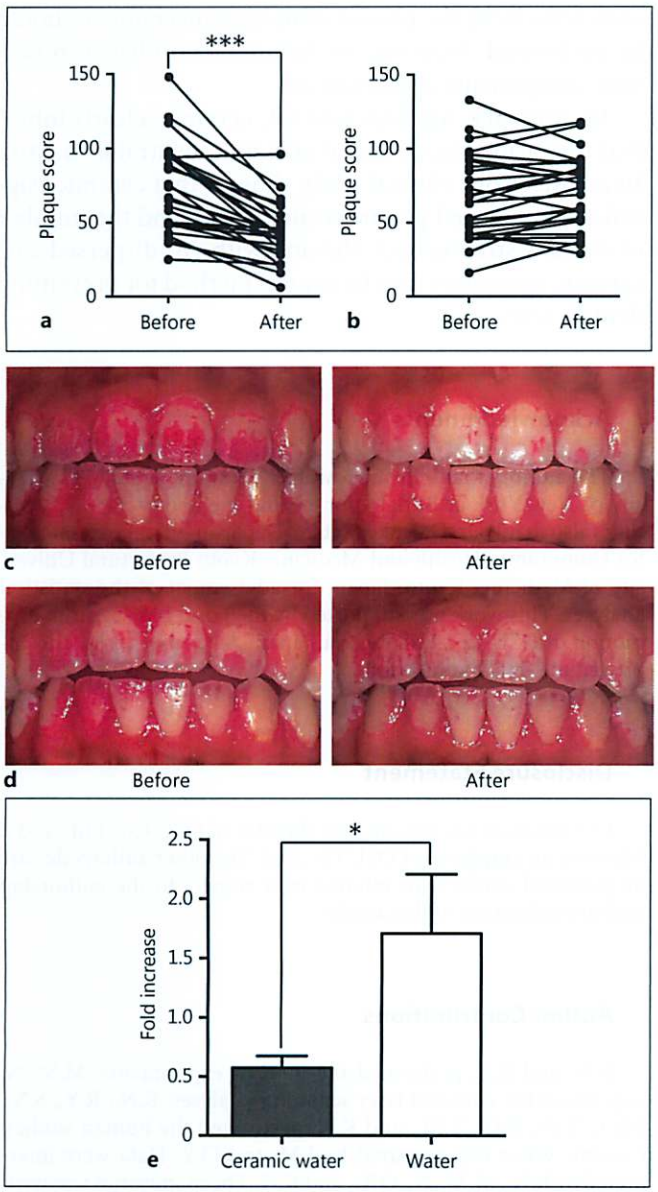

Fig. 7. Clinical study of rinsing with ceramic water (**a**) or untreated water (**b**) evaluated by plaque score. Significant differences were determined using the Student *t* test. *** $p < 0.001$. Representative images of plaque accumulation stained by erythrocin before and after rinsing with ceramic water (**c**) and untreated water (**d**). **e** Fold increases of the total number of *S. mutans*, which were compared before and after rinsing with ceramic water or untreated water. Significant differences were determined using the Student *t* test. * $p < 0.05$.

of polyphenols on plaque deposition following rinsing with oolong tea extracts containing polymerized polyphenols in human studies, which resulted in a significant inhibition of plaque deposition [Ooshima et al., 1994]. Based on our previous research on oolong tea, we attempted to develop a novel oral self-care method and focused on ceramic water, which produces Ag ions as well as methyl and hydroxyl radicals. Though our previous study demonstrated significant plaque deposition inhibition upon rinsing with oolong tea extracts, no significant effects were observed on the number of mutans streptococci. The numbers of mutans streptococci before rinsing with oolong tea extracts was 1.6×10^5 CFU, which was not reduced after rinsing with the extracts (2.1×10^5 CFU). However, in this study, rinsing with ceramic water significantly inhibited both dental plaque accumulation and the number of mutans streptococci. The average number of the total mutans streptococci after rinsing with ceramic water was 8.2×10^4 CFU, which was approximately half as low as that before rinsing with that water (1.6×10^5 CFU). These results may indicate that ceramic water has greater killing effects on mutans streptococci compared with oolong tea. We previously reported that polyphenol components such as epigallocatechin gallate extracted from oolong tea showed strong antibacterial activity [Sasaki et al., 2004]. It is thus important to determine the antibacterial activity induced by each ceramic component in future studies.

Metal ions have been shown to be involved in the inhibitory effects on bacterial colonization [Burne et al., 1987]. Ag ions are effective against both gram-positive and gram-negative bacteria [Burne et al., 1987; Bellantone et al., 2002; Hipler et al., 2006]. Ag ions are also reported to have a negative effect on the fructosidase activity of *S. mutans* [Burne et al., 1987]. Silver diamine fluoride has been widely used to prevent dental caries development whereby Ag ions denature bacterial enzymes [Castillo et al., 2011]. In addition, several dental restorative materials such as fillers in composite resins have been developed which slowly release Ag ions [Kawashita et al., 2000].

Recently, the combination of hydrogen peroxide (H_2O_2) and UV light has been used to induce hydroxyl radicals, which have been utilized for the treatment of dental caries and periodontitis [Feuerstein et al., 2006]. The exposure of H_2O_2 to UV light showed antibacterial effects against *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *S. mutans*, which could be used as a minimal dental treatment [Feuerstein et al., 2005]. Another study showed that hydroxyl radicals were generated from H_2O_2 by irradiation with a 405-nm laser from an ultrasonic scaler [Ikai et al., 2013]. The combination of photo-irrigated H_2O_2 and polyphenolic compounds showed antibacterial activity against both *S. mutans* and *P. gingiva-*

lis. Though the products that release Ag ions or hydroxyl radicals described above are useful for dental caries prevention, these materials are mainly used by dentists and require special attention for use. Therefore, we attempted to create Ag ion releasing ceramics that could be safely and easily used at home.

When ceramics are added to water, Ag ions in addition to methyl and hydroxyl radicals are generated, resulting in antimicrobial activity against various bacteria. However, there is no information concerning the antimicrobial activity of ceramics when added to solvents other than water. In the present study, ceramics added to PBS exhibited antimicrobial activity, indicating that ceramics did not produce any undesirable reactions with low concentrations of sodium chloride, disodium hydrogen phosphate 12-water, potassium chloride, and potassium dihydrogen phosphate, major components of PBS. In addition, ceramics added to BHI broth exhibited activity as growth inhibitors as well as reducing sucrose-dependent adhesion. However, these later effects were not as significant as the antimicrobial activity. In addition, inhibitory activity was prominently observed at the lower BHI concentrations. According to these results, we hypothesized that ceramics may mainly act through antimicrobial activity rather than growth inhibition. Alternatively, the BHI components may weaken the efficacy of ceramics. Further studies regarding the relationship between the components of chemical reagents and the efficacy of ceramics should be performed. However, ceramics drastically inhibited *S. mutans* biofilm formation, which exhibited a more prominent effect than that in the sucrose-dependent adhesion assay, though *S. mutans* was cultured in BHI broth in both analyses. The most significant difference between these assays was that saliva was only added in the biofilm assays. Thus, ceramics may change the disposition of salivary components, which may make it difficult for oral bacteria to form biofilms. Further research to clarify the precise inhibition mechanism should be performed, focusing on the interaction between salivary components and ceramics.

In summary, Ag-dispersed TiC ceramics clearly inhibited the cariogenic potential of *S. mutans* strains in vitro. In addition, our clinical study showed that ceramics significantly reduced plaque accumulation and the number of mutans streptococci. Rinsing with Ag-dispersed TiC ceramic water may thus be a novel method for preventing dental caries.

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Disclosure Statement

O. Yamada is a representative director of OSU Co., Ltd., and J. Maruo is an employee of OSU Co., Ltd. The other authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Author Contributions

R.N. and R.Y. performed the in vitro experiments. M.M.-N. supervised the confocal laser scanning analyses. R.N., R.Y., S.N., M.O., Y.O., R.H., Y.M., and K.N. performed the human studies. Ceramic water was prepared by J.M. and O.Y. Data were interpreted by R.N., M.M.-N., O.Y., and K.N. The manuscript was written by R.N. and O.Y. under the supervision of K.N.

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