Abstract: Denture plaque is a biofilm composed of various microorganisms aggregated with saliva. Various denture cleansers and cleaning apparatuses have been developed and studied. However, the optimum water temperature for denture cleaning is unknown. Therefore, the present study investigated the effects of water temperature during ultrasonic denture cleaning. In vitro, resin disks with artificial Candida albicans biofilm were pressed onto Candida GE media after ultrasonic cleaning with water at different temperatures for 5 min. The media were subsequently cultured at 37°C for 24 h. The colonies formed were observed and colony areas were quantified using ImageJ software (US National Institutes of Health, Bethesda, MD, USA). In situ, the bacterial count and degree of cleanliness on the tissue surface of maxillary dentures were measured before and after ultrasonic cleaning with water at different temperatures for 5 min. Changes in bacterial counts and cleanliness were calculated for each temperature. The ratio of the area occupied by bacterial colonies in vitro and reduction rates in situ after cleaning with warm water were markedly less than those observed after cleaning with cold water. Therefore, ultrasonic denture cleaning with warm water is more effective.

Keywords: temperature; denture plaque; ultrasonic denture cleaning; Candida albicans.

Introduction

Many Asian (particularly Japan) and European countries presently face challenges related to an aging population. The number of elderly and medically vulnerable individuals who need special support and care has been increasing. The importance of oral hygiene has also been well-recognized since Yoneyama et al. (1) reported that good oral hygiene prevents aspiration pneumonia in the elderly. In particular, the preventive effects of perioperative oral management and infection control have also been reported in patients with medication-related osteonecrosis of the jaw and in those undergoing surgical therapy, chemotherapy, and radiation therapy for malignant tumors (2-5). Denture plaque control (i.e., denture cleaning) is essential for maintaining oral hygiene in patients who wear dentures.

Denture plaque is a biofilm composed of microorganisms aggregated with saliva, as seen in dental plaque. Denture plaque is associated with bad breath, dental caries, periodontal disease, denture stomatitis, and aspiration pneumonia (6-8). In addition to the conventional evaluation methods based on bacterial counts, a novel method that uses adenosine triphosphate (ATP) + adenosine monophosphate (AMP) wiping for evaluating denture cleanliness was evaluated. Together, these two methods can comprehensively evaluate denture hygiene.

Many reports recommend a combination of mechanical cleaning with a denture brush and chemical cleaning with denture cleansing agents for effective control of denture plaques (9,10). Various chemical cleaning methods, such
as the use of denture cleaners, disinfectant agents, and water, have been developed, and their effects have been reported (11-16). The relationship between use of high-temperature cleaning water and bacterial counts has been investigated by Glass et al (17); however, little is known about the effects of warm-temperature cleaning water (30-40°C) on bacterial counts. Therefore, in the present experiment, the influence of water temperature used during ultrasonic denture cleaning on bacterial counts and cleanliness was examined in both in vitro and in situ experiments.

Materials and Methods

In vitro biofilm analysis
Preparation of Candida albicans biofilm model (Fig. 1)
C. albicans biofilm model was prepared using the method described in our previous report (18). C. albicans CAD1, which is a clinical strain isolated from denture plaque, was used for this purpose. The strain was cultured in BHI medium (Brain Heart Infusion medium, Becton, Dickinson and Company, NJ, USA) at 37°C for 24 h and then adjusted to a concentration of 1.0×10⁸ CFU/mL by centrifugation. C. albicans suspension (1.0×10⁶ CFU/mL) was prepared by adding 40 μL of prepared C. albicans to 40 mL of Yeast Nitrogen Base medium (YNB, Difco, MI, USA) supplemented with 100 nmol/L glucose and 2.5 mol/L N-acetyl glucosamine.

C. albicans biofilm was grown on an acrylic resin disk (13.5 mm in diameter), which was made of a denture base resin (Acron, GC, Tokyo, Japan). The surface of the resin disk was sterilized with 70% ethyl alcohol and ultraviolet irradiation. Thereafter, the surface of the resin was treated with mucin (Sigma-Aldrich Co. LCC, St. Louis, MO, USA). For this purpose, 0.5 mg/mL of mucin was placed on the resinous surface and incubated at 37°C for 10 min under centrifugation at 75 rpm. C. albicans was initially adhered to the surface. The resin disk was cleaned twice with phosphate buffered saline (PBS). Next, 3 mL of C. albicans suspension was added and incubated at 37°C for 60 min under centrifugation at 75 rpm. Initially, C. albicans was found to be adhered to the resin disk. Thereafter, the resin disk was cleaned twice with PBS, replaced with fresh YNB medium, and incubated at 37°C for 48 h under centrifugation at 75 rpm. An in vitro C. albicans biofilm was thus formed on the resin disk.

Ultrasonic cleaning
The resin disk with the C. albicans biofilm was immersed in and cleaned with distilled water at three temperatures, including 16°C, 30°C, and 40°C, for 10 min using an ultrasonic cleaner (40 kHz in frequency and 70 W in output; Shofu Ultrasonic Cleaner SUC-70, Shofu, Kyoto, Japan). As a control, the resin disk was immersed in distilled water for 10 min. During ultrasonic cleaning, the temperature of the water was confirmed with a digital thermometer. The water temperature remained lower than that which could damage the acrylic resin because of low power and short cleaning time.

Observation of colonies and measurement of colony areas
The treated resin disks were cleaned twice with PBS and dried. The resin disks were lightly pressed onto the Candida GE medium (Nissui, Tokyo, Japan) for 1 s and removed. The media were incubated at 37°C for 24 h. After incubation, the colonies on the media were photographed with a digital camera, and their areas were calculated from the images obtained using the image processing software Image J (US National Institutes of Health, Bethesda, MD, USA). The colony occupancy area ratio (area of the colonies / dish area × 100%), which
is the ratio of the area of the colonies to the dish area, was compared for each temperature condition.

**In situ analysis**

Twenty-four maxillary complete dentures were investigated in this study before routine denture cleaning in the prosthodontic division of Tokushima University Hospital. The region of interest (ROI) in the right molar of the denture tissue surface was cleaned with a sterile cotton swab, and the bacterial count on the swab was electrically measured with a bacteria-counting apparatus (Bacteria counter, Panasonic Healthcare, Tokyo, Japan). ROI in the right premolar was also swabbed with an inspection kit (Lucipak Pen, Kikkoman Bio Chemiphar, Tokyo, Japan), and the degree of cleanliness was measured with an ATP+AMP inspection device (Lumi Tester PD-30, Kikkoman Bio Chemiphar), as shown in Fig. 2.

Then, the denture was immersed in distilled water and cleaned for 5 min using an ultrasonic cleaner. Three temperature conditions (16°C, 30°C, and 40°C) were used for cleaning. Eight dentures were randomly used for each temperature condition. After cleaning, the dentures were dried, and the bacterial count and degree of cleanliness were measured using the same method as that used before cleaning. The measurement sites after cleaning were on the left premolar and molar, i.e., contralateral to the sites measured before cleaning (Fig. 2). The reduction rate for each measured value was calculated using the following equation: Reduction Ratio = (value before cleaning-value after cleaning) / value before cleaning × 100 (%). The reduction rates observed with ultrasonic cleaning at different temperatures of water were compared. In addition, the reduction rate and personal information were not linked.
Statistical analysis
Kruskal-Wallis test with Dunn’s post hoc test and Mann-Whitney U test were used for statistical analysis. All statistical analyses were conducted with a significance level of 0.05 using the SPSS software (version 24.0, IBM Corp. Armonk, NY, USA).

Results

In vitro analysis
Figure 3-A shows colonies of *C. albicans* on resin disks subjected to different temperature conditions. On macroscopic observation, colony formation was found to be suppressed in the ultrasonic cleaning group compared with the stationary group. In particular, colony formation was suppressed with higher-temperature water (i.e., 30°C and 40°C) rather than with lower-temperature water (16°C).

Figure 3-B shows the colony occupancy area ratio for each temperature condition. The area ratio in the ultrasonic cleaning group was markedly less than that in the stationary group. The colony occupancy area ratio was markedly less when the dentures were cleaned with high-temperature water (30°C and 40°C) than when cleaned with lower-temperature water (16°C). In the stationary group, the colony occupancy area ratio was significantly lower among dentures cleaned with water at 30°C than among those cleaned with water at other temperatures.

In situ analysis
Figure 4 shows bacterial count reduction rates and degree of cleanliness before and after ultrasonic denture cleaning under each temperature condition. The group subjected to ultrasonic cleaning with cold water (16°C) had the lowest decrease in bacterial count. Those who underwent ultrasonic cleaning with warm water (40°C) showed the highest decrease in the bacterial count and highest degree of cleanliness. Among these, a marked reduction in the degree of cleanliness was observed among the groups treated with cold (16°C) and warm water (30°C and 40°C). No difference in bacterial count reduction was observed in the groups, although the reduction tended to be higher in the warm-water ultrasonic cleaning groups.

Discussion
Denture plaque is a biofilm composed of colonies of various streptococci; aerobic bacteria, including staphylococci; and facultative anaerobic bacteria attached to a saliva-derived pellicle on the denture surface, extracellular polysaccharides produced by microorganisms, various blood components, and bacterial co-aggregations (19).

In this experiment, two methods were used: electrical bacterial count measurements and ATP+AMP wiping measurements. In the electrical bacterial count measurement technique, the bacterial count was determined by applying the dielectrophoretic impedance measurement method (DEPIM), which measures the bacterial concentration (CFU/mL) by capturing bacteria on the electrode and noting the change in the impedance. These measurements are frequently performed for the elderly in the clinical setting (20-22). On the other hand, in the ATP+AMP wiping test, the amount of luminescence produced by luciferase, a luminescent enzyme, and the amount of ATP+AMP that gets converted were measured. Using the concept that ATP+AMP is commonly present in the living tissue, the degree of contamination was
measured by measuring the amounts of microorganisms and food residue. According to Watanabe et al. (23), ATP wiping measurements are simple and rapid methods for assessing contamination in dental unit water lines. Furthermore, ATP wiping inspection is simple and useful for assessing endoscope cleanliness according to Gillespie et al. (24). In this experiment, both bacterial counts and ATP+AMP wiping measurements were performed to accurately assess the cleanliness of the denture surface.

Proper denture cleaning requires mechanical cleaning with a brush and chemical cleaning with denture cleansers. Ultrasonic denture cleaning is a mechanical method of cleaning that uses micro ultrasonic vibrations and is very effective in removing denture plaque. The effectiveness of combining ultrasonic cleaning and denture cleansing agents for the removal of fungal species from denture surfaces has been reported by Cruz et al. (25). Additionally, the use of various sterilized solutions, such as chemical cleaning agents (i.e., denture cleansers), electrolyzed water, and an aqueous solution of sodium hypochlorite, have been reported for the removal of Candida species (12,13,15,16).

The basic properties of ultrasonic cleaning and denture cleansing solution immersion, especially the effect of temperature of the cleaning solution (i.e., an appropriate water temperature), have not been thoroughly examined in previous studies. Therefore, the differences in ultrasonic denture cleaning due to differences in the temperature of the cleaning water were evaluated in this study through in vitro and in situ experiments. In the in vitro experiment, C. albicans biofilm on a resin disk was used to evaluate the effects of temperature. Denture plaques are characterized by the presence of C. albicans, which has both yeast and filamentous forms (26,27). The C. albicans biofilm enables clinical-like evaluation of denture cleaning in vitro. For the in situ experiment, the influence of water temperature during ultrasonic cleaning was evaluated using bacterial counts and ATP+AMP activity on chairside denture plaque.

The results of the in vitro and in situ experiments suggest that water temperature affects the resultant cleanliness. This effect is the most discernible for water at higher temperatures (40°C). The acrylic resins used for denture bases are resistant to temperatures higher than 40°C. The highest temperature of water used in the present study was 40°C to prevent the possible deformation of the acrylic resin and to approximate the temperature of conventional warm water that would be used in the clinical setting.

Although the notable effect observed with water at 40°C has not been explained, the fact that higher temperature inhibits the formation of filaments of C. albicans caused by cinnamaldehyde action has been proposed by Taguchi et al. (28). Moreover, although 40°C is suitable for the growth of microorganisms, intercellular proteins and linkages that exist between the biofilm’s microorganisms may temporarily loosen during growth, consequently enhancing the effect of denture cleaning due to ultrasonic vibrations.

It can thus be concluded that ultrasonic denture cleaning with warm water (approximately 30-40°C) is more effective than cleaning with cold water. This was evaluated by analyzing the bacterial counts and cleanliness post-cleaning. Enhanced cleaning may be a simple and economical way of promoting oral hygiene and carries broader implications for medical and nursing care.

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Conflict of interest

The authors have no conflict of interest to disclose.

References

1. Yoneyama T, Yoshida M, Matsui T, Sasaki H (1999) Oral care and pneumonia. Oral care working group. Lancet 354, 515.
2. Choi SE, Kim HS (2012) Sodium bicarbonate solution versus chlorhexidine mouthwash in oral care of acute leukemia patients undergoing induction chemotherapy: a randomized controlled trial. Asian Nurs Res 6, 60-66.
3. Devi S, Singh N (2014) Dental care during and after radiotherapy in head and neck cancer. Natl J Maxillofac Surg 5, 117-125.
4. Soutome S, Yanamoto S, Funahara M, Hasegawa T, Komori T, Yamada SI et al. (2017) Effect of perioperative oral care on prevention of postoperative pneumonia associated with esophageal cancer surgery: a multicenter case-control study with propensity score matching analysis. Medicine (Baltimore) 96, e7436.
5. Ohnishi Y, Ito K, Kitamura R, Funayama S, Hori K, Inoue M (2017) Importance of professional oral hygiene in preventing medication-related osteonecrosis of the jaw. Int J Qral-Med Sci 15, 85-92.
6. Coulthwaite L, Verran J (2007) Potential pathogenic aspects of denture plaque. Br J Biomed Sci 64, 180-189.
7. Inuma T, Arai Y, Abe Y, Takayama M, Fukumoto M, Fukui Y et al. (2015) Denture wearing during sleep doubles the risk of...
8. Hannah VE, O’Donnell L, Robertson D, Ramage G (2017) Denture stomatitis: causes, cures and prevention. Prim Dent J 6, 46-51.

9. Paranhos HF, Silva-Lovato CH, Souza RF, Cruz PC, Freitas KM, Peracini A (2007) Effects of mechanical and chemical methods on denture biofilm accumulation. J Oral Rehabil 34, 606-612.

10. Nishi Y, Seto K, Kamashita Y, Take C, Kurono A, Nagaoka E (2012) Examination of denture-cleaning methods based on the quantity of microorganisms adhering to a denture. Gerodontology 29, e259-266.

11. Nagamatsu Y, Tajima K, Kakigawa H, Kozono Y (2001) Application of electrolyzed acid water to sterilization of denture base part 1. Examination of sterilization effects on resin plate. Dent Mater J 20, 148-155.

12. Yilmaz H, Aydin C, Bal BT, Ozcelik B (2005) Effects of disinfectants on resilient denture-lining materials contaminated with Staphylococcus aureus, Streptococcus sobrinus, and Candida albicans. Quintessence Int 36, 373-381.

13. Vieira AP, Senna PM, Silva WJ, Del Bel Cury AA (2010) Long-term efficacy of denture cleansers in preventing Candida spp. biofilm recolonization on liner surface. Braz Oral Res 24, 342-348.

14. Jnanadev KR, Satish Babu CL, Shilpa Shetty S, Surendra Kumar GP, Sheetal HS (2011) Disinfecting the acrylic resin plate using electrolyzed acid water and 2% glutaraldehyde: a comparative microbiological study. J Indian Prosthodont Soc 11, 36-44.

15. Pyo KR, Yoo YS, Baek DH (2015) Antifungal effect of electrolyzed hydrogen water on Candida albicans biofilm. J Dent Rehabil Appl Sci 31, 212-220.

16. Kurt A, Erkose-Genc G, Uzun M, San T, Isik-Ozkol G (2016) The effect of cleaning solutions on a denture base material: elimination of Candida albicans and alteration of physical properties. J Prosthodont, doi: 10.1111/jopr.12539.

17. Glass RT, Conrad RS, Bullard JW, Goodson LB, Mehta N, Lech SJ et al. (2011) Evaluation of cleansing methods for previously worn prostheses. Compend Contin Educ Dent 32, 68-73.

18. Li J, Hirota K, Goto T, Yumoto H, Miyake Y, Ichikawa T (2012) Biofilm formation of Candida albicans on implant overdenture materials and its removal. J Dent 40, 686-692.

19. Teughels W, Van Assche N, Slipeen I, Quirynen M (2006) Effect of material characteristics and/or surface topography on biofilm development. Clin Oral Implants Res 17, 68-81.

20. Kikutani T, Tamura F, Takahashi Y, Konishi K, Hamada R (2012) A novel rapid oral bacteria detection apparatus for effective oral care to prevent pneumonia. Gerodontology 29, e560-565.

21. Kikutani T, Tamura F, Tashiro H, Yoshida M, Konishi K, Hamada R (2015) Relationship between oral bacteria count and pneumonia onset in elderly nursing home residents. Geriatr Gerontol Int 15, 417-421.

22. Funahara M, Hayashida S, Sakamoto Y, Yamamoto S, Kosai K, Yanagihara K et al. (2015) Efficacy of topical antibiotic administration on the inhibition of perioperative oral bacterial growth in oral cancer patients: a preliminary study. Int J Oral Maxillofac Surg 44, 1225-1230.

23. Watanabe A, Tamaki N, Yokota K, Matsuyama M, Kokeguchi S (2016) Monitoring of bacterial contamination of dental unit water lines using adenosine triphosphate bioluminescence. J Hosp Infect 94, 393-396.

24. Gillespie E, Sievert W, Swan M, Kaye C, Edridge I, Stuart RL (2017) Adenosine triphosphate bioluminescence to validate decontamination of endoscopes. J Hosp Infect 97, 353-356.

25. Cruz PC, Andrade IM, Peracini A, Souza-Gugelmin MC, Silva-Lovato CH, de Souza RF et al. (2011) The effectiveness of chemical denture cleansers and ultrasonic device in biofilm removal from complete dentures. J Appl Oral Sci 19, 668-673.

26. Bulad K, Taylor RL, Verran J, McCord JF (2004) Colonization and penetration of denture soft lining materials by Candida albicans. Dent Mater 20, 167-175.

27. Cavalcanti IM, Nobbs AH, Ricomini-Filho AP, Jenkinson HF, Del Bel Cury AA (2016) Interkingdom cooperation between Candida albicans, Streptococcus oralis and Actinomyces oris modulates early biofilm development on denture material. Pathog Dis, doi: 10.1093/femspd/ftw002.

28. Taguchi Y, Hasumi Y, Hayama K, Arai R, Nishiyama Y, Abe S (2012) Effect of cinnamaldehyde on hyphal growth of C. albicans under various treatment conditions. Med Mycol J 53, 199-204.