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Original Article

Efficacy of newly developed denture cleaning device on physical properties of denture material and Candida biofilm

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KEYWORDS
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Abstract *Background/purpose:* Electrolyzed water has antimicrobial activity against oral microbes. The purpose of this study was to investigate the effects of a denture cleaning device that uses electrolyzed water on Candida biofilm on denture base-material and the physical properties of the denture material.
Materials and methods: Denture base-resin disks were prepared with Polymethyl methacrylate. After the formation of *Candida albicans* biofilm on the resin disks, the antimicrobial activity of the denture cleaning device and the chemical cleanser against *C. albicans* biofilm was compared. The resin disks were also treated with the cleaning device and the chemical cleanser for 150 days, and the physical properties were analyzed by an atomic force microscope, Vickers hardness tester, and colorimeter.
Results: The denture cleaning device and the chemical cleanser reduced the levels of *C. albicans* biofilm on the denture resin. Upon immersing of the resin disks for 150 days, the electrolyzed water of the denture cleaning device did not significantly change the surface roughness of the specimens, but significantly reduced its Vickers hardness compared to the initial value. The color changes of the resin disk were 0.477 ± 0.076, 0.612 ± 0.095 and 0.562 ± 0.096 after treating with tap water, the chemical cleanser, and the denture cleaning device, respectively.
Conclusion: The denture cleaning device may be suitable for use by the elderly to clean dentures without side effects caused by the misuse of chemical cleanser.

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Introduction

Dentures are used by people with missing teeth. Furthermore, the use of dentures is steadily increasing as it moves into the aging society. Dentures provide various benefits, such as an attractive look and a feeling of confidence in denture wearers. However, they also have disadvantages, such as being uncomfortable for daily use and inconvenient while eating and talking. Denture cleansers have been used to prevent denture-related stomatitis. In the elderly and immunocompromised patients, denture stomatitis is the most frequent manifestation of oral candidiasis.[1] Furthermore, among *Candida* species, *Candida albicans* has been reported to be the most relevant to denture stomatitis.[2] *C. albicans* is a normal flora in the oral cavity and is a diploid fungus that has blastoconidia and hyphae form.[3] Infection of *C. albicans* initiates by the formation of a biofilm, and the fungus changes from blastoconidia to hyphae form in the biofilm.[3,4] As compared with the blastoconidia, hyphal *C. albicans* in the biofilm expresses infection-associated genes[5] and penetrates into the host tissue by means of hydrolytic extracellular enzymes.[6] The fungi in the biofilm are more resistant to antimicrobial agents due to the barrier function of the extracellular matrix which contains polysaccharides, proteins and nucleic acids.[7,8] Therefore, denture cleansers cannot totally remove the *C. albicans* biofilm from the denture surface. In addition, the misuse of denture cleansers has been reported to occasionally cause gastric ulcers, allergic reactions, and burns.[9] Moreover, the long-term use of denture cleansers brings about physical changes to the dentures affecting the surface roughness, flexural strength and surface hardness[10] and enhances the formation of the Candida biofilm on the surface of denture materials due to the increasing surface roughness.[11]

Electrolyzed water (EW) is generated by an electric current passed though water using metal electrodes. When tap water is electrolyzed by metal electrode, the physical and chemical properties of EW differ from those of raw tap water. Water electrolyzed with a metal electrode has hypochlorous acid, free chloride, radical oxygen, and reactive hydrogen.[12,13] EW has antimicrobial activity against Gram-positive and −negative bacteria.[14,15] EW also has the antifungal activity against *C. albicans*.[16] Furthermore, EW has been reported to provide benefits to human health such as antitumor and anti-inflammation effects.[17,18] Therefore, denture storage and cleaning device that uses EW was recently developed. In this study, the EW generated by this device was investigated to determine the antifungal effect on the hyphal *C. albicans* grown on denture base-material and the effects on the physical properties of denture base-material after long-term use.

Materials and methods

Fungal strain and cultivation

C. albicans ATCC 10231 was used in this study and cultivated in trypticase soy broth (TSB) (BD Biosciences, San Jose, CA, USA), and was cultured at 37 °C in aerobic conditions. Also, to generate hyphae form of *C. albicans*, *C. albicans* was cultivated in Ham's F-12 medium (HyClone, Logan, UT, USA) at 37 °C in a 5% CO_2 condition.

Preparation of denture base-resin

In order to form *C. albicans* biofilm on the surface of the denture material, specimens (20 mm diameter × 3 mm height) of a polymethylmethacrylate (PMMA) material as a denture base-resin (Lucitone 199, Dentsply International, York, PA, USA) were prepared. The disk-shaped wax template was flasked with Type III dental stone (Snow Rock Dental Stone, DK Mungyo, Gimhae, Korea) to obtain a pattern for the specimens. Acrylic resin specimens were processed according to the manufacturer's instructions. The flask was submerged in water at 74 °C for 90 min and at 99 °C for 30 min. After deflasking, the PMMA disks were equivalent in size. Disks were placed in distilled water at room temperature until used.

Investigation of antifungal activity against *C. albicans* biofilm

The denture base-resins were placed into a 12-well polystyrene plate (SPL LifeSciences), and 2 ml of Ham's F-12 medium was then dispensed into the wells. *C. albicans* was inoculated into the prepared 12-well plate, and the plates were incubated at 37 °C in a 5% CO_2 incubator for 72 h. The medium was changed every day with fresh Ham's F-12 medium. To investigate the antifungal activity against a Candida biofilm on surface of denture base-material, after washing the denture base-resins with phosphate buffered saline (PBS) (pH 7.2), the Candida biofilm-formed denture was transferred to Natural Denture Plus® (Ebioteco Co., Seoul, Korea) as an EW denture cleaning device, which included tap water (300 ml). The device was run to generate EW for 5 min with DC 24 V, and *C. albicans* biofilm-formed denture base-resin was incubated for 30 min. To compare the antifungal activities, *C. albicans* biofilm-formed denture base-resin was soaked in Polident® (GSK Korea Co., Seoul, Korea), a popular denture cleanser, for 5 min and transferred into filtered tap water with a PVDF filter (0.2 μm pore size), followed by incubation for 30 min. Both denture base-resins were placed into 12-well plate containing 1 ml of TSB, and the biofilm were mechanically disrupted with a scraper. The suspensions were transferred into 1.5 ml tubes, which were vortexed for 1 min. The suspension was diluted serially 10-fold to 10^7 with TSB. Then, 50 μl of the diluted suspension was spread on trypticase soy agar plate. After incubating the agar plate at 37 °C for 24 h, the colonies of *C. albicans* were counted.

Analysis of physical properties

In another experiment, the denture base-resins were stored in artificial saliva at 37 °C during daytime and in the denture cleaner at night after running the device for 5 min. Also, in case of the chemical cleanser, the resins were immersed in the cleanser solution for 5 min, washed with filtered tap water, and then stored in tap water at night. These procedures were repeated for total of 150 days. The physical properties of the denture base-resins were then analyzed. The surface roughness of the specimen was assessed by stylus and optical based methods on an atomic force microscope (SPM-9700, Shimadzu, Kyoto, Japan) and three different spots on each sample were measured.

The surface harnesses of the specimens were measured by a micro Vickers hardness testing machine (HM-200, Mitutoyo, Kawasaki, Japan). Air was blown over the specimens to remove surface solution, and the specimens were analyzed at a load of 500 gf for 20 s using a pyramid-shaped diamond indenter. Three indentations were made on the surface of the specimens at separated locations, spaced at least 1 mm from adjacent indentations or the specimen periphery. The diagonal of the resulting indentations was measured using a microscope, and the Vickers hardness values displayed on the digital readout of the machine were recorded. A Vickers hardness number (VHN) was then calculated for each specimen. Finally, the colorimetric analysis of the specimens was performed using a portable colorimeter (VITA Easyshade®, VITA Zahnfabrik, Bad Sackingen, Germany). The measurements were performed in the CIE L*a*b* system, and mean values for the specimens were calculated. The L* value is a level of the whiteness or brightness of the specimen. The a* value is the level of redness or greenness, and the b* value is the level of yellowness or blueness. The color changes (ΔE) were calculated using the following equation; $\Delta E^* = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$ and were quantified by the National Bureau of Standards (NBS) units via the formula (ΔE x 0.92) to relate a change to the clinical environment. The critical levels of color differences according to the NBS are shown in Table 1.

Investigation of *C. albicans* attachment on the disk with physical changes

The biofilm formation capacities of *C. albicans* were investigated for the denture base-resins treated with the chemical cleanser and the denture cleaning device. After treating the specimens with the chemical cleanser and the denture cleaning device for 150 days, the specimens were placed into each well of a 12-well plate, and 1 ml of *C. albicans* suspension (1×10^6 cell/ml) was inoculated in the well. The plates were incubated at 37 °C for 4 h in 5% CO_2 incubator to investigate the initial adherence of *C. albicans* on the disks treated with the chemical cleanser and the denture cleaning device for 150 days.

Table 1 Critical marks of color difference by national bureau of Standards.

Critical marks of color difference	Textile terms (NBS unit)
Trace	0.00–0.5
Slight	0.5–1.5
Noticeable	1.5–3.0
Appreciable	3.0–6.0
Much	6.0–12.0
Very much	>12.0

Evaluation of cytotoxicity of EW generated by denture cleaning device

The cytotoxicity of the EW generated by the denture cleaning device and the chemical cleanser solution were investigated. The EW and the chemical cleanser solution were treated on human gingival fibroblast (HGF-1) for 30 min, and the cell viability was investigated by the MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Statistical analysis

Statistically significant differences between the untreated control and the EW- or the chemical cleanser-treated samples were analyzed by the Kruskal-Wallis test and Mann-Whitney U-test by IBM SPSS Statistics ver. 22 software (IBM, Armonk, NY, USA). *P*-values less than 0.05 were considered to be statistically significant.

Results

Antifungal activity of denture cleaning device against *C. albicans* biofilm

A hyphal *C. albicans* biofilm on the surface of the denture base-resin was observed with a phase contrast microscope after disrupting the biofilm (data not shown). After the biofilm formed, the denture base-resin was immersed into the denture cleaning device, the device operated, and the resin disk was stored in the device overnight. The levels of *C. albicans* biofilm on the surface of the resin disk were significantly reduced ($P < 0.05$) (Fig. 1). Moreover, when the resin disk was treated with the chemical cleanser according to the manufacturer's manual and washed with sterilized tap water followed by storing in a glass beaker containing tap water overnight, the chemical cleanser also significantly reduced the levels of *C. albicans* biofilm on the resin disk.

Effect of denture cleaning device on physical properties of denture base-resin

Next, we investigated the effect of the denture cleaning device on the physical properties of the resin disk. The average of the surface roughness values is presented in Table 2. Upon immersion of the resin disk in denture cleaning device and the chemical cleanser, the surface roughnesses of the specimens did not show significant differences (Fig. 2 and Table 2). For all denture resins investigated, the changes in surface roughnesses were less than 20 μm. For the surface hardness, the chemical cleanser significantly decreased the levels of the Vickers hardness in the resin disk after 150 days compared to the initial hardness ($P < 0.05$). Tap water and denture cleaner also significantly reduced the Vickers hardness with similar differences between the initial and 150 days treatment hardnesses ($P < 0.05$) (Table 3). However, when the surface roughness was compared between samples immersed in tap water and the EW denture cleaning device or the

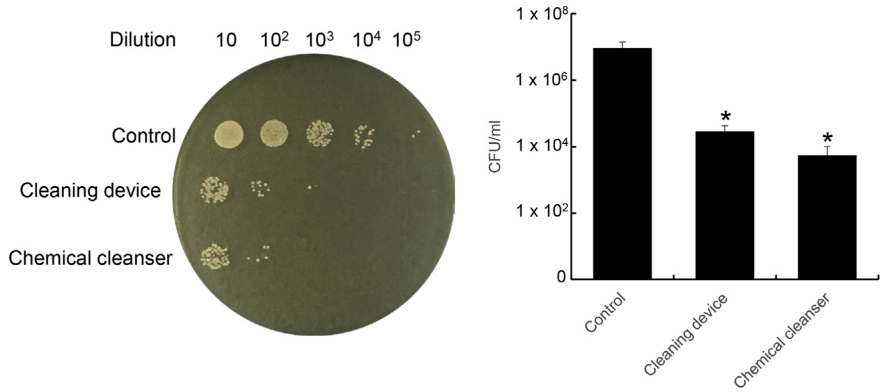

Figure 1 Antibiofilm activity of the denture cleaning device and the chemical cleanser against *C. albicans* biofilm. *C. albicans* biofilm was formed on the denture base-resin, and the biofilm formed the resins were soaked in the denture cleaning device and the chemical cleanser solution. After disrupting the biofilm, the fungal suspension was inoculated on TSA plate. The plates were incubated, and the colonies were counted. Asterisk (*) was indicated significant difference compared to untreated control group ($P < 0.05$).

Table 2 The investigation of surface roughness of denture base-resin.

	Ra (Mean ± STDEV)	
	Initial	After 150 days
Control (Tap water)	307.45 ± 16.49 nm	312.28 ± 20.54 nm
Chemical cleanser	311.07 ± 21.55 nm	329.12 ± 28.16 nm
Denture cleaning device	308.26 ± 22.34 nm	318.32 ± 24.94 nm

Table 3 The change of surface hardness of denture base-resin

	VHN (Mean ± STDEV)	
	Initial	After 150 days
Control (Tap water)	18.49 ± 0.52	17.02 ± 0.45[a]
Chemical cleanser	18.34 ± 0.48	15.63 ± 0.33[a,b]
Denture cleaning device	18.42 ± 0.41	16.95 ± 0.37[a]

[a] Significant difference between initial and after 150 days.
[b] Significant difference compared to control.

chemical cleanser, the levels between tap water and EW denture cleaning device did not show a significant difference, but those between tap water and the chemical cleanser differed significantly ($P < 0.05$). The color changes of the denture base-resin were 0.477 ± 0.076, 0.612 ± 0.095, and 0.562 ± 0.096 upon treatment with tap water, the chemical cleanser, and the denture cleaning device, respectively. When the color changes were computed in NBS units, the denture cleaning device and the chemical cleanser showed "Slight" color differences (Table 4).

Evaluation of initial attachment of *C. albicans* on the resin disk

We examined *C. albicans* colonies on the denture base-resin treated with the chemical cleanser and the denture cleaning device for 150 days. Initial adherence of *C. albicans* showed higher levels on the chemical cleanser-treated denture base-resin than on the denture cleaning device-treated specimen (Fig. 3). Furthermore, *C. albicans* adherence exhibited significant differences between the tap water and the chemical cleanser-treated resin

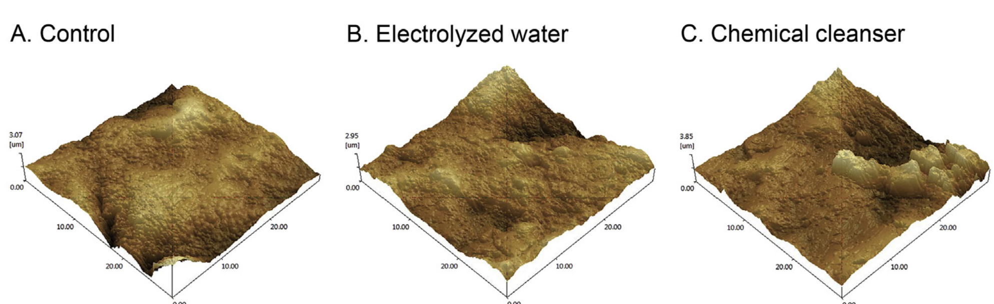

Figure 2 Effects of EW and chemical cleanser on surface roughness of denture base-resin. The denture base-resins wee immersed in the EW from denture cleaning device and the chemical cleanser solution for 150 days, and the surface roughness of the denture base-resin was analyzed by atomic force microscope.

Table 4 The color change of denture base resin after treatment for 150 days.

	ΔE	SDTEV	NBS Unit
Control (Tap water)	0.477	0.076	0.443
Chemical cleanser	0.612[a]	0.095	0.578
Denture cleaning device	0.562	0.096	0.542

[a] Significant difference compared to control group.

($P < 0.05$). However, there were not significant differences between specimens treated with the tap water and the denture cleaning device.

Examination of cytotoxicity

Finally, the chemical cleanser and the EW generated by the denture cleaning device were examined cytotoxicity. The chemical cleanser significantly reduced HGF viability, whereas the EW did not affect the viability (Fig. 4).

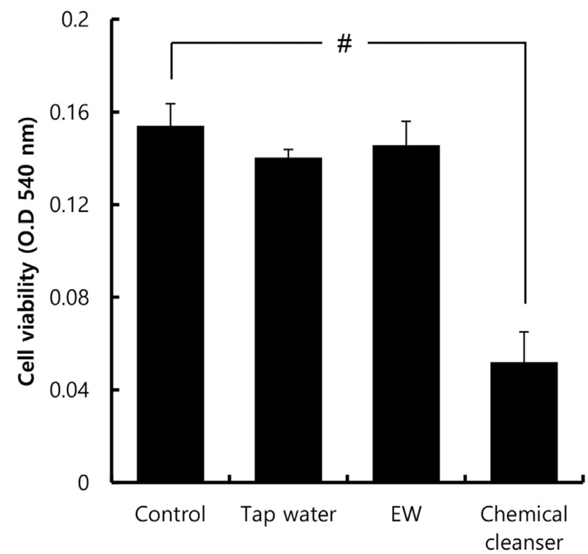

Figure 4 Cytotoxicity assay of EW and the chemical cleanser. Human gingival fibroblast were treated with EW and the chemical cleanser solution. The cell viability was examined by MTT assay. Sharp (#) was indicated significant difference compared to untreated control group ($P < 0.05$).

Discussion

C. albicans is a normal flora and forms a biofilm with hyphae form in the oral cavity, and a denture surface provides favorable conditions for the formation of *C. albicans* biofilm. Furthermore, since *C. albicans* is frequently detected in denture stomatitis patients, this fungus is considered to be a denture stomatitis related pathogen. Moving into an aging society, use of dentures has increased. Therefore, denture hygiene is considered very important, especially for the elderly with weak immune systems. For these reasons, denture cleansers have been developed for effective oral hygiene. Recently, a device using EW for cleaning denture was developed. In this study, the antifungal activity of the denture cleaning device was investigated after the formation of *C. albicans* biofilm on the denture base-resin, and the changes of the physical properties of the denture base-resin by denture cleaning device were examined.

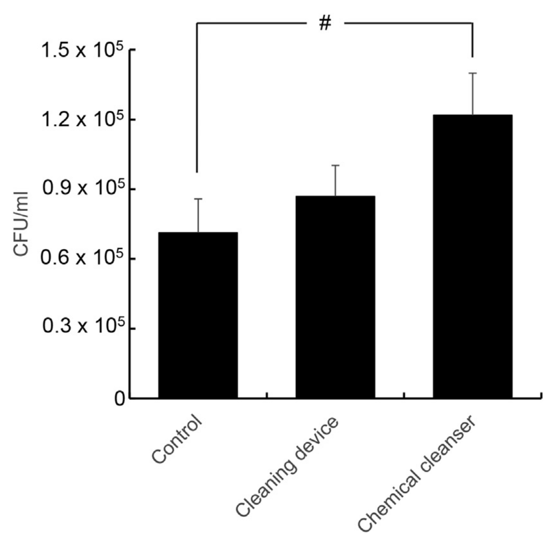

Figure 3 Comparison of *C. albicans* binding on EW and the chemical cleanser treated the resins. The resins were treated with EW and the chemical cleanser solution for 150 days, and the treated resins were reacted with *C. albicans* suspension for 12 h in a Co_2 incubator. The binding *C. albicans* was detached and inoculated on TSA plate. The plate was incubated, and the colonies were counted. Sharp (#) was indicated significant difference compared to untreated control group ($P < 0.05$).

EW has been reported to possess the antimicrobial activity against various pathogens as well as bacterial biofilms.[14,19,20] For these reasons, the electrolyzed water has been used in many food industries for washing vegetables to remove bacteria. Recently, a device using EW has also been developed in the dental field, and denture cleaning device and gargle solution generators have been produced. When the denture cleaning device was investigated the antifungal activity against *C. albicans* biofilm after formation of the biofilm on the resin disk, the denture cleaning device reduced the count of *C. albicans* on denture base-resin. Compared to the popular denture cleanser as Polident, the denture cleaning device showed weaker antifungal activity. EW with tap water has no reported side effects, and EW has been reported to provide benefits for human health, such as antitumor and anti-inflammatory effects,[17,18] whereas occasional side effects of chemical denture cleansers have been reported due to misuse.[9,21] Therefore, considering that older people mainly use denture cleansers, a denture cleaning device that can be used safely may be better, even with a slightly weaker antifungal activity. In particular, the denture cleaning device showed more antifungal activity upon adding 0.001% sodium chloride and completely killed *C. albicans* biofilm on denture base-resin upon adding 0.009% sodium chloride (data not shown). However, at concentrations above 0.02% sodium chloride, the device did not work due to its built-in overcurrent protection.

The problem with conventional denture cleansers is that they change the physical properties of the denture materials when used for long periods.[22–24] PMMA disk as a denture base-resin was treated with the chemical cleanser and the denture cleaning device for 150 days, and the physical properties of the denture base-resin were analyzed. Comparing the chemical cleanser and the denture cleaning device, the denture cleaning device-treated disk showed a smaller increased of surface roughness and decreased of surface hardness compared to the chemical cleanser-treated disk. Furthermore, the change of surface hardness and surface hardness were not significantly different between the denture cleaner and tap water. In case of color change, the difference between the chemical cleanser and the denture cleaning device did not show. The denture cleaning device may have less effect on the physical properties of denture base-resin than the chemical cleanser. High conductivity of a palladium electrode may produce oxygen radicals (O^-), free chlorine (Cl-), hypochlorous acid (HClO) and reactive hydrogen (H) due to the negative oxygen reduction potential (ORP) and high concentration of dissolved hydrogen. The chemical reactions in electrolysis of tap water are $H_2O \rightarrow 1/2\ O_2 + 2H^+ + 2e^-$, $2Cl^- \rightarrow Cl_2 + 2e^-$ and Cl_2 (aq) + $H_2O \leftrightarrow HClO + HCl$ at the anode, and $H_2O + 2e^- \rightarrow 1/2H_2 + OH^-$ at the cathode. Among the components, hypochlorous acid has the strongest antimicrobial activity.[25] Also, *C. albicans* is susceptible to acids.[26,27] Therefore, hypochlorous acid may also have antifungal activity against *C. albicans*. In this study, the EW with added NaCl stronger antifungal activity against *C. albicans* than the pure EW. The EW containing NaCl may have contained higher quantities of hypochlorous acid, and more antifungal activity. Although, sodium hypochloride (NaOCl) was generated by electrolyzing tap water with NaCl, NaOCl is unstable in aqueous condition and rapidly reacts with H_2O, followed by the production of HOCl and NaOH ($NaOCl + H_2O \rightarrow HClO + NaOH$).[28] The effects of the physical changes of the resin disk caused by the denture cleaning device and the chemical cleanser on the early colonization of *C. albicans* were investigated. The adherence of *C. albicans* had higher values on the chemical cleanser-treated the resin than on the denture cleaning device-treated resin disk. A significant difference of *C albicans* adherence between tap water and the denture cleaning device was not found. *C. albicans* adherence on denture acrylic resin is correlated with surface roughness of the acrylic resin.[29] Therefore, the increased surface roughness caused by the chemical cleanser may have increased *C. albicans* adherence and biofilm formation when the chemical cleanser was used for washing denture, whereas *C. albicans* adherence on the denture cleaning device-treated resin did not exhibit significant differences compared to that of the non-treated resin. Based on these results, when the denture cleaning device is used to clean dentures, the dentures may be used hygienically for long periods because there is little physical change. Finally, the EW is known to be beneficial, not harmful for human health.[17,18] Therefore, this EW by the denture cleaning device was investigated cytotoxic effect on human oral tissue. The EW did not affect human gingival fibroblasts.

Base on the above results, the denture cleaning device showed satisfactory results for cleaning denture materials due to its antifungal activity hyphal *C. albicans* biofilms on a denture base-resin. Furthermore, this device had little effect on the physical properties of the denture base-resin, such as surface roughness, surface hardness, and color. Therefore, the denture cleaning device may be suitable for use by the elderly without the side effects caused by the misuse of chemical cleansing agent.

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