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Research article

Removal effect of *Candida albicans* biofilms from the PMMA resin surface by using a manganese oxide nanozyme-doped diatom microbubbler

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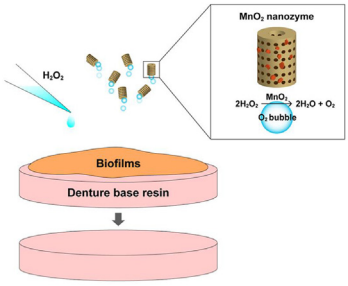
GRAPHICAL ABSTRACT



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ABSTRACT

To prevent oral candidiasis, removal of the *Candida* biofilms from dentures is important. However, common denture cleaners are insufficiently effective in removing biofilms. A manganese oxide (MnO_2) nanozyme-doped diatom microbubbler (DM) can generate oxygen gas microbubbles by a catalase-mimicking activity in hydrogen peroxide (H_2O_2). DM can invade and destroy biofilms with the driving force of continuously generated microbubbles. In this study, the *Candida* biofilm removal efficiency by co-treatment of DM and H_2O_2 was investigated. Diatom particles were reacted with (3-aminopropyl)triethoxysilane to prepare amine-substituted diatom particles. These particles were reacted with potassium permanganate to fabricate DMs. The morphology and components of DM were analyzed by using a scanning electron microscope (SEM). Four types of denture base resin specimens on which biofilms of *Candida albicans* were formed were treated with phosphate-buffered saline (PBS group), Polident 5-Minute (Polident group), 0.12% chlorhexidine gluconate (CHX group), 3% H_2O_2 (H_2O_2 group), and co-treatment of 3 mg/mL of DM and 3% H_2O_2 (DM group). The biofilm removal effect of each group was quantitatively analyzed by crystal violet assay, and the results were visually confirmed by SEM images. After each treatment, the remaining *C. albicans* were stained with Hoechst 33342/propidium iodide, and observed with confocal laser scanning microscopy (CLSM) to evaluate the viability. MnO_2 nanozyme sheets were successfully doped on the surface of the fabricated DM. Although biofilms were not effectively removed in the Polident and CHX groups, CLSM images showed that CHX was able to effectively kill *C. albicans* in the biofilms on all resin specimen types. According to the crystal violet analysis, the H_2O_2 groups removed the biofilms on

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heat-activated and 3D-printed resins ($P < .01$), but could not remove the biofilms on autopolymerizing and milled resins significantly ($P = .1161$ and $P = .1401$, respectively). The DM groups significantly removed *C. albicans* from all resin specimen types ($P < .01$).

1. Introduction

Oral candidiasis is denture-related stomatitis, the most common human fungal infection [1]. This oral disease is caused by the *Candida* species [2]. *Candida* can penetrate the oral mucosal tissue, evade the host's defensive mechanisms, and have several virulence factors [3]. *Candida albicans* is a primary microbe in denture-related stomatitis. [2], and there is a greater number on the dentures' surface covering the mucous membrane than on the patient's mucosa itself, indicating that the dentures can become infection reservoirs [4, 5].

Polymethyl methacrylate (PMMA) denture base is porous and rough, so *Candida* can easily adhere and form a biofilm [6, 7]. The presence of biofilms on dentures has been associated with denture stomatitis, as well as with systemic conditions, especially in elderly patients [8]. To prevent denture stomatitis caused by *Candida* and protect the patients' health, it is necessary to remove the biofilm formed on the denture using an appropriate cleaning procedure [9, 10].

Dentures can be cleaned mechanically and chemically, as well as with a combination of the two [11]. Mechanical methods generally use brushing with or without dentifrice [12]. Since it is difficult for the elderly or disabled patients to use correct mechanical cleaning methods, the use of chemical methods is recommended [13, 14]. Chemical denture cleaners such as hydrogen peroxides, chlorhexidine digluconate, and commercial effervescent denture cleaners have been conventionally used [15, 16, 17, 18] However, these cleaners are insufficiently effective in removing matured biofilms, especially those formed by *Candida.* [19, 20] The *C. albicans* biofilm on dentures has resistance to denture cleaners and antifungal drugs [21, 22].

Manganese oxide nanozyme-doped diatom microbubbler (DM), a recently developed material used as an active cleaning agent, is a hollow cylinder-shaped diatom biosilica with manganese oxide (MnO_2) nanozyme sheets [23]. DM generates oxygen gas bubbles by rapidly decomposing H_2O_2 with the catalase-mimicking activity of MnO_2 in H_2O_2. In a previous study, DM invaded and destroyed *Escherichia coli* biofilm with the driving force of continuously generated microbubbles, and H_2O_2 molecules diffused into the biofilm, effectively removing it [23].

This research aimed to evaluate the feasibility of using DM as a novel denture cleaner. The effect of DM on *C. albicans* biofilms' removal formed on heat-activated, autopolymerizing, 3D-printed, and milled acrylic resin specimens was studied and compared to conventional denture cleaners. This study's null hypothesis is that the effectiveness of the denture cleaners tested would be similar.

2. Materials and methods

2.1. Fabrication of the DMs

DMs were fabricated as described previously [23]. For preparing amine-substituted diatom particles, 2 g of diatom particles (2 g) and 60 mL of toluene were placed in a three-necked round-bottom flask with a reflux condenser, a thermometer and an N_2 gas tube. After that, 0.6 mL of distilled water was mixed and stirred at room temperature for 2 h. Then, 3.4 mL of (3-aminopropyl)triethoxysilane was added to the mixture and refluxed for 6 h at 60 °C. After cooling the mixture, it was washed three times sequentially with toluene, 2-propanol, and distilled water. After drying in a vacuum desiccator for 2 days, 0.1 g of amine-substituted

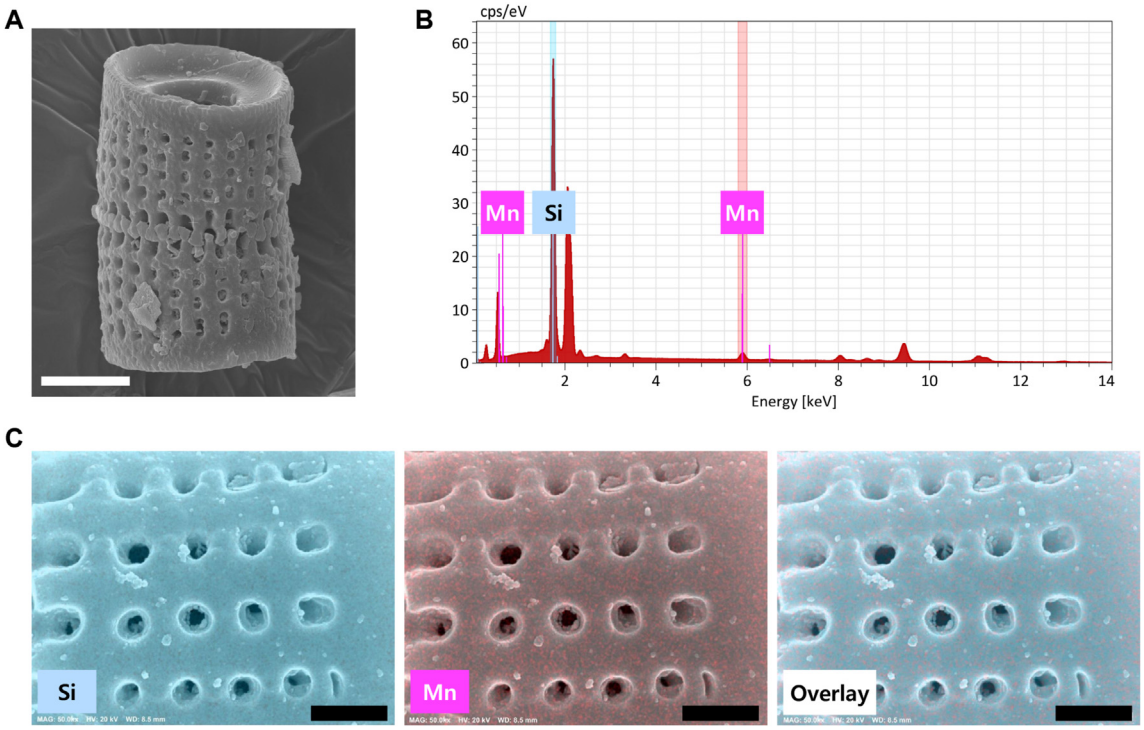

Figure 1. Fabrication of a MnO_2 nanozyme-doped diatom microbubbler (DM). A, Scanning electron microscopy images of a DM (magnification: 15000 times, white scale bar = 5 μm). B, Representative elemental spectrum of a DM obtained from an energy-dispersive spectrometer. C, EDS digital images of element Si. D, EDS digital images of element Mn. E, Overlay EDS digital images of elements Si and Mn (black scale bar = 800 nm).

diatom particles was added to 1 mL of potassium permanganate solution (50 mM) and sonicated at room temperature for 30 min. Finally, the samples were washed three times sequentially with distilled water and ethanol, and dried in an oven at 60 °C for 1 day.

2.2. Physicochemical characterization of DMs

The scanning electron microscopy (SEM) images for observing the morphology of DMs were obtained with Apreo S (Thermo Fisher Scientific) operating at 10.0 kV. The element mapping of DMs was analyzed using an energy-dispersive spectrometer coupled with the SEM system at 20.0-kV acceleration voltage.

2.3. Preparation of denture base acrylic resin specimens

A total of 240 disk-shaped (Ø10 × 2-mm) denture base acrylic resin specimens were prepared by using four fabricating techniques as follows (60 disks for each fabricating technique): autopolymerization (Vertex Self-Curing; Vertex Dental), heat-activated polymerization (Meliodent Heat Cure; Heraeus Kulzer GmbH), milling (Pink PMMA BLOCK; Huge Dental Material), and 3D printing (Denture Plus ARUM 5.0; ARUM Dentistry). The specimens were designed with a CAD software program (Meshmixer, Autodesk; San Rafael) to prepare the same design and size regardless of the manufacturing techniques. Autopolymerizing and heat-activated resin specimens were fabricated by using a conventional flasking and pressure-pack technique. Milled resin specimens were fabricated with a milling machine (DEG-5X100; ARUM Dentistry). The 3D-printed resin specimens were fabricated by using a digital light processing 3D printer (ASIGA MAX UV; ASIGA) and post-polymerized with an ultraviolet light-polymerization unit (PURE PRO; U-Dent) according to the manufacturer's instructions. The prepared resin specimens were rinsed for 5 min in an ultrasonic cleaner and immersed in distilled water for 24 h [24]. After that, the specimens were sterilized under ultraviolet light for 8 h per side and then stored in sterile bags [16].

2.4. Biofilm formation on resin specimens

To create a biofilm, resin specimens were coated with saliva [25]. Unstimulated saliva was collected in sterile plastic tubes at least 1.5 h after eating, drinking, or tooth brushing. The ethical approval for this research was obtained from the Institutional Review Board (IRB) of Seoul National University Dental Hospital (CRI 22008). Besides, written informed consent was obtained from all the participants prior to the study. Collected saliva was centrifuged (12000 rpm, 10 min, 4 °C), and only the supernatant was mixed with PBS (pH = 7.4) with 1:1 (v/v) ratio. This mixture was filtered through a 0.2-μm pore size Minisart syringe filter (Sartorius Stedim Biotech GmbH). The sterile resin specimens were placed in a 24-well tissue culture plate and incubated with a filtered saliva/PBS mix for 2 h at 37 °C. After removal of saliva, 1 mL of *C. albicans* (ATCC 18804) cultured in yeast-malt extract broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1.0% dextrose,

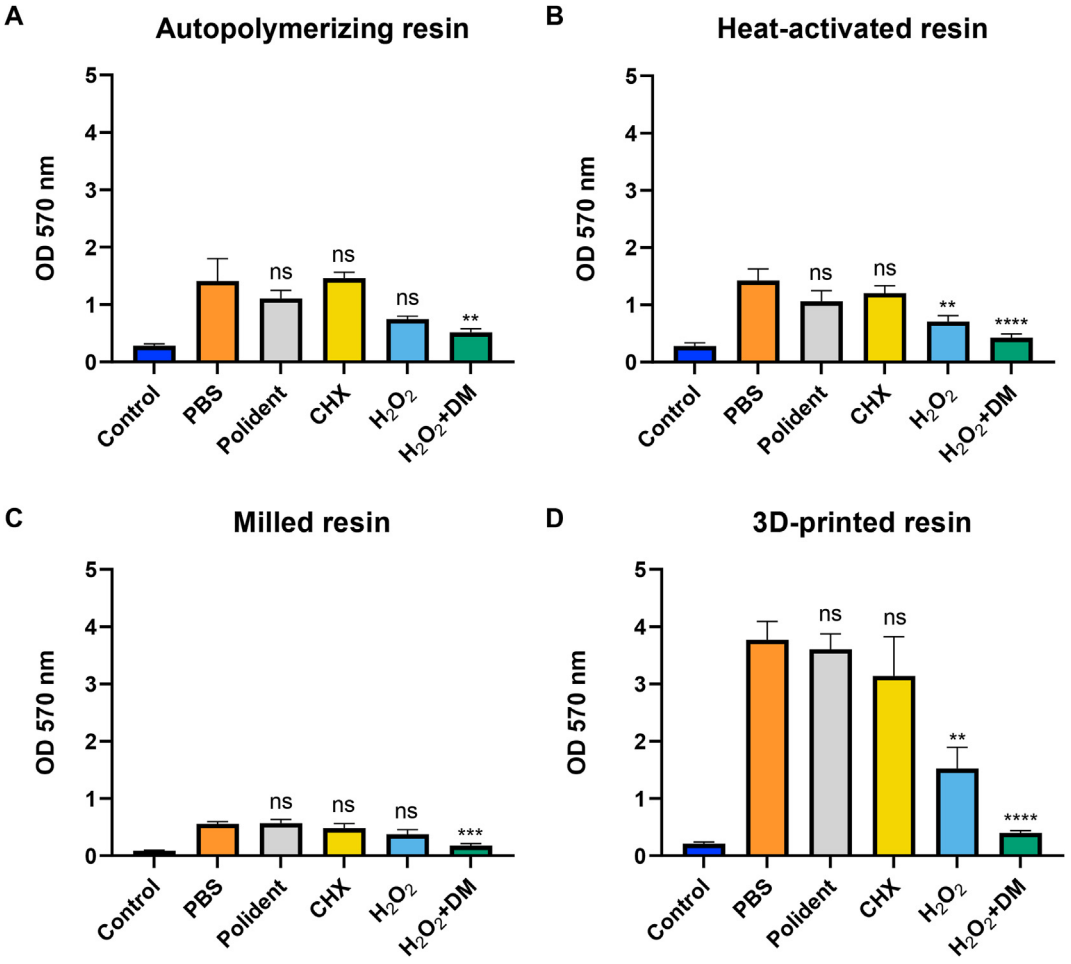

Figure 2. *Candida albicans* biofilm removal effect evaluated using crystal violet analysis. Denture base acrylic resin specimens were prepared by using four fabricating techniques. A, Autopolymerizing resin. B, Heat-activated resin. C, Milled resin. D, 3D-printed resin. Data are expressed as mean values ± SEMs. The Kruskal–Wallis test is performed with Dunn's multiple comparisons test. The following symbols represent statistically significant differences between the PBS and experimental groups (ns: not significant, *: $P < .05$, **: $P < .01$, ***: $P < .001$, and ****: $P < .0001$).

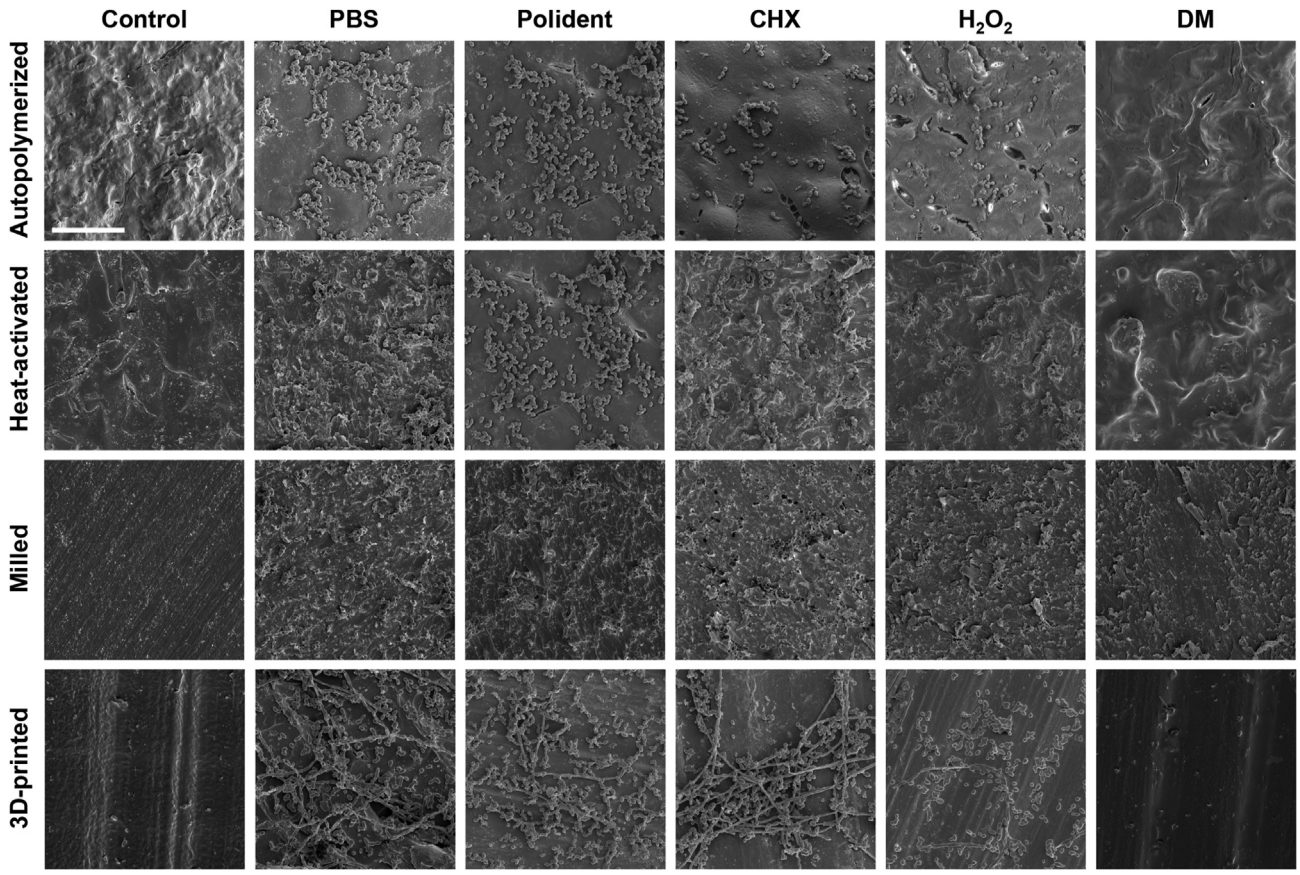

Figure 3. Scanning electron microscopy images of denture base acrylic resin specimens after each biofilm removal treatment. Each resin type was arranged in each row, and each treatment type was arranged in each column (magnification: 2000 times, scale bar = 50 μm).

concentration 1×10^6 cells/mL) was added and incubated aerobically for 24 h at 37 °C.

2.5. Biofilm removal treatments and crystal violet assay

The 60 resin specimens prepared with the same fabrication technique were randomly assigned to six groups (n = 10). After biofilm formation, the resin specimens were gently washed with PBS and treated according to each group's corresponding protocol. The concentration and application time of each group's agents were determined based on previous studies or the manufacturer's instructions: PBS group, PBS for 10 min; Polident group, Polident 5-Minute (GlaxoSmithKline) for 5 min (manufacturer's instructions), CHX group, 0.12% (w/v) CHX for 10 min; H_2O_2 group, 3% (v/v) H_2O_2 for 10 min; DM group, co-treatment of 3 mg/mL of DM and 3% H_2O_2 for 5 min; and negative control (no contamination to verify asepsis of the experiment), PBS for 10 min [16, 23].

After each treatment, the remaining biofilms were quantified by crystal violet assay [26]. The resin specimens were washed with PBS and incubated with 1 mL of 1% (w/v) crystal violet solution (Junsei Chemical) for 10 min to stain the remaining biofilm on the resin specimens. After that, the specimens were rinsed three times with PBS to remove the residual dye. The remaining crystal violet dye in the biofilms was extracted by using 95% ethanol. The dissolved crystal violet dye's optical density was quantified using a microplate reader (Epoch 2; Bio-Tek Instruments) at 570 nm.

2.6. SEM analysis

To visually confirm the crystal violet assay results, SEM analysis was performed as described previously [27]. After each treatment, the resin specimens were fixed with 1 mL of 4% paraformaldehyde for 4 h and washed three times with 1 mL of PBS for 15 min. After that, the specimens were fixed with 1 mL of 1% osmium tetroxide for 60 min and rinsed three times with 1 mL of PBS for 15 min. The specimens were dehydrated in a successively increasing ethanol concentration for 15 min each at 70%, 80%, 90%, 95%, and 100%. Subsequently, the resin specimens were treated in 1 mL of 100% hexamethyldisilazane for 20 min. After the specimens were completely dried, platinum coating was performed. Each resin specimen was examined by SEM at a 10-kV voltage.

2.7. Biofilm analysis by confocal laser scanning microscopy (CLSM)

The viability of *C. albicans* remaining after each treatment was evaluated by using the CLSM. The specimens were stained in broth containing 5 μg/mL Hoechst 33342 (Invitrogen-Life Technologies) and 5 μg/mL propidium iodide (Invitrogen-Life Technologies) for 30 min at 4 °C as previously described [28]. Each specimen was washed three times with PBS and placed upside-down on glass-bottomed confocal dishes (SPL Life Science) with BacLight mounting oil (Thermo Fisher Scientific). The images were obtained by using a CLSM instrument (LSM700; Carl Zeiss) equipped with 405- and 555-nm excitation lasers.

2.8. Statistical analysis

All data are presented as mean value ±SEM (standard error of the mean). The significance of the differences among groups was determined by the Kruskal–Wallis test followed by Dunn's multiple comparisons test ($\alpha = .05$), since the data's normality and homoscedasticity assumptions had been violated. GraphPad Prism 9 (GraphPad) was used for statistical analyses.

3. Results

3.1. Physicochemical characterization of DMs

Fossilized *Aulacoseira* diatom particles in the form of hollow cylinders (approximately 10 μm in diameter and 18 μm in length) with many holes (approximately 500 nm in diameter) on the surfaces were used in this study (Figure 1A). Elemental analysis through SEM revealed that MnO_2 nanozymes were uniformly doped on the diatom particles' silica surfaces (Fig. 1B and C).

3.2. Crystal violet assay

The crystal violet assay results represent the total *C. albicans* biofilms remaining on the acrylic resin specimens after each treatment (Figure 2). In the case of autopolymerizing acrylic resin, OD values of DM group (0.51 ± 0.06) showed significantly lower than the PBS group (1.41 ± 0.39) ($P < .01$), but Polident, CHX, and H_2O_2 groups showed no significant difference from the PBS group (Figure 2A). Even in the case of milled resin, only DM Group (0.17 ± 0.04) showed significantly lower OD values than PBS Group (0.56 ± 0.04) ($P < .001$), but the other treated groups showed no significant difference from the PBS group (Figure 2B). In the case of OD values of heat-activated acrylic resin specimens, the H_2O_2 and DM groups had significantly lower values (0.71 ± 0.10 and 0.43 ± 0.07, respectively) than the PBS groups (1.42 ± 0.20) ($P < .01$ and $P < .0001$, respectively), but Polident and CHX groups showed no significant difference from the PBS group (Figure 2C). In the case of 3D-printed resin, OD values of H_2O_2 group (1.52 ± 0.37) and DM group (0.40 ± 0.04) were significantly lower than the PBS groups (3.77 ± 0.32) ($P < .01$ and $P < .0001$, respectively), the other treated groups showed no significant difference from the PBS group (Figure 2D).

3.3. SEM analysis

To visually confirm the *C. albicans* biofilm removal efficiency, four types of acrylic resin specimens were subjected to SEM imaging after each treatment (Figure 3). The *C. albicans* cluster was similarly observed in the PBS, Polident, and CHX groups. In the H_2O_2 groups, a relatively small number of *C. albicans* was observed. In the DM groups, very few *C. albicans* and fragmented or unrecognizable biofilm remnants were observed.

3.4. Biofilm analysis by CLSM

According to the CLSM images of all acrylic resins types (Figure 4), *C. albicans* stained with propidium iodide were more clearly observed in the CHX groups. In the H_2O_2 group, a relatively small number of cells was observed compared to that in the PBS group, and there were few remaining cells in the DM group.

4. Discussion

The present study's results demonstrated that each tested denture cleaner showed a different biofilm reduction on the acrylic resin specimens; therefore, the null hypothesis was rejected. In the crystal violet assay results, the Polident and the CHX groups did not show a significant difference from the PBS group (Figure 2). Polident and CHX could not effectively remove the biofilms of the denture base resin specimens. H_2O_2 effectively removed the biofilms of heat-activated and 3D-printed resin specimens and showed no significant difference from the PBS group in other resin types (Figure 2). When compared to the PBS groups, DM is the only group to have significantly removed more *C. albicans* from the four kinds of acrylic resin specimens (Figure 2).

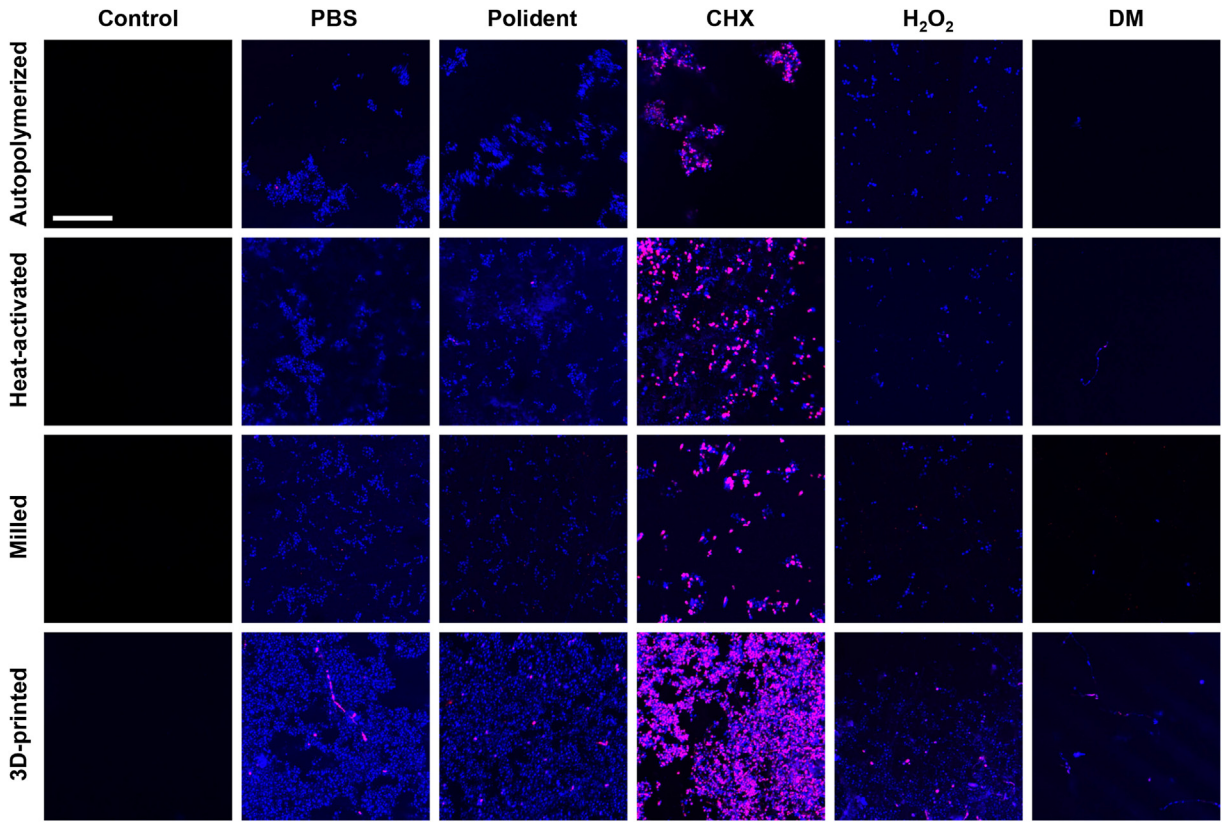

Figure 4. Confocal laser scanning microscopy images of the remaining biofilms on the denture base acrylic resin specimens after each biofilm removal treatment. The dual-staining method using Hoechst 33342/propidium iodide was used to evaluate the viability of the remaining *Candida albicans* after treatment. Both live and dead cells are stained with Hoechst 33342, and dead cells are stained with propidium iodide. Each resin type was arranged in each row, and each treatment type was arranged in each column (scale bar = 50 μm).

The SEM images were observed to visually confirm the biofilm removal efficiency, which supported the crystal violet assay results (Figure 3). The remaining biofilms of the Polident and CHX groups were similar to those of the PBS groups, but relatively few biofilms were observed in the H_2O_2 groups. In the DM groups, only fragmented or unrecognizable biofilm remnants were observed. To evaluate the viability of *C. albicans* remaining after each treatment, the specimens were stained and observed with CLSM (Figure 4), and a dual-staining method using Hoechst 33342/propidium iodide was used [28, 29]. Hoechst 33342 can cross cell membranes and stain the DNA of living and dead cells; in contrast, propidium iodide selectively labels dead cells as it only enters cells with damaged plasma membranes [29]. The CHX groups did not significantly remove biofilms, but effectively killed *C. albicans* (Figure 4).

Polident is a denture cleaning tablet that has been reported in previous studies to be effective [30]. However, in this study, Polident could not effectively remove the biofilms or kill *C. albicans*, and these results are similar to those reported by previous studies [18, 31]. CHX has been reported to show high effectiveness against *C. albicans* [32], but this agent did not effectively remove the biofilms according to the crystal violet assay result. Da Silva et al. evaluated the effect of CHX on killing *C. albicans* by culturing biofilms, and the same results can be confirmed with the CLSM images in the present study. Therefore, CHX can kill *C. albicans*; however, the dead *C. albicans* and exopolymeric substances were not removed. H_2O_2 could remove biofilms on heat-activated and 3D-printed resins, but did not show a significant effect on the other two resin types. Martínez-Serna et al. reported that the effect of reducing *C. albicans* was insufficient when H_2O_2 was used alone [16].

The generated O_2 gas bubbles nucleate and form microbubbles inside the diatoms' hollow space [23]. As the bubbles build up the pressure, the DM particles continuously eject microbubbles and move randomly, propelled by driving forces. DM particles penetrate the biofilms, and the continuous generation of O_2 gas and the diffusion of H_2O_2 become possible within the biofilms [23]. Consequently, the biofilms could be removed more effectively in the DM than in the H_2O_2 groups. Practitioners and patients can perform cleaning procedure by placing a denture sprinkled with DM particles in a denture case containing 3% H_2O_2 solution.

In this study, resin specimens were prepared by four fabricating techniques to evaluate the effect of removing biofilms on various denture base acrylic resin types used in general dental practice. Unpolished resin specimens were used in this study because the *C. albicans* main reservoir is the dentures' surface covering the mucosa, and *C. albicans* can more easily penetrate the unpolished surface [33, 34]. Regardless of the resin fabricating technique, DM effectively removed the biofilms.

In this study, the DM concentration was set to 3 mg/mL, and the treatment period was 5 min according to the previous study [23]. Co-treatment of 3 mg of DM and 3% H_2O_2 solution could effectively decrease the cell viability a lot within 5 min [23]. This study is the first to report the possibility of DM application as a novel candidate for denture cleaning. Further studies are needed on the appropriate DM concentration and application time for denture cleaner use in clinical practice. It is also necessary to study the effect of removing multispecies biofilms and the biofilms on the dentures used by patients. DM can be helpful in dental clinical situations where biofilm removal is required. This material has the potential to be used for cleaning other prostheses, mouthwash for orthodontic patients, root canal treatment, and treatment of periodontitis and peri-implantitis.

5. Conclusions

Based on the findings of this in vitro study, the following conclusions were drawn:

1. Co-treatment of DM and H_2O_2 effectively removed *C. albicans* biofilms formed on autopolymerizing, heat-activated, milled, and 3D-printed denture base resin specimens.
2. H_2O_2 effectively removed the *C. albicans* biofilms of heat-activated and 3D-printed resin specimens, but showed no significant difference from the PBS group in autopolymerizing and milled resin.
3. CHX killed *C. albicans*, but did not effectively remove biofilms from the resin specimens.

Declarations

Author contribution statement

Eun-Hyuk Lee: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yun-Ho Jeon: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sun-Jin An: Contributed reagents, materials, analysis tools or data.

Yu-Heng Deng; Hyunjoon Kong: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ho-Beom Kwon; Young-Jun Lim: Conceived and designed the experiments.

Myung-Joo Kim: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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