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Research Article

In Vitro Antifungal Activity against Oral *Candida* Species Using a Denture Base Coated with Silver Nanoparticles

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Although oral *Candida* easily adheres to denture base materials, many denture detergents are effective only against bacteria but not against *Candida*. Silver nanoparticles (AgNPs), which are known to have potent antibacterial and antifungal activity, have been used in the prevention of oral candidiasis (OC). We evaluated the adherence of *Candida albicans* and *Candida glabrata* on a heat-cured Acron resin piece supported by AgNPs by low-vacuum scanning electron microscopy (SEM) and measuring colony-forming units. *C. albicans* and *C. glabrata* increasingly adhered to the resin surface of the control piece over time, but the adhesion AgNP of both *Candida* species to the AgNP-coated surface was significantly inhibited ($P < 0.001$). Low-vacuum SEM revealed that *C. albicans* and *C. glabrata* on the resin surface of control pieces appeared as oval colonies, with a major axis of 3-4 μm and a smooth cell wall, but those on the AgNP-coated resin surface were less abundant than the control and showed swollen yeast features, with a major axis of more than 5 μm and a corrugated cell wall. Our results suggest a way to prevent denture-associated OC by using denture base materials processed by AgNPs.

1. Introduction

Because oral care decreases the disease prevalence of respiratory tract infections of perioperative and elderly patients, as well as persons requiring nursing care [1], the importance of oral care is widely recognized. Progress in social aging has led to an increased population of denture-wearers, but denture cleaning is not easy for the elderly. Although the tedious care required renders denture wearing cumbersome, to such a patient or a person requiring social care, denture wearing is indispensable for food intake, as well as to maintain nutritional status. *Candida*, which easily adheres to denture base material, serves as a hotbed for mycotic stomatitis [2]. However, there are very few commercially available denture-cleaning agents effective against *Candida*. Metal ions such as silver, copper, and zinc have bactericidal action. The antibacterial activities and safety of silver have become apparent in recent years, resulting in its growing use in the fields of food and medicine. Silver nanoparticles (AgNPs) have high antimicrobial activity and have shown potent inhibition of *Candida*, equivalent to that of amphotericin B [3–5]. Studies on the targets and mechanisms of AgNPs have indicated that reactive oxygen species (ROS) and hydroxyl radicals produced by AgNPs disrupt the mitochondria and cell membrane of a fungus, leading to apoptosis [4, 6]. However, although AgNP-containing denture resin prevents the adhesion of *Candida albicans* to the resin [7], it does not influence the adhesion of other fungi or biofilm formation, despite antifungal activity [8]. In order to demonstrate the anticandidiasis action of AgNPs, we evaluated the application of AgNP-processed denture base AgNP for the prevention of oral candidiasis (OC).

2. Materials and Methods

2.1. Preparation of Silver-Incorporated Heat-Cured Resin. A heat-cured acrylic resin for denture base, Acron, was used in the study. A paraffin wax sheet (146 × 74 mm), with a 1.0 mm thickness (Bite Wax, Quest Corp., Japan), was embedded with

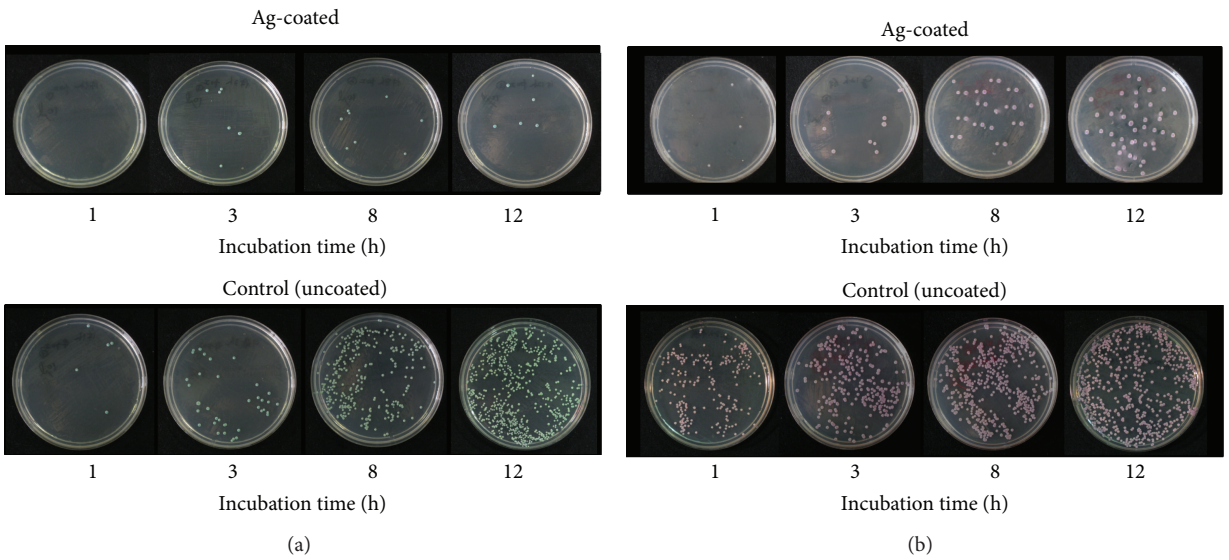

Figure 1: Macroscopic observation of (a) *Candida albicans* (*C. albicans*) and (b) *Candida glabrata* (*C. glabrata*) growth on CHROMagar *Candida* medium, in which the Acron pieces (AgNP-coated and uncoated) were rinsed with 10 mL PBS solution and incubated with the yeast suspension for 1, 3, 8, and 12 h.

plaster and washed with hot water after curing. A mixture of poly(methyl methacrylate) powder and solution (PMMA; Acron, GC Co. Ltd., Japan), at ratio of 100 g : 46 mL, was used to infill the gap in the plaster at 100°C and 15 MPa for 15 min. The plaster was carefully removed to obtain a heat-cured resin sheet with a 1.0 mm thickness, and the Acron piece was cut into 10 mm × 10 mm pieces to be used in the study. AgNPs were obtained by microwave irradiation of a solution mixture containing silver nitrate, polyethylene glycol, ethanol, water, and 24%/5% w/w aqueous ammonia in the presence of the Acron piece, yielding an Acron-AgNP composite [9]. An Acron piece without AgNP coating was used as the control.

2.2. Preparation of Yeast Culture Solution. The *Candida* standard strains used in the study were *C. albicans* (ATCC18804) and *C. glabrata* (ATCC90030). The suspension solution of the *Candida* standard strains was prepared in RPMI 1640 culture medium adjusted to a McFarland factor of 0.5 (absorbance at 530 nm).

2.3. Adhesion Assay and Analysis. Candidal adhesion to Acron with (processed) or without (control) silver incorporation was assayed in sterile microplates (12 wells/plates). Each piece was placed in a well containing 1 mL of the yeast suspension (5×10^5 cells/mL), in order to cover the piece completely, and then incubated at 36°C for 1, 3, 8, and 12 h. Five processed pieces and 5 control pieces were used for each incubation time. Colony-forming capability, assessed by colony-forming units (CFU)/mL, was determined as follows: the test piece was removed from the cell suspension, rinsed 3 times with phosphate-buffered saline (PBS) to remove non-adherent *Candida*, and transmitted to a new sterile petri dish. Adherent *Candida* was then removed by shaking for 10 min with 1 mL 0.1% TritonX-100 PBS solution. A 10 μL aliquot of the solution was inoculated on CHROMagar Candida medium (Nippon Becton and Dickinson Company, Ltd., Japan) and incubated for 48 h to determine CFU/mL. Each piece was rinsed 3 times with PBS solution, fixed with 5% glutaraldehyde solution for 60 min, and incubated with 2% osmic acid for 30 min. The surface of the test pieces were observed with a low-vacuum scanning electron microscope (SEM, TM-1000, Hitachi, Japan) with magnification ratios of 1,000x and 10,000x.

2.4. Statistical Analysis. The numbers of *C. albicans* and *C. glabrata* adherent to the surface of Acron pieces with and without silver coating were evaluated by two-way analysis of variance, followed by Tukey's test. A significance level of 0.05 was used for all analytical tests.

3. Results

3.1. Adhesion of Yeast to the Acrylic Denture Base Resin. As shown in Figure 1, 48 h incubation of the rinse fluid on CHROMagar Candida medium exhibited time-dependent increases in *C. albicans* and *C. glabrata* colony formation. For the control uncoated piece, *C. albicans* increased to 7.50×10^4 CFU/mL with a 12 h incubation, whereas with the AgNP-coated piece, *C. albicans* increased to only 3.83×10 CFU/mL, showing statistical significance between the control piece and the AgNP-coated piece ($P < 0.001$, Figure 2(a)). A similar result was obtained with *C. glabrata*, which increased to 6.99×10^5 CFU/mL with a 12 h incubation on the control pieces versus 2.18×10^2 CFU/mL on the AgNP-coated piece ($P < 0.001$, Figure 2(b)).

3.2. Low-Vacuum SEM Observation (1,000x) of the Surface of the Acron Piece. Low-vacuum SEM observation of the

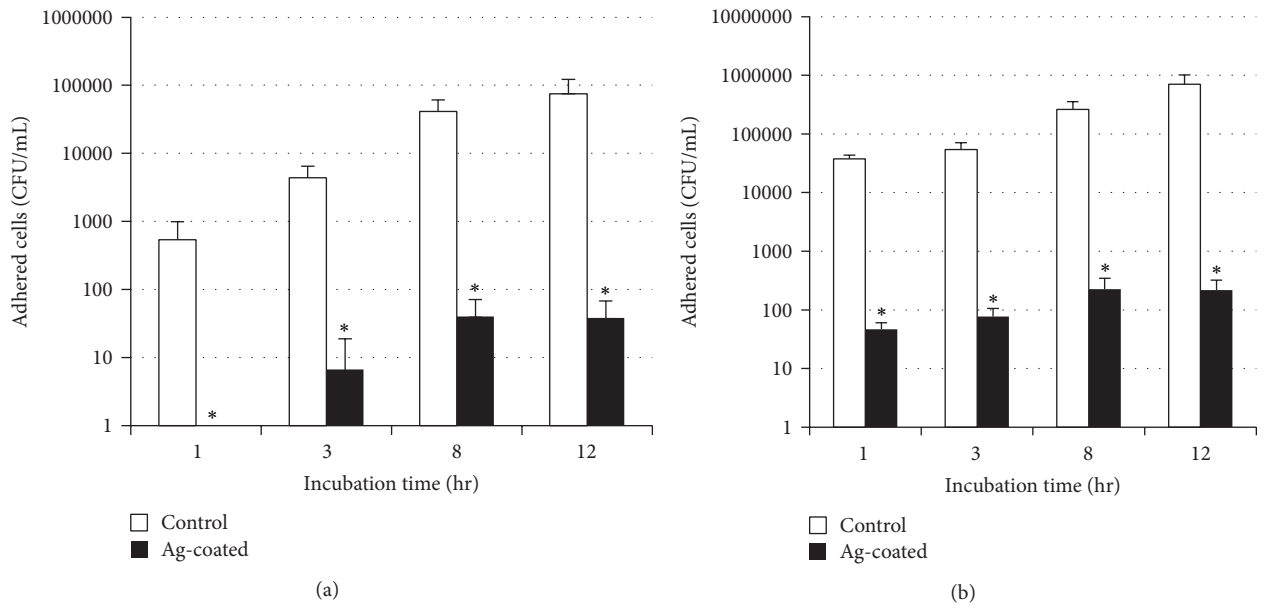

Figure 2: Number of adherent cells on an Acron piece coated with AgNPs (Ag-coated, $n = 5$) or without AgNPs (control, $n = 5$), as measured by counting colony forming units (CFU) on CHROMagar *Candida* medium, where 10 mL of PBS rinse solution from the Acron pieces (Ag-coated or uncoated) were incubated with (a) *C. albicans* and (b) *C. glabrata* for 1, 3, 8, or 12 h. *Significantly different from control ($P < 0.001$).

Acron piece surface with a magnification ratio of 1,000x demonstrated that a similar number of *C. albicans* adhered to both AgNP-coated and control pieces after 1 h of incubation (Figure 3(a)). Three hours after the start of incubation, *C. albicans*, in the form of yeast, began to aggregate and adhere to the control pieces, whereas very few *C. albicans* adhered to the AgNP-coated pieces in the form of yeast. The difference in the number of adherent *C. albicans* between AgNP-coated and control pieces became remarkably larger over time. A similar finding was observed for *C. glabrata* (Figure 3(b)).

3.3. Low-Vacuum SEM Observation (10,000x) of the Surface of the Acron Piece. Low-vacuum SEM with a magnification ratio of 10,000x was performed on uncoated and AgNP-coated Acron pieces. The Acron pieces were incubated for 3 h with *C. albicans* and demonstrated oval yeast cells with smooth cell walls and a major axis of 3–4 mm for the control, but on the AgNP-coated pieces, both yeasts showed deformed features, such as swollen cells with shriveled cell walls, a major axis of over 5 mm, and in some cases, leakage of cellular contents (Figure 4), indicating a loss of normal yeast cell function for adhering to the host.

4. Discussion

For the treatment of superficial mycoses such as OC and deep-seated mycosis, antifungal azoles, amphotericin B (AMPH-B), and micafungin are primarily used. These drugs are not prescribed prophylactically but administered following a definitive diagnosis of candidiasis. Oral care is recommended as a usual prophylactic measure, especially for elderly people, the population of whom has been increasing. However, the daily practice of oral care, particularly cleaning of dentures, is difficult for elderly people. In a study by Dağistan et al. [2], 70% of stomatitis cases were denture induced, and in 68% of them, culture results were positive for fungi, with *C. albicans* being the most frequently isolated species. We have also demonstrated in a previous paper that *Candida* was detected in 78% of denture-wearing patients, who showed high pathogenic episodes of stomatitis and oral candidiasis, and ultrasonic cleaning of the dentures eliminated *Candida* [10]. However, it is difficult for elderly patients to perform daily ultrasonic cleaning of their dentures. Several attempts to fabricate a denture base material to prevent the adhesion of *Candida* have been made. He et al. investigated in vitro adhesion of *C. albicans*, *C. krusei*, and *C. dubliniensis* to 4 different denture base materials and found that there was a significant difference in candidal adhesion among the types of denture base material, *Candida* species, and presence of absence of heat curing [11]. Adherence of microorganisms varied with different surface textures of the denture base [12], with significantly greater adhesion of *C. albicans* on rough rather than smooth surfaces. Additionally, precoating the denture base materials with saliva reduced *Candida* adhesion [13]. Treatment of a denture base with acrylic resin by glow-discharge plasma [14], ROS [15], or mannan [16] can also prevent *Candida* adhesion. Thus, several attempts to modify denture base materials have shown promising results but have not reached practical use in the clinical setting. Silver and its compounds have long been known to be effective against microorganisms and to demonstrate an antifungal activity equivalent to or more potent than AMPH-B and fluconazole (FLCZ) [3–5, 17]. A novel adduct of AgNPs to peptide hydrolysates from collagen, GX-95, was found to possess potent antifungal activities against *C. albicans*, including

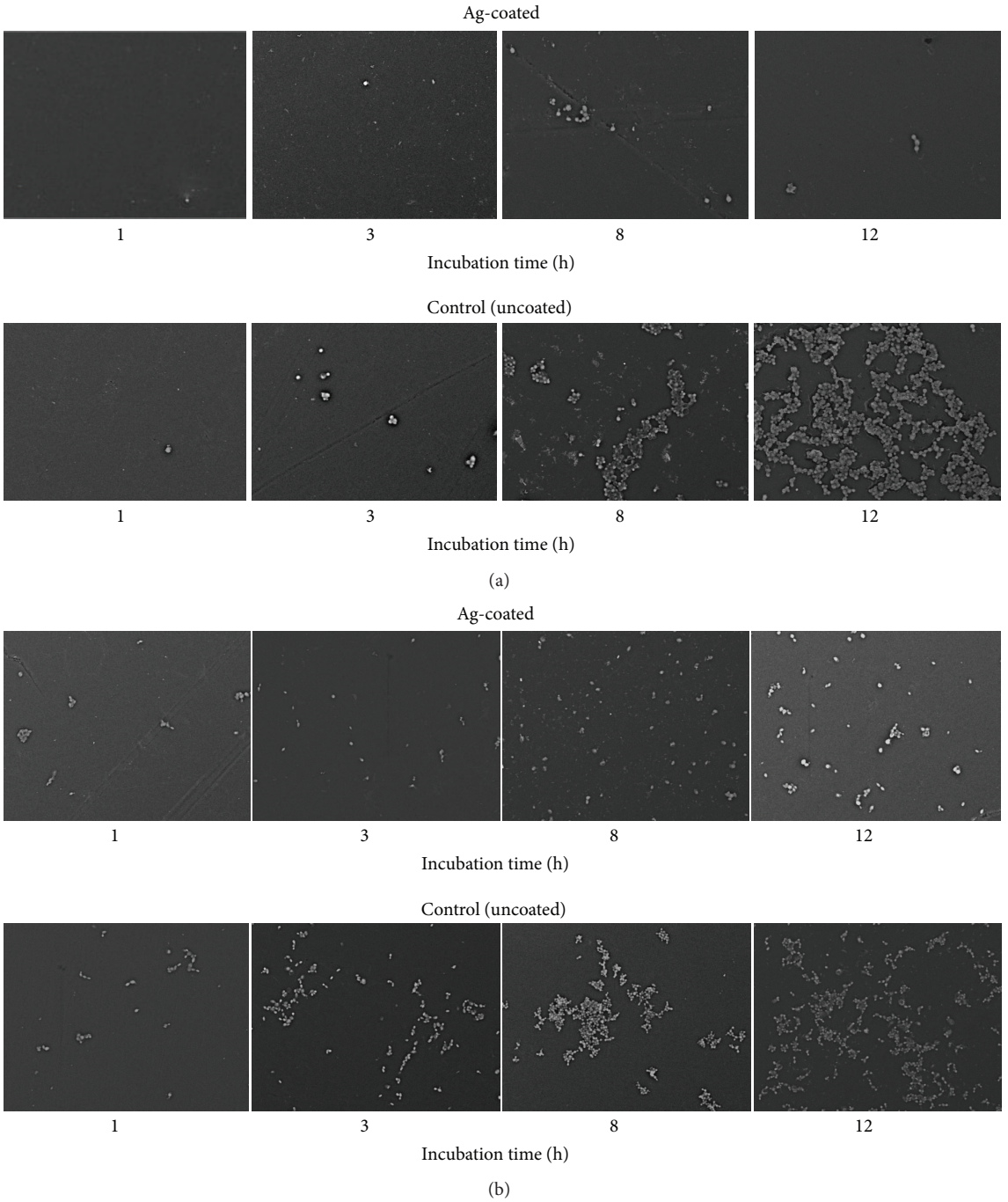

Figure 3: Low-vacuum scanning electron microscopic observation (1,000x) of the surface of an AgNP-coated and uncoated Acron piece. The Acron piece was incubated with (a) *C. albicans* or (b) *C. glabrata* for 1, 3, 8, or 12 h. (a) Although similar numbers of *C. albicans* adhered to both AgNP-coated and control pieces at 1 h, *C. albicans* began to aggregate and adhere to the control pieces in the form of yeast at 3 h, whereas only a few *C. albicans* adhered to AgNP-coated pieces in the form of yeast. The difference in the number of adherent *C. albicans* increased between the AgNP-coated and control pieces with incubation time. (b) A similar observation was observed with *C. glabrata*.

strains resistant to FLCZ, itraconazole, and flucytosine [18]. In addition, the cytotoxicity of silver, especially of AgNPs, to human cells was found negligible in comparison with that to fungal cells [5, 19]. The antifungal effects by silver are attributable to the disruption of the structure of the fungal cell membrane due to the destruction of membrane integrity, resulting in leakage of intracellular ions and other materials and to the inhibition of the normal budding process by affecting the cell cycle at the G2/M phase [4]. *C. albicans* exposed to AgNPs exhibited increased ROS and hydroxyl radical production, resulting in mitochondrial dysfunction and apoptosis [6]. Because intracellular accumulation of ROS

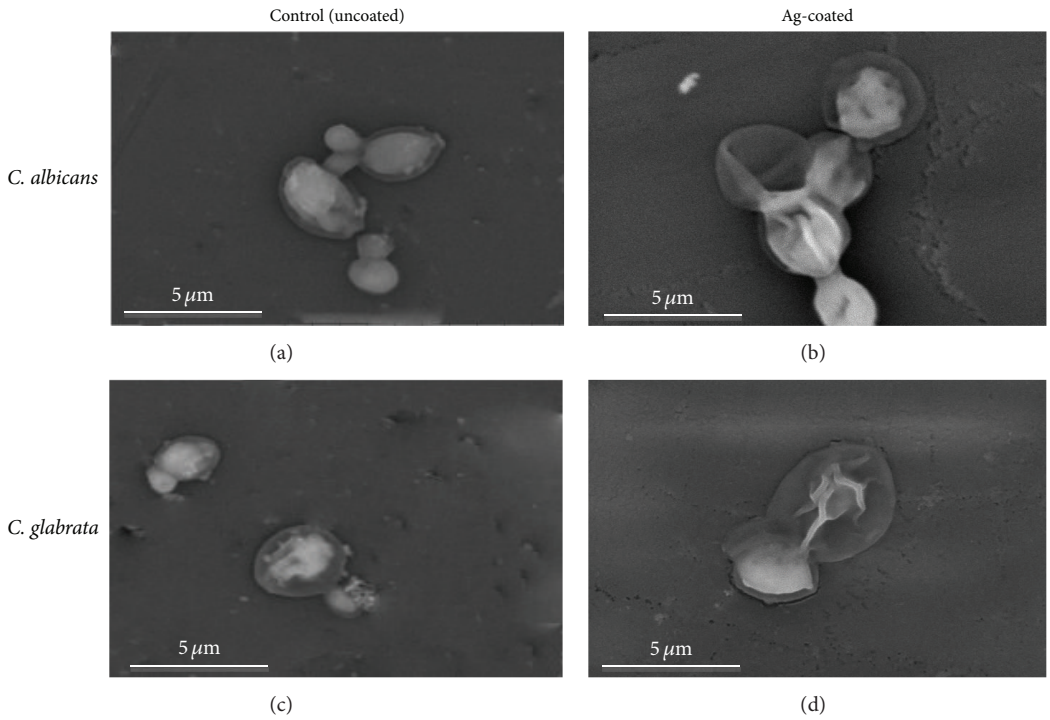

Figure 4: Low-vacuum scanning electron microscopy (10,000x) of the surface of Acron pieces coated with (Ag-coated) and without (uncoated) silver nanoparticles. Acron pieces were incubated with (a) *C. albicans* and (b) *C. glabrata* for 3 h. *C. albicans* and *C. glabrata* had oval yeast cells with smooth cell walls and a major axis of 3-4 mm in the control, but the Ag-coated piece showed swollen yeast cells with shriveled cell walls and a major axis of over 5 mm, with leakage of cellular contents in some cases.

is accompanied by a mutation in CDC48 or by expression of mammalian bax, which are triggers of apoptosis, the generation of oxygen radicals is a key event in the ancestral apoptotic pathway of yeast [20]. Application of silver, especially AgNPs, to denture base materials inhibits the adhesion of *Candida* to the denture surface and hence prevents OC. Acosta-Torres et al. reported that PMMA-AgNP discs significantly reduced the adherence of *C. albicans* in vitro, presenting a new biocompatible antifungal PMMA denture base material [7]. Chladek et al. found in vitro antifungal efficacy of 16.3–52.5% by modifying the soft lining of dentures with AgNPs [21]. Wady et al. demonstrated that an AgNP solution with antifungal activity against *C. albicans* showed no effect on *C. albicans* adherence and biofilm formation after its incorporation into a denture base resin [8]. Thus, potent antifungal activity of AgNPs has been commonly observed but inhibitory activity against *Candida* adhesion to a denture base has not. In the present study, adherence of *C. albicans* and *C. glabrata* to the surface of heat-cured Acron resin pieces modified by AgNPs was significantly lower than that of the control Acron piece, based on CFU. By SEM observation, *C. albicans* and *C. glabrata* on the surface of the control Acron piece showed oval yeast cells with smooth cell walls and a major axis of 3-4 μm, but they were remarkably deformed by the AgNP coating, as evidenced by swollen cells with shriveled cell walls, a major axis of over 5 μm, and leakage of cellular components. The latter effect resulted in a loss in ability of yeast cells to adhere to the host. Thus, AgNPs-coated denture base material has several advantages in inhibiting candidal adhesion on the denture surface comparing AgNPs-contained denture base material, such as easy preparation and high exposure of silver ions on the denture surface.

In conclusion, potent antifungal effects of AgNP coating of denture base material were demonstrated in vitro, as shown by inhibition of *Candida* adherence to the denture material surface and deformation of the normal morphology of *Candida*. Further attempts to apply AgNP-coated denture base materials for clinical use are expected.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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