Effect of zinc oxide nanoparticles incorporated into tissue conditioner on antifungal, physical, and mechanical properties

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This study evaluated the antifungal, physical, and mechanical properties of tissue conditioner incorporated with different amounts of zinc oxide nanoparticles (ZnOnps) at different storage times (0, 7, and 14 days). Specimens of 0, 5, 10, 15 wt% ZnOnps, or 15 wt% nystatin incorporated into tissue conditioner were fabricated (control, 5Zn, 10Zn, 15Zn, and Nys). The direct contact test (n=6) was performed to evaluate the antifungal effect against C. albicans suspension. The penetration depth (n=6) and tensile bond strength (n=8) were evaluated following ISO 13139. The 15Zn significantly reduced C. albicans cell number compared with control at all storage times (p<0.001). The penetration depths and tensile bond strengths of the 5Zn, 10Zn, 15Zn, and Nys were not significantly different compared with control at all storage times (p>0.05). In conclusion, the 15Zn provides antifungal effect up to 14 days without adverse effects on penetration depth and tensile bond strength.

**Keywords:** Antifungal effect, Penetration depth, Tissue conditioner, Tensile bond strength, Zinc oxide nanoparticles

INTRODUCTION

Removable dental prostheses are commonly used in patients who require replacement of missing dentition. The advantages of wearing dentures include improved masticatory function, esthetic, phonetic, and oral health that related to the quality of life. However, long term use of a denture or poor denture hygiene may lead to opportunistic oral infections, especially in immunocompromised and disabled geriatric patients⁹.

Denture-induced stomatitis is the most common inflammatory reaction of the oral mucosa underneath a denture⁹. The etiology of this disease is multifactorial. An ill-fitting denture, poor denture hygiene, and microbial colonization on the denture surface and oral mucosa substantially increase the risk of denture-induced stomatitis⁸. The principle pathogens of denture-induced stomatitis are Candida species, especially Candida albicans. Candida adheres to the oral mucosa and denture surfaces in filamentous growth form⁴⁻⁶. Moreover, the roughness and hydrophobicity of denture surfaces also promote microbial attachment, leading to biofilm development. Denture-induced stomatitis is usually painless and asymptomatic, however, taste alteration, burning sensation, and mucosal bleeding can develop in some individuals⁹.

The treatments of denture-induced stomatitis, including systemic antifungal medication, topical antifungal therapy, oral hygiene care, and soaking denture in disinfectants, have been reported⁷. However, long-term soaking in disinfectants, e.g. 1% sodium hypochlorite or 2% glutaraldehyde, alters the mechanical properties of denture materials⁸. A successfully simple method is to discontinue wearing the denture in combination with or without the administration of an antifungal drug, but this method might not be accepted by patients who could not discontinue wearing their denture for a long period due to esthetic and masticatory concerns. Eliminating the fungal reservoir in an acrylic denture base by grinding off the superficial tissue surface of the denture followed by relining with tissue conditioner has been suggested⁹. This method disrupts the colonization of pathogens.

Tissue conditioner consists of polyethyl methacrylate (PEMA) powder, plasticizers, and ethyl alcohol. It is used as relining material to improve stability of ill-fitting denture, provide a cushion effect, and equalize functional pressure onto the denture bearing tissues⁹. This allows traumatized denture bearing tissues to recover to its healthy condition before the new denture is made⁹. It is normally recommended for short period of usage. Aging of tissue conditioner leads to an increase in surface roughness and enhances reestablishment of microbial colonization¹¹.

Several in vitro and in vivo studies focused on incorporating antimicrobial/antifungal agents into tissue conditioners to prevent microorganism attachment¹²⁻¹³. Most studies incorporated different antifungal drugs, especially nystatin and fluconazole, into tissue conditioners¹⁴⁻¹⁷. A previous study investigated the effect of incorporating inorganic antimicrobial agents, such as silver nanoparticles, into tissue conditioners¹⁸. Organic or natural agents, such as organum oil and poly(acryloyxethyltrimethyl ammonium chloride)-grafted chitosan, have been mixed with tissue conditioners¹⁹⁻²⁰. However, the incorporation of such antimicrobial agents alters the physical or mechanical properties of tissue conditioners²¹⁻²².

Recently, nano-scaled metal oxides have been used in several applications. Zinc oxide nanoparticles
(ZnOnps) appear as white powder. These nanoparticles are odorless, good thermal stability, and low soluble in water. The advantages of ZnOnps are UV protection and anti-microbial activities. ZnOnps can reduce the viability of pathogenic bacteria and yeast in a concentration-dependent manner\(^{23,24}\). A previous study compared the antibacterial activities of three metal oxides nanoparticles and concluded that ZnOnps showed the highest of growth inhibition against various bacteria\(^{25}\). ZnOnps has been used in many dental products, such as dental restorative materials, luting materials, and root canal filling materials\(^{26-28}\). To date, the antifungal, physical, and mechanical properties of a tissue conditioner incorporated with ZnOnps have not been investigated.

The objective of this study was to evaluate the antifungal, physical, and mechanical properties of a tissue conditioner incorporated with different amounts of ZnOnps at different storage times. The null hypothesis was that there were no significant differences in antifungal effect, penetration depth, or tensile bond strength of the tissue conditioner incorporated with different amounts of ZnOnps at different storage times.

### MATERIALS AND METHODS

The tissue conditioner used in the present study was GC Soft-liner. Details of the materials used and their chemical compositions are shown in Table 1. The material preparation used throughout this study was prepared as follows. A pure mixture of PEMA powder and liquid part at a powder/liquid ratio of 2.2 g/1.8 g, according to the manufacturer’s recommendation, was homogenously mixed for 90 s and served as the control group. Five, 10, 15 wt% ZnOnps powder, and 15 wt% nystatin, calculated from the weight of the PEMA powder, were separately mixed with the liquid of tissue conditioner for 30 s to make suspension. The PEMA powder was then added into the suspension and homogenously mixed for 90 s. The ratio of powder (ZnOnps powder and PEMA powder) or (Nystatin and PEMA powder) and liquid was kept constant of 2.2 g/1.8 g.

#### Antifungal evaluation

Eighteen disc-shaped specimens (12 mm in diameter and 2 mm in thickness) per group were fabricated. The mixture of each group was prepared, poured into a metal mold, covered with an un-plasticized polyester film, and pressed flush. After the material set, the excess was removed. A photograph of a specimen in each group is shown in Fig. 1.

*C. albicans*, ATCC 90028, cells were cultured on Sabouraud Dextrose agar (SDA) (HiMedia Laboratories, Mumbai, India) for 24 h. Three 1-mm-diameter distinct colonies were isolated and transferred into a fresh glass tube containing 3 mL of Sabouraud Dextrose broth (SDB) (HiMedia Laboratories). The suspension was homogenously vortexed and incubated for 24 h at 37°C. After incubation, the suspension was vortexed for 15 s and then transferred into a new glass tube containing 3 mL of SDB. The turbidity of the new suspension was adjusted to achieve a 0.5 McFarland standard turbidity that is equivalent to 0.3 optical density at a 530 nm wavelength using a UV-vis spectrophotometer (Nicolet Evolution 500, Thermo Electron, Madison, WI, USA). The 0.5 McFarland suspension, approximately 1.5×10^6 cells/mL, was diluted twice with SDB to reach a final concentration of 1×10^4 cells/mL. Next, 100 µL of diluted suspension was spread on SDA plates to confirm the initial amount of *C. albicans* cells before each testing.

The antifungal assay was modified from a previous study\(^{24}\). Eighteen disc-shaped specimens per group

![Fig. 1 Photograph of a specimen in each group in the antifungal assay: (A) control, (B) 5Zn, (C) 10Zn, (D) 15Zn, and (E) Nys.](image-url)

| Material/product name | Manufacturer | Batch number | Composition |
|-----------------------|--------------|--------------|-------------|
| Tissue conditioner/ GC Soft-liner | GC, Tokyo, Japan | 1806052 | Powder: polyethyl methacrylate (PEMA) Liquid: ethyl alcohol 15%, butyl phthalyl butyl glycolate 85% |
| Zinc oxide nanoparticles/ ZnOnps | Nano Materials Technology, Chonburi, Thailand | 190411 | Zinc oxide: (purify>99.5%, average particles size 20–40 nm.) |
| Heat-cured denture base material/SR Triplex Hot | Ivoclar Vivadent, Schaan, Liechtenstein | XT 0628 | Powder: polymethyl methacrylate (PMMA) Liquid: methyl methacrylate 50-100%, ethylene glycol dimethacrylate 3-<10% |
| Nystatin oral suspension/ Nyst Oral | Continental-Pharm, Bangkok, Thailand | 014 | Nystatin 100,000 unit |
Tensile bond strength
Forty-eight acrylic resin plates (25×25×3 mm³) were prepared for each group using a heat-cured denture base material (SR Triplex Hot, Ivoclar Vivadent, Schaan, Liechtenstein) according to the manufacturer’s recommendation. After polymerization, the acrylic resin plates were wet-polished using 600-grit silicon carbide paper with a polishing machine (Nano 2000, Pace Technologies, Tucson, AZ, USA) and stored in deionized water at 37±1°C for 7 days. A plastic collar (10 mm in internal diameter and 3 mm in height) was placed at the center of the prepared acrylic resin plate. The mixture of each group was prepared, poured into the collar, followed by a 2-kg-weight load. One hour after placing the tissue conditioner on the acrylic resin plate, the bonded specimens in each group were equally divided into 3 subgroups (n=8) and stored in deionized water at 37±1°C for 24 h, 7 and 14 days. The tensile bond strength was measured using universal testing machine (EZ-SX, Shimadzu, Kyoto, Japan) at a crosshead speed of 10 mm/min until failure according to ISO specification 10139-2:2016. The failure modes were observed and classified as adhesive (total debonding at the interface between the lining material and acrylic resin plate), cohesive (total rupture within the tissue conditioner) and mixed mode.

Statistical analysis
The number of C. albicans cells and tensile bond strength data were analyzed by two-way ANOVA followed by Tukey’s post-hoc test for multiple comparison (p<0.05). The penetration depth data were analyzed by two-way repeated measures ANOVA followed by Bonferroni post-hoc test for multiple comparison (p<0.05). All statistical analyses were performed using SPSS 22.0 (IBM, Armonk, NY, USA).

RESULTS
Two-way ANOVA of the antifungal assay results demonstrated significant differences on two main factors (group and storage time) and their interaction (p<0.001). The means and standard deviations of the number of C. albicans cells are shown in Fig. 2. ZnO nanoparticles incorporated into the tissue conditioner dose-dependently reduced C. albicans cell number at day 0. The antifungal effect in the 15Zn group resulted in a significant reduction in C. albicans cell number compared with the control group at all storage times (p<0.001). The antifungal effect in the Nys group demonstrated the greatest reduction only at day 0 compared with the control group (p<0.001). However, the antifungal effect in the Nys group at day 7 (p=0.594) and day 14 (p=0.156) was not significantly different compared with the control group.

Two-way repeated measures ANOVA of the penetration depth demonstrated significant differences (p<0.05).
Table 2 The means and standard deviations of the penetration depth (mm) in each group at different storage times

| Group | Day 0       | Day 7       | Day 14      |
|-------|-------------|-------------|-------------|
| Control | 1.22 (0.03) | 0.70 (0.01) | 0.64 (0.01) |
| 5Zn    | 1.24 (0.02) | 0.70 (0.02) | 0.62 (0.02) |
| 10Zn   | 1.23 (0.02) | 0.69 (0.02) | 0.62 (0.01) |
| 15Zn   | 1.24 (0.02) | 0.69 (0.01) | 0.62 (0.01) |
| Nys    | 1.21 (0.02) | 0.67 (0.03) | 0.61 (0.02) |
| Average (95% CI) | 1.226a (1.217–1.235) | 0.686b (0.675–0.697) | 0.622c (0.617–0.626) |

Values with the different superscript letters indicate significant differences between storage times ($p<0.05$). 95% CI=95% Confidence interval.

Table 3 The means and standard deviations of the tensile bond strength (MPa) in each group at different storage times

| Group | Day 0       | Day 7       | Day 14      |
|-------|-------------|-------------|-------------|
| Control | 0.25 (0.02) | 0.26 (0.04) | 0.29 (0.03) |
| 5Zn    | 0.26 (0.03) | 0.26 (0.03) | 0.28 (0.03) |
| 10Zn   | 0.24 (0.02) | 0.26 (0.02) | 0.27 (0.04) |
| 15Zn   | 0.24 (0.02) | 0.26 (0.03) | 0.28 (0.02) |
| Nys    | 0.26 (0.03) | 0.29 (0.02) | 0.29 (0.02) |
| Average (95% CI) | 0.250a (0.241–0.259) | 0.268b (0.259–0.277) | 0.282b (0.273–0.290) |

Values with the different superscript letters indicate significant differences between storage times ($p<0.05$). 95% CI=95% Confidence interval.

only between storage times ($p<0.001$). The main factor of group ($p=0.055$) and the interaction between group and storage time ($p=0.527$) of the penetration depth were not significantly different. The means and standard deviations of the penetration depths are shown in Table 2. The penetration depth of all groups significantly reduced over time ($p<0.001$).

Two-way ANOVA of the tensile bond strength demonstrated significant differences only storage times ($p<0.001$). The main factor of group ($p=0.052$) and the interaction between group and storage time ($p=0.781$) of the tensile bond strength were not significant different. The means and standard deviations of the tensile bond strengths are shown in Table 3. The mean tensile bond strength of all groups at day 7 and 14 were significantly higher compared to day 0 ($p<0.001$). In addition, all specimens demonstrated a cohesive mode of failure.

**DISCUSSION**

The aim of the present study was to evaluate the antifungal, physical, and mechanical properties of a tissue conditioner incorporated with different amounts of ZnOnps at different storage times. Statistical analysis indicated that there were significant differences on two main factors (group and storage time) and their interaction in the antifungal effect. There were significant differences only between storage times in the penetration depth and the tensile bond strength. Therefore, the null hypotheses were partially rejected.

GC Soft-liner (tissue conditioner) was selected in the present study because it is widely distributed. Tissue conditioner is recommended to be changed every few days, typically for up to 7 days29), with the aim of rapid revitalizing the oral mucosa to a healthy condition. However, the maximum 14-day period was chosen in the present study because this interval mimics the protocol for treating denture-induced stomatitis using nystatin31). The concentration of ZnOnps incorporated into the tissue conditioner was increased up to 15 wt% because this concentration was the minimum concentration which still provided antifungal property after 14-day water immersion in our pilot study.

There are several methods for evaluating an antimicrobial medication. The agar diffusion or disc diffusion test is a well-known procedure due to its simplicity and low cost32). However, the agar diffusion test is limited by the solubility of the tested materials diffusing into the surrounding aqueous media. Therefore, this method is not appropriate to evaluate the...
antimicrobial effect of ZnOnps. The direct contact test was chosen in the present study because it is suitable for evaluating the antimicrobial effect of close contact between the tested materials and microorganisms on solid materials.

The present study found that ZnOnps incorporated into the tissue conditioner reduced dose-dependently C. albicans cell number at day 0. These findings are in line with previous studies that found that ZnOnps has a concentration-dependent antimicrobial effect. The 5Zn and 10Zn groups demonstrated a significant reduction in C. albicans cells only at day 0 (89% and 99%, respectively). After 7 and 14-day water immersion, the 5Zn and 10Zn did not demonstrate a significant reduction in C. albicans cells compared to control group. This phenomenon might be attributed to the leaching out of ZnOnps into the water which resulted from the lack of chemical bond between ZnOnps and any compositions in tissue conditioner. The 15Zn group showed a significant reduction in C. albicans cells at day 0 (99%), 7 (98%), and 14 (92%), compared with the control group at each storage time. However, it should be considered that the antifungal effect of the 15Zn group at day 7 and 14 was modest compared with its effect at day 0. Thus, the reduced C. albicans cell number in vitro might not warrant performing in vivo study. Clinical studies are needed to find the effective degree of antifungal effect of tissue conditioner.

Nystatin is an antifungal agent for treating denture-induced stomatitis that is directly applied to the oral lesions 3–5 times daily. The antifungal mechanism of nystatin results from nystatin binding to ergosterol on the cell membrane of Candida species, leading to increased permeability, causing cell death. The present study demonstrated that the Nys group had the greatest reduction in C. albicans cells only at day 0. Previous studies have demonstrated that the antifungal effect of nystatin decreased over time. The deterioration in the antifungal effect of nystatin may occur because nystatin was exposed to water for 7 and 14 days. Moreover, the difference in the water solubility between nystatin (0.36 g/L at 24°C) and that of ZnOnps (0.0042 g/L at 18°C) might play some role in the sustainable effect of ZnOnps compared to nystatin.

The penetration depth test and the tensile bond strength test were selected to evaluate whether the addition of ZnOnps would affect the physical and mechanical properties of tissue conditioner. The increased amounts of ZnOnps up to 15 wt% into the tissue conditioner did not affect the penetration depth and the tensile bond strength compared with the tissue conditioner alone at each storage time. This might be due to the small amount of additive and nanosized particle of ZnO incorporated into the tissue conditioner. Moreover, the cylindrical penetrator with a 1-mm-diameter vertical rod in the penetration depth test might not be able to detect the differences of each group containing nanosized particles of ZnO at the same storage time.

The mean penetration depth of all groups showed a decrease in value over time. However, the penetration depth of all groups after 7-day water immersion was over 0.5 mm which was within the ISO criteria. The mean tensile bond strength of all groups at day 7 and 14 were significantly higher when compared to day 0. The results of both penetration depth and tensile bond strength might be attributed to the leaching out of ethyl alcohol from the tissue conditioner, especially at day 7, leading to the hardening of tissue conditioner over time. The failures in all groups showed cohesive failure as also demonstrated in the previous studies. This suggested low intermolecular force within tissue conditioner compared to the interfacial strength between tissue conditioner and acrylic denture base. The strong mechanical retention at the interface between the tissue conditioner and acrylic denture base results from the chemical components of the tissue conditioner. The ethyl alcohol in liquid part of tissue conditioner swells the polymer particles of the acrylic denture base and allows the plasticizer to penetrate between the swollen particles. Moreover, the powder of tissue conditioner is methacrylate-based component, similar to denture base material.

This is the first study to evaluate whether ZnOnps incorporated into a tissue conditioner enhanced its antifungal property. The antifungal property of the tissue conditioner containing 15 wt% ZnOnps sustained for a longer period, while retaining its physical and mechanical properties. These findings could supplement the treatment protocol of denture-induced stomatitis by reducing the number of pathogens, promoting an appropriate environment for healing, and decreasing the frequency of replacing the tissue conditioner. Although using systemic or topical antifungal drugs such as ketoconazole, nystatin is the standard treatment for denture-induced stomatitis, modifying the tissue conditioner with ZnOnps might be used as an adjuvant treatment. However, the limitations of the present study in terms of the type of microorganisms and tissue conditioners should be addressed in future studies. The effect of ZnOnps on mixed biofilms that mimic the oral environment might exhibit different results. The safety of ZnOnps incorporated into tissue conditioner should be addressed because tissue conditioner made direct contact with oral mucosa. The cytotoxicity of tissue conditioner containing ZnOnps both of in vitro and in vivo should be further investigated.

CONCLUSION

Within the limitations of this study, it was concluded that 15 wt% ZnOnps incorporated into the tissue conditioner provides an antifungal effect up to 14 days without adverse effects on the penetration depth and tensile bond strength of the tissue conditioner.

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