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Original Article

In Vitro Shelf-Life for the Antifungal Effects of Nano Chitosan Integrated With Tissue Conditioners on *Candida albicans*

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Abstract

Introduction: The growing number of elderly individuals worldwide, including Iran, has increased the edentulous population. Using dental prosthesis including complete denture is a common treatment method that causes denture stomatitis. In addition, the tissue conditioner (TC) used to improve the fit and comfort of dentures leads to more microbial especially *Candida* colonization. Therefore, the present study aimed to evaluate the *in vitro* shelf-life of chitosan nanoparticles (ChNPs) incorporated into a TC for the control of *Candida albicans*.

Methods: In the current experimental study, nano chitosan was developed and added to the discs of the TC and then used to perform its antifungal susceptibility properties through disk agar diffusion and broth micro dilution susceptibility tests during 1–7 days. Finally, data were analyzed using $SPSS_{20}$ ($P=0.05$).

Results: The prepared nano chitosan was first analyzed and verified by Fourier-transform infrared spectroscopy and electron microscope. Only disks containing 5% and 2.5% nano chitosan inhibited *C. albicans* growth in 24 hours and 48 hours. There were no significant statistical differences between nystatin with 2.5% and 5% nano chitosan during 1–2 days. The antifungal properties of nano chitosan dramatically decreased over time, and there was no antifungal effect after 2 days.

Conclusion: Our results revealed that the addition of ChNPs to a TC can completely control the colonization of *C. albicans* in the first 2 days.

Keywords: Tissue conditioner, Nano chitosan, *Candida albicans*, Shelf-life

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Introduction

The globally increasing number of elderly individuals, including Iran, is primarily attributed to rising life expectancy. This demographic shift has significant implications for oral health, due to the prevalence of edentulousness (complete tooth loss) among older adults. Although research indicates that such tooth loss can lead to a decline in the quality of life, it is frequently not taken seriously by the general population (1).

In an extensive community-based cohort study involving 3,054 elderly participants aged over 65 years, Musacchio et al reported that 44% of the edentulous participants identified smoking, menopause, serum albumin levels less than 40 g/L, and living alone as the most significant predisposing factors (2).

In spite of the lack of a properly structured epidemiological study on edentulousness and tooth loss in Iran, Khazaei et al found that the prevalence of edentulousness ranges from 0.3% in children aged 3–5 to 70.7% in adults aged more than 65 years, indicating an increase with aging (3).

The dental prosthesis, including complete removable denture therapy, has evolved into a reliable and effective treatment method for managing the consequences of edentulousness. However, it can lead to denture stomatitis (DS), which is a chronic inflammatory condition resulting from the colonization of normal oral flora microorganisms, particularly *Candida albicans*, on the palatal and alveolar oral mucosa. This colonization can even result in the creation of *Candida* biofilm on the denture base material (4).

Reports in the scientific literature indicate that the prevalence of DS among complete denture wearers ranges from 15% to over 70%, especially in institutionalized populations (5).

Although DS is commonly asymptomatic, it may result in bleeding, burning, and troublesome taste disorders. Moreover, DS usually results from a defect in the adaptation of complete dentures on the mucosal-bearing tissue. Tissue conditioners (TC) are typically used in prosthodontics to repair injured tissues supporting dentures, improve the fit and comfort of dentures, and serve as palliative materials. However, they can also act as a reservoir for microorganisms due to their micro porous nature, which can lead to microbial colonization, particularly by *C. albicans* (7).

Chitosan is a biopolymer derived from chitin, which is predominantly found in the exoskeletons of crustaceans and certain fungi. In addition, it is composed of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine units. This biopolymer can be extracted through the deacetylation of chitin, which can be sourced from various filamentous fungi, including *Mucor rouxi*, *Absidia coerulea*, *Absidia blakesleeana*, *Gongronella butieri*, and *Phycomyces blakesleeanu* (8).

Saeed et al, in a study examining the incorporation of chitosan into TC, demonstrated that this combination presents a promising approach for the prevention and management of DS. They concluded that chitosan effectively inhibited the growth of *C. albicans* for 24 hours (9). Chitosan nanoparticles (ChNPs) have shown a large spectrum of antimicrobial actions against viruses, fungi, and bacteria, exhibiting minimal toxicity to humans and animals. Their enhanced antimicrobial efficacy is attributed to their increased surface area and their ability to efficiently interact with the cell membranes of bacteria and fungi. Additionally, these NPs increase microbial cell membrane permeability. This phenomenon occurs due to the positive charge of chitosan particles, which reacts with the negatively charged components of pathogenic microbial cell membranes. This interaction enhances the permeability of the cell membranes, leading to the increased leakage of intracellular components (10).

The findings of one study by Wang et al (11) revealed that ChNps successfully inhibited the attachment and biofilm development of *C. albicans* on various surfaces, including human epithelial cells. The researchers attributed the antifungal activity of nano chitosan to its ability to disrupt the cell membrane integrity of *C. albicans*, leading to cell death. A recent study performed by Shree Abiraami et al (12) indicated that adding probiotics to ChNPs significantly enhances their antifungal activity against *Candida* species. This combination not only increases the antifungal efficacy but also improves the stability and transport of NPs to infection sites, emphasizing the potential of nano chitosan in combating fungal infections within clinical environments (11).

This *in vitro* experimental study seeks to assess the antifungal shelf-life of GC TCs incorporated with 5% and 2.5% ChNPs against *C. albicans* colonization, comparing their efficacy with nystatin.

Materials and Methods

Sample Selection and Methodology

After approval by the faculty's research council, this *in vitro* experimental study was conducted in the prosthodontics (faculty of dentistry), medical parasitology, and mycology (faculty of medicine) departments.

Considering the 95% confidence level, 80% power, and 1.5 standard deviations for the number of colonies, as well as the difference between the averages of 10 colony units, the minimum sample size of 7 disks was calculated for each test group in the present study. Furthermore, the average colony counts of each tested disk group were collected and analyzed using the Mann-Whitney test with SPSS software, version 20.

Preparation of Chitosan Nanoparticles

ChNps were developed as explained in previous research (13). First, the 400 mg chitosan powder (molecular weight of 50–190 kDa, De-acetylation degrees of 75%; Sigma Aldrich, St. Louis, MO, USA) was dissolved in 400 mL of the sterile solution of 2% V/V acetic acid (Merck, Germany). Then, distilled water was vigorously mixed with sodium triphosphate at 25°C on a BS-Es-60 electric shaker (Biomarker, China), setting at the pH of 5.6 using sterile sodium hydroxide (Merck, Germany). ChNPs were prepared using the ionic gelation method by adding 24 mL of triphosphate pentasodium ($Na_5P_3O_{10}$, molecular weight of 367.86 g/moll) to the 60 mL of chitosan solution and then vigorously blended on a magnetic stirrer (Labtron, Iran; 700 RPM for 30 minutes at room temperature) to form ChNPs in the mixture. The mixture was centrifuged (Behdad, Iran) at 2,500 rpm (round per minute) for 30 minutes.

The precipitated ChNPs were rinsed three times with sterile phosphate-buffered saline (PBS), then dried and frozen using a freeze dryer (Pharm Laboratory, India) for further tests. It should be noted that this process is crucial for preparing NPs for subsequent characterization and application.

Analyzing of Chitosan Nanoparticle Specification

The collected nano chitosan was analyzed using scanning electron microscopy and Fourier-transform infrared spectroscopy (FTIR) methods at the Nano Structured Coatings Institute, Payam-e-Noor University, Shahedieh, Yazd, Iran (14).

Candida albicans Inoculation Preparation

A standard clinical isolate strain of *C. albicans* (PTCC 5027), obtained from the Iranian Research Organization for Science and Technology (Iran), was cultured on Sabouraud dextrose agar plates (Merck, Germany) and incubated at 30°C for 24 hours. Following incubation, a freshly isolated *C. albicans* colony was dissolved in 1 mL of the sterile saline solution (sodium chloride 0.85%)

and diluted to reach a concentration of $1–5 \times 10^6$ colony-forming units per milliliter (CFU/mL), which is equivalent to a half McFarland standard. This preparation was then used for further antifungal susceptibility testing (15).

Antifungal Susceptibility Testing of Chitosan Nanoparticles Using Broth Micro Dilution Test

To evaluate the antifungal effects of ChNPs, the broth micro dilution method was employed based on the Clinical and Laboratory Standards Institute (CLSI M27-3A) protocol using 24-well enzyme-linked immunosorbent assay micro plates. This method is widely recognized for determining the minimum inhibitory concentration of antifungal agents against various pathogens, including *C. albicans* (15).

Overall, 5 g of the chitosan nanoparticle was dissolved in the RPMI 1640 broth (Merck, Germany), and the volume reached 100 mL (a 5% solution) by adding RPMI. First, serial double dilutions of the prepared ChNPs (5–0.625%) were prepared in the sterile RPMI 1640 broth. Next, 100 µL from each dilution was poured into micro-plate wells triplicate, and 100 µL of the 1×10^6 CFU/mL of *C. albicans* cell suspensions were added to each well and sealed by putting a micro-plate's lid. Moreover, the nystatin suspension (100,000 IU/mL) and sterile PBS solution (100 µL) were employed in triplicate as positive and negative controls instead of the ChNP solution in this study. The micro-plate was incubated at 30°C for 48 hours on a reciprocal shaker set at 100 rpm, and 10 µL of each resulting solution was then added to 90 µL of sterile PBS and finally cultured on Saboroaud dextrose agar (Oxide, UK) containing 50 mg/mL chloramphenicol (Hakim Pharmaceutical Company, Iran) plates. The cultivated plates were incubated at a 30°C incubator (Behdad, Iran) for 48 hours, and the isolated *Candida* colonies were counted in each culture. Each isolated colony was explained as a viable *C. albicans* cell in this test based on the CLSI M27-3A protocol (15).

The average number of *Candida* colonies for each concentration of ChNPs, as well as for the positive and negative controls, was calculated as viable colony-forming units per milliliter (CFU/mL). Additionally, the minimum inhibition concentration of ChNPs, defined as the lowest concentration that completely inhibits fungal growth, was determined by comparing the results from the wells containing ChNPs to those of the negative and positive control wells (15).

Preparation of Tissue Condition Disks Containing Chitosan Nanoparticles

First, 250 mg of low molecular weight ChNP (41.5 nm) was mixed with 3 g of TC powder (GC TC, GC Dental products, Japan), and dissolved in 2 mL of the supplied TC solution based on the manufacturer's instruction to prepare 5% ChNP disks. The prepared TC dough was instantly pressed on the sterile surface to reach 1 mm thickness, which was pierced to 5 mm diameter dough using a sterile round cutter, and dried up in an incubator at 40°C for 20 minutes. After drying, TC disks were gently brought out that uniformly formed into 1 mm thickness and 5 mm diameter discs, used for *C. albicans* biofilm formation and antifungal susceptibility testing (Figure 1). TC disks containing 2.5% and 1.25 % nano chitosan were also prepared as explained for 5% TC disks.

TC disks containing100,000 IU nystatin (Emad Pharmaceutical Company, Iran), by the addition of 5% %wt./wt. of nystatin (16) to GC TC powder before dissolving in the TC diluent, were used instead of ChNPs, and TC disks without any additives (PBS solutions) were used as positive and negative controls (n = 4) in the current study, respectively.

Determination the Effectiveness of Tissue Conditioner Disks Containing Chitosan Nanoparticles in Controlling Biofilms of Candida albicans

All disk groups were sterilized with UV rays, placed in the wells of six-well micro-plates, and separately immersed in synthetic artificial saliva (compositions: Kcl. Kscn, KH_2PO_4, NaCl, NH_4Cl, Urtia, NA_2SO_4, 10 H_2O, pH = 6.75; Nikseram, Razi, Iran). Then, they were shaken on a reciprocal shaker at 100 rpm at 37°C. The artificial saliva in all wells was regularly changed daily (to simulating normal human saliva washing effect and its pH (6.75) was controlled daily) and for up to 7 days. After 1–7 days, the immersed disks were washed twice with a sterile physiological solution (NaCl 0.85%) and used for *Candida* biofilm formation as follows:

A: Broth Micro Dilution Test

The immersed disks of each experimental group were put in 24 dwelling plate's wells, which contained 900 µL of the RPMI 1640 (Merck, Germany) broth separately and were contaminated with a 100 µL of 1×10^6 CFU/mL *C. albicans* cell suspensions. The plate was incubated for 48 hours on a reciprocal shaker at 30°C. All contaminated disks were then rinsed with a sterile PBS solution to wash the *Candida* cells that were no attached to the disks (15).

Each test and the control disk group were placed in a 15 mL sterile Falcon tube containing 10 mL of the PBS solution and glass pearls and then subjected to an ultrasonic bath (Elma, Germany) at 45 kHz for 5 minutes to remove the adhered *C. albicans* from the biofilm on the TC disks. Finally, 100 µL of the washed solution was

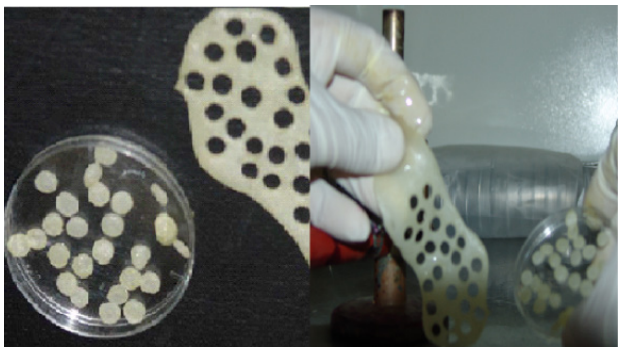

Figure 1. Preparation of Tissue Conditioner Disks Containing Nano Chitosan for *in Vitro* Biofilm Formation

cultured on a plate containing Sabouraud dextrose agar (Oxoid, UK) using a sterile glass spreader. All cultures were incubated in a 30°C incubator for 48 hours, and the growing *Candida* colonies were enumerated.

B: Performing Disk Diffusion Test (15)

Sabouraud dextrose agar plates were inoculated by pipetting 0.5 mL of the *C. albicans* suspension that was uniformly spread across the surface using a sterile spreader and allowed to dry at room temperature for 30 minutes. The test (TC disks containing ChNPs, n = 7) after 24 hours, 48 hours, 72 hours, and 7 days of incubation in the artificial saliva and the negative (TC disks without any ChNPs) and positive (containing nystatin as a gold standard, n = 4) controls were placed onto each seeded agar plate (15 mm from the edge and 25 mm from the center of the other disk) and aerobically incubated at 37°C for 24–48 hours. The diameters of the inhibition zones for *C. albicans* growth were assessed with a caliper. The average diameters of the inhibition zone were taken for each test and the control group disks after each established time.

Statistical Analysis

The obtained data were analyzed using SPSS (Statistical Package for Social Sciences) software, version 20. The confidence interval was set at 95%, and *P*-values < 0.05 were considered statistically significant. Eventually, the mean values of data were analyzed using the Kruskal-Wallis and Mann-Whitney tests.

Results

ChNPs prepared in this study were examined by scanning electron microscopy (Figure 2C), which showed NP dimensions ranging from 18 nm to 97.5 nm (Figure 2B), with an average size of 41.54 nm (Figure 2A).

ChNPs prepared in this study were analyzed using FTIR (17). FTIR analysis indicated that the amide I peak, associated with NH_2 bending, shifted from 1647 cm^{-1} to 1651 cm^{-1}. Additionally, the new peaks observed at 1238 cm^{-1} (C–O–C stretch) and 1555 cm^{-1} (amide II) are depicted in Figure 3. These changes suggest the formation of a complex due to electrostatic interactions between the NH_3^+ groups of chitosan and the phosphoric groups of tripolyphosphate within the nano chitosan.

The results of the broth micro dilution susceptibility testing for ChNP solutions are summarized in Table 1 and shown in Figure 4. This testing was performed to determine the minimum inhibitory concentration of the ChNPs against *C. albicans*.

Based on the results (Table 1 and Figure 4A-B), the concentrations of 5% and 2.5% ChNPs in TC disks completely inhibited the growth of *C. albicans*, the same as 100,000 IU of nystatin. Therefore, only TC disks containing these two concentrations were used for further susceptibility testing using the disk diffusion test. Moreover, the 2.5% ChNP and 1.25% ChNP solutions were determined as the minimum fungicidal and

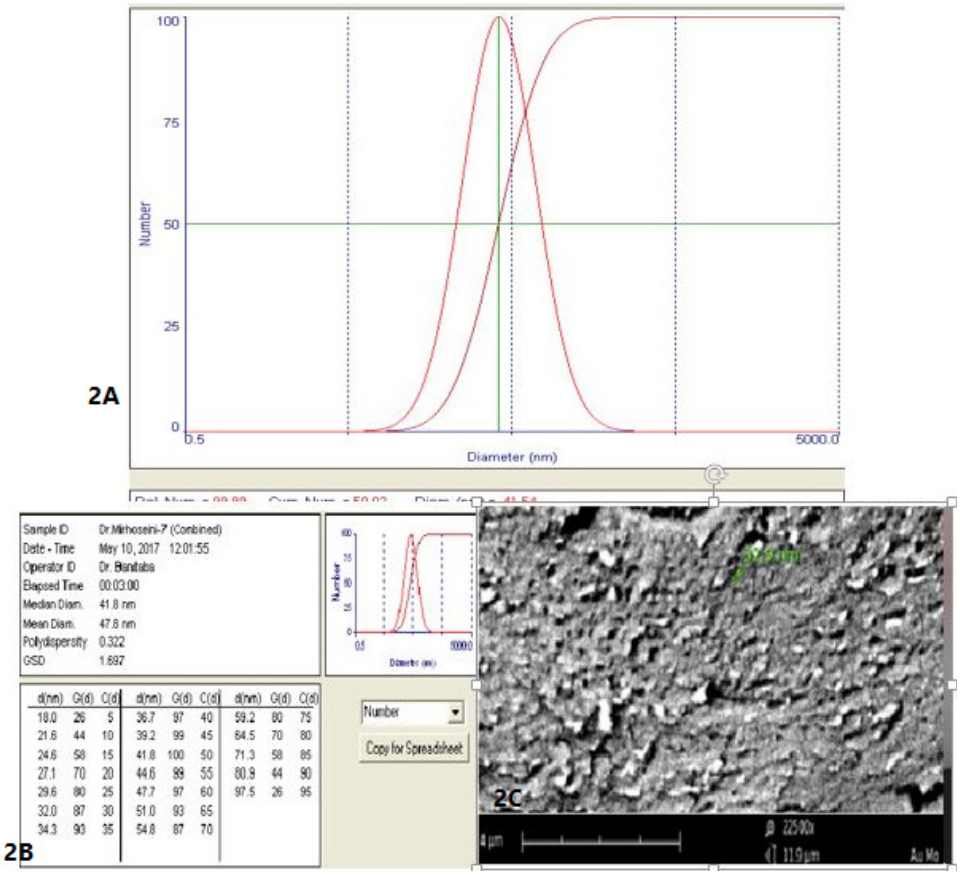

Figure 2. The Diagram for Determining the Size of Synthesized Chitosan Nanoparticle **(A)**, Particle Size **(B)**, and Its Aspects by SEM **(C)**
Note. SEM: Scanning electron microscopy

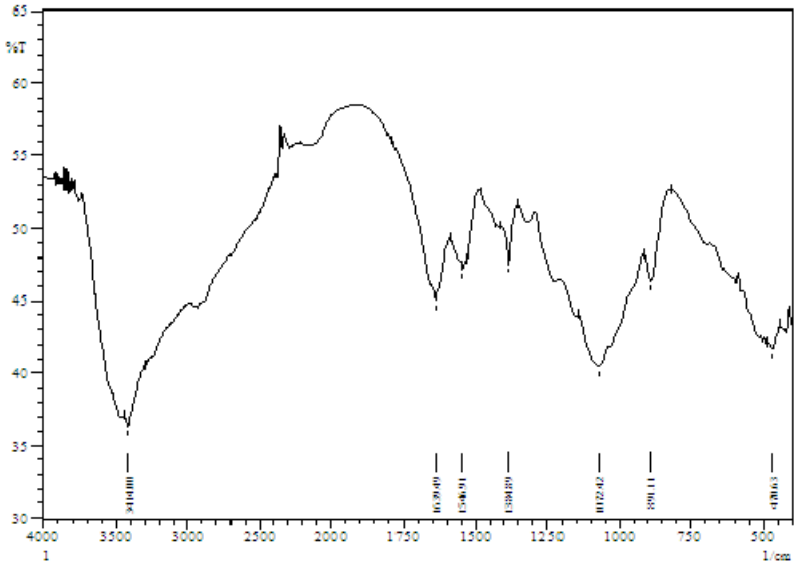

Figure 3. The FTIR Diagram of the Prepared Chitosan Nanoparticle
Note. FTIR: Fourier-transform infrared spectroscopy

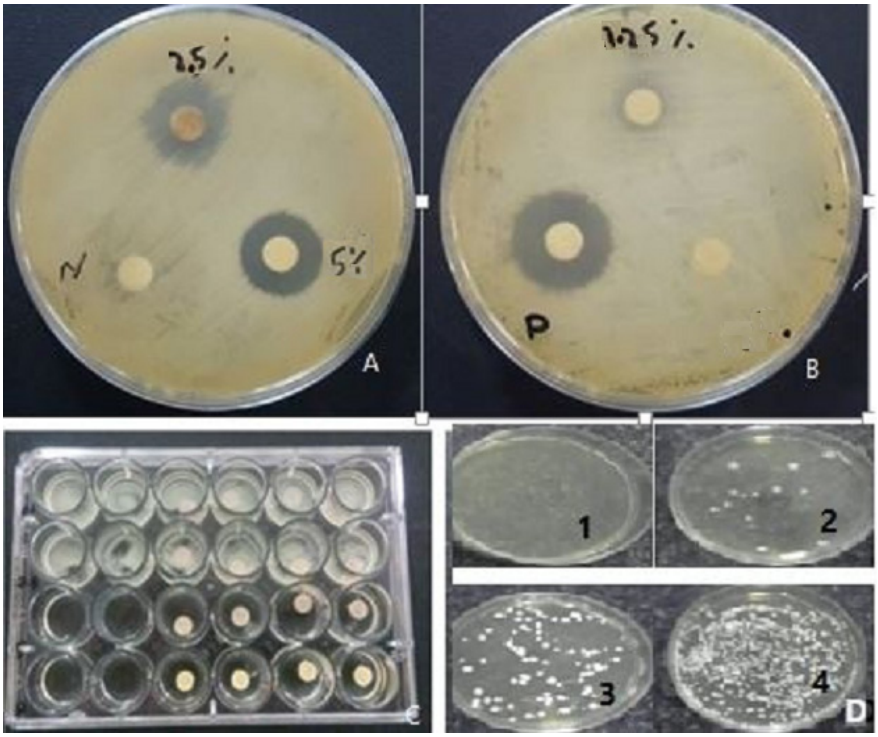

Figure 4. Antifungal Susceptibility Testing of TC Disks Containing ChNPs
Note. TC: Tissue conditioners; ChNP: Chitosan nanoparticle; PBS: Phosphate-buffered saline.
A: B: Disk diffusion test (N: Negative control; P: Positive control, 5%, 2.5%, and 1.25% ChNP disks)
C: Broth microdilution test of TC disks
D: Cultures of broth microdilution results (test groups)
D_1: Culture from the ChNP 5% disk solution (48 hours), D_2: Culture from 2.5% ChNPs (48 hours), D_3: Culture from 1.25%; D4: Negative control (TC disks without any ChNPs and antifungals immersed in PBS)

minimum inhibition concentrations, respectively, which inhibited 90% growth of *C. albicans* in comparison with the negative control in the present study.

Our results confirmed that TC disks containing 5% and 2.5% nano chitosan concentrations could entirely inhibit the growth of *C. albicans* within the first 24 and 48 hours. No statistical differences (Mann–Whitney test) were found between these concentrations of ChNPs and nystatin ($P=0.0001$) in the first 24 and 48 hours.

After 48 hours, the antifungal property of ChNPs was dramatically reduced, and after 72 hours, there were no reductions in the cultivated *C. albicans* colonies compared to nystatin ($P=0.127$).

Discussion

The increasing population of elderly complete denture users and the development of related complications have led to the application of TCs as temporary soft liners to

Table 1. Results of Broth Micro Dilution Susceptibility Testing for Chitosan Nanoparticle Solutions

ChNPs (conc)	5%	2.5%	1.25%	0.625	Nys. (Con+)	Saline (Con-)
[1]Mean±SD	0	0	79±18	918±81	0	1250±252

Note. SD: Standard deviation; *C. albicans*: *Candida albicans*; TC: Tissue conditioner; ChNP: Chitosan nanoparticle; PBS: Phosphate-buffered saline; Nys: Nystatin.
[1] Number of isolated colonies (viable *C. albicans*) counted after culturing microplate wells' test results. Test groups: TC disks containing 5% and 2.5% ChNPs; Positive control: Nystatin (100000 IU); Negative control: PBS solution

Table 2. Results of the Mean Diameter Zone of Inhibition (mm) Around the Tested Disks

Test Groups	N	Mean±SD	Minimum	Maximum
5% disks (24 hours)	7	2.1±0.75	1.2	3.2
(48 hours)	7	1.8±0.6	1	2.5
(>48 hours)	7	0	0	0
2.5% disks (24 hours)	7	1.5±0.48	1	2
(48 hours)	7	1.2±0.35	0.9	1.6
(>48 hours)	7	0	0	0
Nystatin (24 hours)	4	2.9±0.9	1.5	4
(48 hours)	4	1.8±0.3	1.1	2.3
(>48 hours)	4	0.7±0.1	0.2	1.1
PBS	4	0	0	0

Note. SD: Standard deviation; TC: Tissue conditioner; ChNP: Chitosan nanoparticle; PBS: Phosphate-buffered saline.
Test groups: TC disks containing 5% and 2.5% ChNPs
Positive control: Nystatin (100000 IU)
Negative control: PBS

Table 3. Results of Broth Micro Dilution Susceptibility Testing for Chitosan Nanoparticle Solutions

Test Groups	n	[1]Mean±SD	Minimum	Maximum
5% solution (24 hours)	7	0	0	0
(48 hours)	7	0	0	0
(>48 hours)	7	0	0	0
2.5% solution (24 hours)	7	0	0	0
(48 hours)	7	0	0	0
(>48 hours)	7	58±13	18	89
<2.5% solution (24 hours)	7	10245±2150	890	1386
(48 hours)	7	Many (uncountable)		
(>48 hours)	7	Many (uncountable)		

Note. SD: Standard deviation.

the base of resin prostheses. TCs are commonly utilized to alleviate DS in elderly denture wearers, many of whom struggle with maintaining acceptable levels of prosthetic hygiene due to age-related conditions.

A low pH and anaerobic environment appear between the bases of acrylic denture or TC and the cover of the mucosa, resulting in *C. albicans* overgrowth and increased enzyme activity (18). Hence, it seems that TC may intensify DS by the implantation of *C. albicans* on its surface. The antifungal incorporation of medications into TCs establishes a potent antimicrobial barrier that effectively controls DS. This approach has been supported by various studies, highlighting the benefits and efficacy of modified TCs in managing this common condition among denture wearers (19, 20).

ChNPs have emerged as highly promising materials that have garnered significant attention over the past few decades. These NPs hold great promise as nano-carriers, capable of encapsulating drugs and active compounds to deliver them to targeted areas within the body, ensuring controlled release (21).

The results of the current study demonstrated that *C. albicans* is a partially sensitive and dose-dependent fungus against the developed ChNPs, which only remained effective during the first 48 hours at 2.5% and 5% concentrations of TCs.

Our results also conform the findings of Wassel et al (22), and Mousavi et al (23), indicating a greater impact in a shorter timeframe when compared to other medications. Specifically, their studies revealed that ChNPs exhibit significant antifungal activities against *C. albicans*, with effective inhibition observed at higher concentrations without any alteration on TC properties (24).

Similarly, Mousavi et al reported the antifungal effect of a 2.5% concentration of ChNPs combined with TCs in inhibiting *C. albicans* growth for the first 24 and 48 hours (23).

Likewise, Safari et al found a sharp reduction in *C. albicans* CFUs compared with the control and nystatin groups when TCs were combined with ChNPs, underscoring the effectiveness of integrating ChNPs into dental materials to enhance their antimicrobial properties (25).

In the current investigation, the antifungal property of ChNPs was compared with nystatin, showing similar antifungal effects on *C. albicans* for 24 hours and 48 hours.

The addition of nano chitosan demonstrated a dose-dependent inhibitory property on the growth of *C. albicans*. Furthermore, immersing the discs in artificial saliva revealed a negative correlation between the duration of immersion and the level of inhibition observed in this study. In line with our findings, those of the study by Radani et al involving the inclusion of miconazole with TC confirmed a dose-dependent inhibitory impact on the growth of *C. albicans* (26). Additionally, a negative correlation was observed between the duration of immersion in artificial saliva and the level of inhibition.

Chitosan-based nanomaterials are at the forefront of research and have garnered significant interest due to their variety of physicochemical characteristics (e.g., biocompatibility, biodegradability, and non-toxicity), making them highly promising for various biological applications. Chitosan, along with its derivatives, is utilized in a range of fields, including biomedical engineering and pharmaceuticals (27).

Poznanski et al, conducting a review that systematizes the information available regarding the parameters that enhance the antifungal activity of chitosan and

ChNPs (28), concluded that fungi can be categorized into chitosan-sensitive and chitosan-resistant types. In sensitive species, the cell membrane becomes permeable due to chitosan, leading to the up-regulation of various genes that encode proteins associated with the cell wall, oxido-reductases, and transport-related proteins.

C. albicans is a sensitive fungus against ChNPs in biofilm formation on denture-based materials under both *in vitro* (29) and *in vivo* conditions (30, 31). Research has demonstrated that ChNPs effectively inhibit the growth of *C. albicans* biofilms, showcasing their potential as an antifungal treatment in dental applications.

Our *in vitro* study had two limitations, including evaluating the negative effects of ChNPs on the physical and mechanical properties of TCs and analyzing their cytotoxicity effect under *in vivo* conditions . Accordingly, it is suggested that future studies analyze the cytotoxicity effect of ChNPs on relevant oral cells (gingival fibroblasts and keratinocytes) and biofilm-specific assays, such as 2,3-bis(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide or confocal laser scanning microscopy).

Conclusion

The results of the current study confirmed that the addition of ChNPs to TCs can be useful in controlling *C. albicans* colonization for the first two days. After this period, it is recommended that the tissue conditioner be replaced with a new layer containing ChNps.

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Authors' Contribution

Conceptualization: A.A . Jafari and M.R. Hakimi-Meybodi
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Investigation:
Methodology: A.A. Jafari and M.H. Lotfi-Kamran
Project Administration: M.H. Lotfi-Kamran, and AA. Jafari
Resources:
Software:
Supervision: A.A. Jafari, M.H. Lotfi-Kamran, and A. Fallah-Tafti
Validation: A.A. Jafari and A. Fallah-Tafti
Visualization: A.A. Jafari
Writing-Original Draft: A.A. Jafari and M.R. Hakimi-Meybodi
Writing-Review and Editing: A.A. Jafari and M.R. Hakimi-Meybodi

Competing Interests

The authors declare no conflict of interests.

Ethical Approval

This study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd (ethical code IR.SSU.REC.1396.159). This approval ensured that the study adheres to ethical standards for conducting research involving human or animal subjects, safeguarding their rights and well-being throughout the research process.

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