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Antifungal Effect and Softness of Denture Tissue Conditioner Incorporated with Chitosan

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Abstract

Tissue conditioners treated oral candidiasis as a cushioning material. Adding Nystatin to the tissue conditioner was one of the treatment plans for treating such cases. Due to their biocompatibility and antimicrobial abilities, chitosan has become one of the promising materials in the treatment of fungal infections. Ninety-two samples were constructed from the tissue conditioner. They were divided into six groups: G1 tissue conditioner without additions, G2 with 3.2% Nystatin addition, G3 with chitosan addition 5%, G4 with chitosan addition 7%, G5 with Nystatin 3.2% and chitosan 5%, G6 with 3.2% Nystatin and chitosan 7%. Two tests were accomplished: the disc diffusion test for fungal inhibition capacity and the hardness test. Statistical analyses were made using ANOVA and Duncan's test using SPSS 18. The results showed that the samples in group G6 had significantly the highest inhibition efficiency without compromising the resilience property of the tissue conditioner, the hardness test showed that the addition of Nystatin and chitosan did not affect the resilience property of the tissue conditioner, also showed that the hardness of the tissue conditioner was increased significantly after seven days. The addition of the Nystatin 3.2% and chitosan 7% demonstrated the most superior inhibition efficacy in one and seven days compared to other groups, including the control group, indicating successful sustained release of Nystatin in addition to the fortified inhibition in the presence of chitosan. The hardness test showed no effect of the added materials on the resilience of the tissue conditioner. The tissue conditioner should be replaced preferably after seven days due to its loss of resilience and intended cushioning ability.

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Introduction

Polymethyl methacrylate (PMMA) has often been the prosthetic material of choice for patients who have lost all or part of their teeth, dominating the prosthesis production industry. Despite broad adoption, standard compression and injection molding methods have drawbacks, like heightened vulnerability to microbial colonization [1,2]. Some inherent properties in the PMMA could encourage this microbial adhesion, like surface porosities, inferior hardness, and roughness. Accordingly, epidemiological research indicates that 70% of patients with detachable prostheses have denture-associated stomatitis, with *Candida albicans* as the primary pathogen [3].

Most cases of denture stomatitis or thrush are associated with oral tissue damage that often needs a treatment of the damaged tissue with tissue conditioners that act as a palliative, and denture tissue surface modifiers. Unfortunately, these materials can be an etiologic factor for microbial adhesion, leading to the aggravation of the situation. For this reason, prosthodontists prefer using antifungal agents in combination with the tissue conditioners [4]. Denture stomatitis can be effectively prevented and controlled by combining antifungal and antibacterial medications with tissue conditioner materials, which restrict microbial plaque formation, particularly candida biofilm [5].

One of the most essential antifungal agents used to treat denture candidiasis is Nystatin, which is used to treat fungal infections successfully. It is an ionophore that binds explicitly to ergosterol to kill fungi.

The primary structural component of the cell is ergosterol, a fungal membrane, which also creates pores in the cell membrane when it is present in sufficient amounts [6]. However, the presence of Nystatin in sufficient amounts on the denture base or tissue conditioner depends primarily on the form of the material, where topical application of Nystatin has a restricted effect since it would be washed away after a period, in addition mixing of the powdered Nystatin to the polymers can reduce its efficiency due to the limited amount that could be released which is sufficient to treat the infection [7].

Chitosan is another antifungal material, a naturally occurring amino polysaccharide with various uses in industry and medicine. Its notable characteristics include low toxicity levels, appropriate biodegradability, favorable biocompatibility, and antimicrobial characteristics [8]. Previous studies have evaluated the efficiency of combining Nystatin with chitosan with each other or with another compound in treating oral candidiasis [5,7,9]. However, no previous information is available about the efficiency of combining Nystatin and chitosan with the tissue conditioner, all in the powder form as a fungicidal, its sustained release, and the effect of this combination on the surface hardness of the tissue conditioner.

Safari et al. (2024) reported that combining chitosan with the green-synthesized silver nanoparticles had a superior efficiency against fungal colonization compared to the effect of Nystatin, chitosan, and silver nanoparticles alone [5]. Ayub et al., 2024 found that the composite of Nystatin-coated CuO nanoparticles had a higher impact on microbial growth inhibition than Nystatin medication alone [7]. Through electrostatic interactions with negatively charged phospholipids, chitosan influences the cell membrane. Chitosan can enter a cell when the cell membrane is broken. This may result in disruption of protein synthesis and suppression of DNA/RNA synthesis [10,11]. Nystatin takes action against fungal infection by specific binding to ergosterol, which is a significant constituent of the fungal cell membrane, leading to pore formation in that membrane, where the pores aid in potassium ion leakage and acidification of the cell, consequently, the death of the fungal cells [12]. The null hypothesis was that combining chitosan and Nystatin powders with the powder of tissue conditioner would not increase the antifungal efficiency and raise the hardness of the tissue conditioner.

Materials and Methods

The total number of samples in the current study was 92 samples distributed to six groups (n =17 where 12 samples were constructed to conduct the disc-diffusion test and five samples to perform the hardness test. The first group (G1) was the negative control group without any addition. The second group (G2) was the positive control group with a 3.2% addition of Nystatin powder. In the third group (G3), chitosan powder was added at 5% to the tissue conditioner without Nystatin. In the fourth group (G4), chitosan was added to 7% of the tissue conditioner without Nystatin. In group five (G5), Nystatin was added at 3.2%, and chitosan at 5% to the conditioner. Lastly, in the sixth group (G6), 3.2% Nystatin and 7% chitosan powders were added to the tissue conditioner powder.

A tissue conditioning (TC) material in the powder and liquid form was used to prepare the samples, COE-comfort (GC AMERICA INC., ALSIP, IL, USA). Nystatin powder FZBIOTECH (Xi'an Fengzu Biological Technology Co., Ltd., Xi'an, China) and chitosan powder (Shaanxi Top Pharm Chemical Co. LTD, Shaanxi, China) were used. The fractions' percentages of the samples' contents were estimated using a PG 503-S MonoBloc digital balance with 0.000g precision (Mettler Toledo Ltd, Switzerland). To prepare the samples of G1, the powder of the TC was mixed with the liquid in a 1:2 ratio. To combine the samples with Nystatin (3.2% equals 0.032/1g TC), or chitosan (5% equals 0.05/1g TC, and 7% equals 0.07/1g TC) alone or with each other the weight of the powder of the G1 was measured and then the weights of the added powders were subtracted from the conditioner's powder to make sure that the same weight was used in the samples between the groups. The powder was formed and mixed after that at a 1:2 powder-to-liquid ratio, as recommended by the manufacturer. The flasks were closed, and the samples were allowed to set. After they set the access material, it was removed carefully using surgical blade No. 12 (Ningbo Greetmed Medical Instruments Co., Ltd, Zhejiang, China). Then all the samples were stored for 24 hours in distilled water before conducting the tests.

To prepare the samples of the disc-diffusion test, a hard plastic sheet, Imprlon (Scheu dental Co., Iserlon, Germany) of 1.5mm thickness was cut into circular discs with a 5mm diameter [13] were poured with elite dental stone (Zhermack Co., Polesine, Roma, Italy). After the stone was set, the plastic discs were removed to create a mold for the samples. Isolating the pathogenic Candida from the oral cavity of a patient with denture stomatitis is necessary to investigate the antifungal activity of chitosan. After obtaining mouth swabs from the tissue surfaces of six old dentures that would be replaced by the patients who were enrolled in the prosthodontics clinic at the University of Mosul College of Dentistry, this isolation was carried out following the approval by the human research Ethics Committee in the College of Dentistry, University of Mosul, under protocol no. (UoM/H.DM.L86/22). The collected swabs were cultured immediately in dishes containing (Sabouraud 4% Glucose Agar) according to the ISO specifications 11133 and 16212 [14,15]. The colonies of the *Candida* were differentiated by the specific color of each colony species using Chrome Center Chromagar (CHROMagar Co., Paris, France). After the incubation at 37°C for 24- 48 hours, the color changes of the fungal colonies were the colony of *Candida albicans* green, the colony of *C. tropicalis* in blue surrounded by a pink halo, the colony of *C. Parapsilosis* was purple, and the colony of *C. krusei* was pink [13,16]. Swabs were collected from the colonies under study *Candida albicans, C. Parapsilosis*, and incubated similarly. The prepared disc-shaped samples were placed in sterile conditions and incubated at 37 °C. The inhibition zones' radii were measured after one day and seven days [13,16,17].

To achieve the hardness test, rigid plastic sheets of Imprelon with a 4.5 mm thickness were cut circularly to create the samples' models with a 4.5mm thickness and 25mm diameter. The model discs were poured with Elite stone inside the flask and removed after the stone had set to produce the mold with the required dimensions. The powder was mixed in the same manner mentioned above for each specific group and allowed to set. The access material was removed with surgical blade 12. Then they were stored in distilled water for 24 hours before conducting the hardness test. A Shore A durometer (PHPSA, Hans Schmidt & Co. GmbH, Waldkraiburg, Germany) was used to measure the hardness of the samples. The mean of three readings for each sample was calculated, and the readings were taken after one day and seven days.

The study was approved by the ethical committee of the scientific research in the College of Dentistry, University of Mosul, with reference protocol no.: UoM.Dent/H.DM.L.86/22 at 26/12/2022.

The statistical evaluations were conducted by the SPSS 18 program (SPSS Inc., Chicago, IL, USA). The ANOVA test, Duncan's post hoc test, and the Shapiro-Wilk test for normality of distribution at $P< 0.05$.

Results

The results of the disc-diffusion test against *Candida albicans* after 24 hours were listed in Table 1. The results demonstrated that the inhibition zones of G6 2.2667±.675mm and G5 2.1667±.437mm were significantly

higher than those of the other groups. Followed by G2 1.5333±.293mm which was significantly higher than G1, G3, and G4 (.2333±.234, .6333 ±.362, .7333±.532 mm) repectively. The inhibition zones of G4 and G3, respectively, with chitosan addition alone were significantly higher than those of G1 without any addition. The results of the disc diffusion test against *Candida albicans* after seven days showed that there was a drop in the means of all groups, however, the same statistical order of 1 day statistics was followed where G1 without addition was still significantly the lowest among the groups followed significantly by G3, which was significantly lower than G4. The addition of Nystatin alone in G2 had significantly higher inhibition efficacy than G1, G3, and G4; however, this efficacy was significantly lower than G5 and G6, where both groups had the highest fungicidal activity among all groups.
Table 1. About here
The inhibition zones against *Candida parapsilosis,* were listed in Table 2, at day one reading showed that G6 2.2000±.008mm and G5 2.1833±.283mm were significantly higher than other groups, followed by G2 1.3000±.003mm which was significantly higher than G4 .9000±.118, whereas G3 .8833±.0134mm and G1 .3000±.0231mm showed significantly the lowest inhibition results among all groups. After seven days results showed that there was a drop in the means of all groups, however, the same statistical order of 1 day statistics was followed.
Table 2. About here
The hardness test results are shown in Figure 1. The results showed that there was an insignificant difference among all groups after 24 hours and seven days, showing that there was no effect of the addition of Nystatin or chitosan, each alone or together, on the hardness property of the tissue conditioning material used in the current study. Also, there was a noticeable increase in the hardness of the tissue conditioner after seven days in all groups.

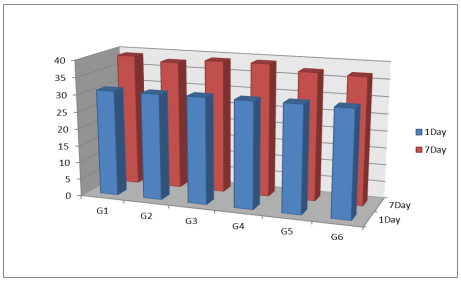

Figure 1. Duncan's multiple range of Hardness test of all groups after 1-day and 7-day.

Discussion

The current study showed a significant increase in inhibition efficiency when Nystatin and chitosan powders were added to the tissue conditioner powder without a significant effect on the hardness property of the material. According to these results, the null hypothesis was rejected. Treatment options for patients with denture stomatitis (DS) involve relining or remaking the denture, removing the triggering cause, prescribing medication, and commencing with oral hygiene directions. To alleviate the signs and symptoms of this condition, conditioning the affected basal seat is crucial. Tissue conditioner lining material was used to line the infected patient's denture [18].
The current study showed a significant inhibition efficacy of Nystatin against microbial species. *Streptomyces noursei* produces the macrolide Nystatin, which exhibits exceptional effectiveness against Candida molds, particularly Candida albicans. However, the side effects of Nystatin, like bitter taste and allergic reaction development, are commonly associated with multiple applications of the drug, especially with gel or liquid forms [18–20]. This study was conducted to minimize the frequent applications of Nystatin by combining the drug with chitosan and the tissue conditioner to ensure better efficiency and sustained release of the medication without adverse effects. The results of the disc diffusion test against the two types of Candida under examination showed that the greatest inhibition efficiency was associated with combining chitosan and Nystatin powders with the tissue conditioning material. These findings might imply that the physical network structure resembling an open cell determines the drug release from chitosan powder. Drug release from the combination happens through the pores of the low polymer concentration, without an effect on the hardness property of the tissue conditioner, up to 7% of chitosan [21–23]. This sustained release may be the outcome of the solubility of Nystatin in the chitosan after adding the tissue conditioner's liquid. These results go with previous studies that found that Nystatin release is increased in combination with chitosan gel up to 5%. In contrast, the rise of chitosan above this concentration may decrease the Nystatin release. This controversy comes from the fact that this study used Nystatin and chitosan in powder form [21,24]. The mechanism of action of the chitosan molecule in inhibiting bacteria was documented in numerous studies. Using confocal microscopy, Park et al. determined that chitosan molecules are present in fungal plasma membranes and inhibit their function [25]. Chien et al. also employed an electron microscope to show that the cell wall of Candida albicans contains chitosan molecules, either with or without photodynamic inactivation [26]. When chitosan powder came in contact with the liquid of the tissue conditioner, it most probably turned into a gel form that contained Nystatin inside the polymeric mass, which explains the sustained release of the drug. These findings are consistent with the findings of former studies which demonstrated that the increase of the gel contents in the polymeric mass is directly proportional to the increase of the drug sustained release [27–30]. Hardness and resilience of the dental materials used in prosthodontics are very important properties to achieve their intended purposes [31], for this reason the hardness test of the tissue conditioner was conducted in this study, where, the loss of resilience of the conditioner may lead to failure of the material to participate successfully in the healing process which can be no more act as a cushion for the inflamed, infected sites of the oral mucosa [32,33]. The results of the current study showed that the tissue conditioner's hardness was not affected by the presence or absence of Nystatin or chitosan over the seven days. On the other hand, the hardness of the material alone or with addition was noticeably increased after seven days, which can be explained by the leaching out of the plasticizing agent molecules to the wet environment of the oral cavity and partial replacement of these molecules by water [34]. The motion or slippage of the polymeric chains over each other is the cause of the resilience owned by the tissue conditioner that gives the cushioning property that helps in the healing process. On the other hand, this motion gives the basis for unstable mass that loses the plasticizer molecules, leading to hardening of this mass after a period [35–37].
It can be seen that the addition of Nystatin alone significantly increased the inhibition ability against fungal infection; however, this inhibition capacity was improved dramatically with the addition of chitosan powder, leading to a higher inhibition capacity of the combination in the first twenty-four hours and after seven days. The Nystatin, chitosan, and tissue conditioner combination showed the highest inhibition efficiency without compromising the resilient property of the tissue conditioner, giving rise to the importance of this study, where high concentrations of Nystatin were ensured over seven days with the help of the presence of chitosan. If this period is not enough for complete healing, replacing the tissue conditioner with a new one is recommended due to the loss of its resilient properties.
One of the limitations of this study was the use of one type of tissue conditioners and one form of the materials (powder form), where another form, like gel or liquid form, was not tested. Future studies shedding light on the effect of this combination on other

fungal types, along with the study of the microscopic behavior of the combination, will be helpful in understanding the exact action of this combination on microbial cells.

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Table 1. Means, statndard deviations, and statistical differences of the inhibition zones (mm) of the groups against *Candida albicans* after one day and seven days.

Group no.	N	Nystatin 3.2%	Chitosan 5%	Chitosan 7%	Mean (mm)±SD after 1 day	Mean (mm)±SD after seven days
G 1	12	--	--	--	.2333±.234^{A}	.2000±.142^{A}
G 2	12	++	--	--	1.5333±.293^{B}	1.2167±.721^{C}
G 3	12	--	++	--	.6333 ±.362^{C}	.4833±.384^{A}
G 4	12	--	--	++	.7333±.532^{C}	.7500± .473^{B}
G 5	12	++	++	--	2.1667±.437^{D}	1.7000±.093^{D}
G 6	12	+	--	++	2.2667±.675^{D}	1.9667±.738^{D}

Where SD is the statndard deviation, mm is millimeters. The identical letters shows insignificant difference within the same coloum.

Table 2. Means, statndard deviations, and statistical differences of the inhibition zones (mm) of the groups against *Candida parapsilosis* after one day and seven days.

Group no.	N	Nystatin 3.2%	Chitosan 5%	Chitosan 7%	Mean (mm)±SD after 1 day	Mean (mm)±SD after seven days
G 1	12	--	--	--	.3000±.0231	.2167±.354^{A}
G 2	12	++	--	--	1.3000±.003	1.2167±.0973^{B}
G 3	12	--	++	--	.8833±.0134	.3833±.203
G 4	12	--	--	++	.9000±.118	.6167±.102
G 5	12	++	++	--	2.1833±.283	1.4333±.009
G 6	12	+	--	++	2.2000±.008	1.8333±.214

Where SD is the statndard deviation, mm is millimeters. The identical letters shows insignificant difference within the same coloum.