ORIGINAL ARTICLE

The Effectiveness of Chitosan Nano-Particles Addition into Soft Denture Lining Material on *Candida Albicans* Adherence

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ABSTRACT

Background: Because of the viscoelastic properties of soft liners , these materials will act as a cushion between the denture and the edentulous ridge to lower and redistribute the occlusal forces over the denture bearing area **.** One of the major problems associated with the soft liner using is the increased risk of invasion of Candida organisms on the soft lined dentures intaglio surface **.**
Aim: To investigate antifungal effect of incorporation of Chitosan nano – particles into heat cured acrylic based soft lining material .
Methods: Thirty samples were prepared and divided into three groups (control group , 1.5wt% , 2wt% experimental group), ten samples for each group, The sterile soft lining samples were deposited in sterilized tubes containing Sabouraud dextrose broth in which a small portion of the isolated yeast were suspended and incubated for 1 hr. at room temperature. After that , the specimens were removed , rinsed with Phosphate buffered saline , dried then fixed by methanol , stained with crystal violet and examined under inverted light microscope.
Results: The result of adherence test showed a highly significant decrease in the number of Candida cells adhered to soft liner after incorporating 1.5wt% , 2wt% chitosan compared to samples of control group.
Conclusion: Chitosan can be regarded as a strong antifungal material and incorporating the chitosan into soft liner can succeed in producing soft lining material with antifungal activity against Candida micro organsims.
Keywords: Chitosan, Nano-Particles, Denture Lining Material, Candida Albicans Adherence.

INTRODUCTION

Acrylic resin denture bases provide an artificial substructure that preserves the location of the denture teeth and an oral mucosal interfacial area in which the occlusal forces are held. The elastic modulus of the acrylic resin denture bases is considerably higher than that of the tissues on which they settle[1].

Due to the friable nature of the supporting mucosa , areas of force intensity or the poor fit of the denture base which can lead to tissue injury and sore spots , manufacturers have established soft denture base liner to mitigate the risk of pain and discomfort arising from the transfer of denture base force to oral mucosa[2].
Soft denture liners are typically used as a barrier between the rigid denture base and the underlying tissues, rendering the oral mucosa less vulnerable to trauma[3].

The soft denture liners showed many problems, such as plaque deposition and susceptibility to fungal and bacterial aggregation and growth, which were found to be more vulnerable to microorganisms adhesion than acrylic resin base materials due to their roughness on the surface , in addition to the physical and chemical association between microbes and the material[4]**.**

One of the significant problems found in the case of poor oral and denture hygiene is the heightened possibility of invasion of Candida species on the intaglio surface of soft lined dentures[5]**.**

The most prevalent type of oral fungal infections is Candida albicans. It is estimated that 93 percent of patients with denture stomatitis are infected with Candida albicans[6].

Because of its multifactorial etiology, the treatment of denture stomatitis associated with candida is complicated . A large number of treatment strategies were used, including rinsing the dentures, stopping the use of dentures overnight, relining or replacing prosthesis and the use of topical or systemic antifungal drugs[7]**.**

Chitosan is a biocompatible , biodegradable natural polymer obtained from crustacean's outer shells, It is ideal to be used in different medical purposes due to its antifungal and antibacterial qualities[8]**.**

It is non toxic (biologically safe) and was recommended as a bioadhesive to the oral mucosa[9]**.**
Chitosan oligomers impede the fungal cell growth by diffusing into hyphae and interacting with enzymes necessary for its growth[10].

MATERIALS AND METHODS

This study include Incorporation of Chitosan nanoparticles (India) into soft liner monomer (Netherland) in concentration (0% control , 1.5% , 2% by wt.) , and the results compared with that of control positive which including the addition of Nystatin (China) 1.4% by wt. to the soft liner monomer **.**[(11)] for each group , 10 specimens were prepared **.**
Specimen's preparation: Plastic patterns (Disk shaped), 10 mm in diameter and 2mm in thickness used for preparing soft liner specimens[12]. Firstly silicone impression material (addition type) was kneaded by the hands and adapted in a circular shape , after that these plastic disks were imbedded in this silicone and wait until complete setting of the silicone occur while these patterns were inside it , then the silicone circle were inserted in the lower half of dental flask which already had dental stone (freshly mixed according to manufacturer 's instructions W / P ratio : 25ml /100g). The excess of stone material was removed and smoothened[13]. When the dental stone was set completely , the stone surface , silicone and plastic disks

were covered with thin layer of separating medium and left to dry , then the upper part of the dental flask was placed over the lower part and filled completely with dental stone (with vibration to eliminate any incorporated air bubbles) and covered with its lid [13]. When the second layer of the stone was completely set, the flask was opened and plastic disks were removed leaving a space in the silicone**.**

Proportioning and mixing of heat cure soft liner: According to the manufacturer's instructions (P / L ratio 1.2 g: 1 ml), The amount of soft liner liquid and powder were determined and mixed in dry clean glass jar and covered with a lid.

Incorporation of Chitosan nanopowder into soft liner

Chitosan nanopowder was first weighted in clean, dry glass container and the soft liner monomer added to it and mixed with probe sonication apparatus to break them into individual micro particles by vibration at 120W and 60 KHz for 3 min. The weight of chitosan nanopowder powder should be subtracted from the soft liner powder weight to keep the same manufacturer's P/L ratio[14,15].

Packing: when the soft lining material reach to the dough stage, it was needed by the hands and placed on the previously prepared mold and covered by sheet of polyethylene , then the upper portion was placed on it and a pressure applied to it by the hydraulic press to ensure equal distribution of soft lining material inside the mold and for expelling the excess material .

After that the flask was removed from the press and opened , the polyethylene sheet was removed as well as the excess material by wax knife and the stone surface coated again with separating medium and allow to dry.

Finally, both pieces of the flask were brought in and secured in a good manner and retained to the press machine and left under Pressure of 100Kg/cm2 for 5 min. , then clamping was done to be ready for curing[14].

Curing and finishing: This process was achieved by placing the packed flask in digital thermostatically controlled water bath. According to the manufacturer's instructions, the curing time was 90 min. at 70°C, then the temperature was raised to 100°C for 30min[15]. The flask removed from the water bath after the completion of curing cycle and allowed to cool at room temperature for 30 min. and then placed for 15 min under the tap water to complete the cooling process, the flask was opened when became completely cold and the specimens removed from the mold[14]. By using sharp blade, the excess material removed from the specimens and finishing was done by fine grit silicone polishing burs and fine grit sandpaper , then the specimens rinsed with distilled water and sterilized by the autoclave (121º/15 pci).

Isolation of *C. albicans:* *C. albicans* was isolated from the mouth of patients with signs and symptoms of denture Stomatitis attended the prosthodontics clinic of Dentistry College - Kufa University for seeking treatment [16]. The oral lesions were scrubbed gently by using a cotton swab and then inoculating sabouraud dextrose agar which is the primary isolation medium [17]. These swabs were cultured and aerobically incubated at 37° C for 48 hrs, and saved in 4° C to be used in the other tests[18].

Identification of *C. albicans*

A- Microscopical examination: A small portion was taken from isolated colony and emulsified in a normal saline drop on the slide to prepare suspension that was spread, allowed to dry at room temperature, and fixed by passing the glass slide over the flame of Bunsen burner several times. This slide was stained according to Gram's Method[19]

B- Germ tube formation: From a single colony, One lope inoculums of yeast cells was taken and suspended in tubes (contained 0.5 ml of serum), then these tubes incubated at temperature of 37^0 C for 3hr. Then a suspension drop was placed on a glass slide and this slide was examined under Light microscope to see the presence of germ tubes[20].

C- Biochemical Identification: VITEK 2 is a fully automated device with a sensitive fluorescence-based technology that performs microorganism identification. Prior to testing, a suspension of cultured yeast was prepared in sterile saline at 2.0 turbidity of Mc Farland standard, as determined by using Densi Chek instrument, the VITEK ID-YST card was filled with the suspension automatically, sealed , and incubated at 35.5 °C for 18 h in the VITEK 2 instrument and optical density readings were taken every 15 min. automatically . The final results were compared with the database, and the unknown microorganism's identifications were obtained[21].

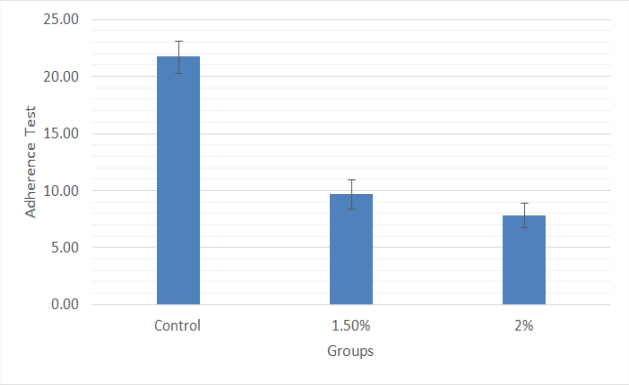
Evaluating the effect of Chitosan / soft liner specimens on adherence of *C. albicans*

Adherence test procedure: According to manufacturer's instructions, **Sabouraud dextrose broth (SDB)** was prepared and poured into sterile tubes and a small portion of the isolated yeast were suspended in the media, and the concentration of the suspension was established equal to (0.5) McFarland standard by using a McFarland densitometer[22]. The sterile soft lining samples were deposited in the previously prepared media in the sterilized tubes and incubated for 1 hr. at room temperature. After that, the specimens were taken out of the incubator and removed from the suspension and rinsed with phosphate buffered saline for one min. with gentle rocking for eliminating the non-adhered yeast cells, and dried with filter paper[14]. The adhered Candida cells on the soft lining specimens was fixed by using methanol and then stained for 60 seconds with crystal violet , and rinsed again for 30 seconds with PBS solution, then dried with filter paper and examined under inverted light microscope[23 ,24]. Under the inverted light microscope, the adherent Candida cells enumerated for each sample in three standardized fields and the mean of such fields was taken for each specimen.

RESULTS

Evaluating the adherence ability of *C. albicans* to soft liners By examining the stained specimens for each group under the inverted light microscope , The highest mean value of control group (21.72) while the minimum mean value is (7.85) for experimental group (2% of chitosan) (Fig. 1).

Figure 1: Bar charts of the mean values and standard deviation of *C. albicans* adherence test



By using **one - way ANOVA test**, Comparison of the means of groups results was highly Significant and listed out within Table 1.

Table1: One - way ANOVA table

	Sum of Squares	Df	Mean Square	F	Sig
Between groups	1132.913	2	566.456	363.139	0.000
Within groups	42.117	27	1.560		
Total	1175.030	29			

In accordance with the results of data homogeneity of Levene's test which indicates non-significant differences among the groups, accordingly, Boneferroni test has been chosen for candida adherence multiple comparisons (Table 2).

Table 2: Bonferroni multiple comparisons test

Tested Groups		Mean Difference (I-J)	Sig.
Control	1.5%	12.000*	0.000
	2%	13.870*	0.000
1.5%	2%	1.870*	0.007

**The mean difference is significant at the 0.05 level.*

DISCUSSION

Because of the viscoelastic properties of soft liners , these materials will act as a cushion between the denture and the edentulous ridge to lower and redistribute the occlusal forces over the denture bearing area [25, 26, 27]. One of the major problems associated with the soft liner using is the increased risk of invasion of Candida organisms on the soft lined dentures intaglio surface [5].

Many studies have attempted to avoid the colonization of fungi by incorporating different antifungal agents in the material of the soft denture liner and the addition of nano particles [7, 28]. An attempt was made in the present study to improve soft lining materials has anti-fungal properties to C. albicans, by including Chitosan nano powder into soft liner. From the study's statistical findings, there has been considerable important decrease in the numbers of C . albicans cells adhered to the surface of the soft lining material containing chitosan nano powder compared with control specimens group.

In healthy humans, Candida albicans is a harmless commensal fungus . Candida albicans , besides, can cause simple infection and may increase the risk of systemic infections in immune compromised patients. Oral candidiasis can occur in the old patients with long lasting use of wide-spectrum antibiotics, corticosteroids and immune suppressants, acquired immunodeficiency syndrome (AIDS) [29]. Antifungal agents are recommended In these cases but *Candida* species showed recently increasing resistance to available antifungal medication. Chitin is the main structural component of crustacean and arthropod shells. The partial deacetylation of chitin results in chitosan which is a polysaccharide composed of glucosamine and N-acetyl glucosamine units linked by glycosidic bonds β(1–4)[30].

Due to its biocompatibility, biodegradability, non toxic properties and antimicrobial activity, there has been growing interest in chitosan modification and application in biomedical field[31].

Antifungal mechanism of action for chitosan include three proposed mechanisms

A. In the first mechanism, plasma membrane of fungi is the main target of chitosan . The positive charge of chitosan enables it to interact with negatively charged phospholipids components of fungi membrane. This will increase the permeability of membrane and causes the leakage of cellular contents, which subsequently leads to cell death [32].
B. Chitosan acts as a chelating agent , binds to trace elements , causing the necessary nutrients unavailable for normal fungal growth **.**[33]
C. Finally, the third mechanism indicated that chitosan could penetrate the fungal cell wall and bind to its DNA. That will inhibit mRNA synthesis and therefore affect the development of essential proteins and enzymes[31].

CONCLUSION

From the research provided, it can be concluded that Chitosan nano powder can be regarded as a strong antifungal material and incorporating the chitosan nano powder into soft liner material can succeed in producing a soft lining material with antifungal activity agiants Candida micro-organisms. Also , 2% experimental group showed a better activity against candida albicans comparing to control and 1.5% experimental group.

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