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Evaluation of the Antifungal Activity of Heat-Cured Soft Denture Liner Impregnated with Chitosan Nanoparticles

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Abstract

The most serious problem of the resilient denture lining materials is colonization and penetration of the soft-liner surface by Candia albicans and related Candida species. So, this research was oriented to check the antifungal effect of Chitosan nanoparticles (CHs NPs) incorporated into heat-cured acrylic soft liners. 60 specimens were prepared, 30 specimens for disk diffusion test and other 30 specimens for viable count of C. albicans test. The specimens split into six groups depending on different percentages of Chitosan NPs addition: 0.5wt%, 1.5wt%, 2.5wt%, 3.5wt%, 4.5wt%, and control group without addition 0%). For disk diffusion test, the autoclaved soft lining samples were fitted into sterilized Petri dish containing Mueller Hinton agar upon which candidal suspension were swabbed then fitted the samples on the agar surface, incubated for 24 hrs. Then the inhibition zones were measured by utilizing digital caliper ruler. For viable count test, the autoclaved samples of soft liner were submersion in sterilized tubes containing Sabouraud dextrose broth in which a small amount of candidal suspension, incubated for 24hrs. The Results of the disk-diffusion test showed the highest mean value of the inhibition zone at 3.5wt% group followed by 4.5wt% group and there was no inhibition zone for control and other experimental groups. Furthermore, the viability test indicated the colony forming units of C. albicans were decrease as the percentage of CHs NPs increased. With limitation of this study, it was concluded the CHs NPs considered as a strong antifungal agent and the impregnation of the chitosan nanoparticles into soft liner that help to form a soft lining material with antifungal activity and minimal candida associated infection.

Introduction:

In spite of the fact that, the implant prosthesis is considered as standard and best way of care for complete edentulous individuals [(1)] but the high cost, anatomical complications and medical conditions are considered a major limiting factors for those reasons, the necessity for acrylic resin denture bases is still as a treatment option [(2)]. During mastication, functional forces are transferred to the basal seat mucosa throughout a hard denture base this led to injury the oral mucosa also producing painful sore spot and further alveolar bone resorption [(3)]. So resilient denture lining materials are intended to be flexible, absorb energy and having a cushion effect between the hard denture bases and the underlying tissues. The lining material having remarkable capabilities in healing the sore tissue and its absorbing and distribution of functional loads on dentures areas and this leading to improve the denture retention and the fitness of the dentures to the tissue [(4)]. There are several conditions need to use flexible lining materials for complete dentures such as non-pliable thin atrophic mucosa, excessive or irregular bone resorption, sharp alveolar ridge, bony protuberances exostosis or painful neurological points in edentulous patients, the patients who suffer from bruxism and xerostomia requires more specific ways for treatment with complete denture [(5,6)].

Despite the many benefits of using the soft denture lining material, it has some problems, water sorption and color change [(7)], but the most serious problems that has been reported is colonization and penetration of the soft liner surface by Candida albicans and related Candida species [(8)]**.** As well as it was reinforced by environmental conditions under the denture, as well as the physical structure of the material itself [(9)].

There are many of studies show that there is a closed relation between denture induced-stomatitis and both of microbial-factors and poor-fitting dentures [(10)]. Due to its multi-factorial etiology, the treatment of denture stomatitis is complex, there is a lot of effective antifungal agents have been used, either topically or systematically [(11)], but it difficult to maintain an efficient dose of antifungals agents intra-orally [(12)]. To avoid these problems, many attempts are made to impregnate the antifungal agents within the soft liner material by addition of Nano particles [(13,14)]. A study was used Chitosan nano powder into soft liner to improve anti-fungal properties to *C. albicans* of soft lining materials [(15)]. Chitosan is a natural polymer, derived from the outer shell of crustaceans, which characterized by its biodegradability and biocompatibility [(16)]. It has been used in different biomedical, pharmaceutical due to its safe profile, biodegradability, and biocompatibility, in addition to its bacteriostatic and fungi-static [(17)]. So, this study was designated to evaluate the antifungal activity of incorporation of different concertation of the Chitosan NPs into soft liner.

Materials and Methods

Heat polymerizing acrylic based soft liner (Vertex™ Soft, Vertex-Dental, Netherlands) was used in this study. Chitosan nanoparticle powder (Us Research Nanomaterials, Inc, USA) was incorporated into soft liner powder in different percentages (0.5%, 1.5%, 2.5%, 3.5% and 4.5% by weight). After that, evaluating the antifungal activity of CHs NPs/PEMA samples by utilizing disk diffusion test and the viable count of *C. albicans* test. The results were compared with the control group (without any addition to the soft liner), 60 samples were prepared, 30 samples for each test.

Specimens Preparation

Plastic Models and Mould Preparation

Samples with specific dimensions for each test were designed by utilizing Auto CAD machine 2015 [(18)]. The disk shape with dimensions of 6mm in diameter and 0.5 mm thickness for disc diffusion test [(19)]. While a plastic pattern for candida viability count test was square in shape about (10×10×2.3 mm) length, width and thickness respectively [(20)]. Firstly, A hard but flexible silicone duplicating material used to duplicate the plastic pattern then

waited until the duplicating material have been completely set, then the mould with plastic patterns was inserted in the lower part of the flask which filled with freshly mixed dental stone, which mixed as instructed by the manufacturer's (W/P ratio: 25ml/100g), when the mixture of dental stone become set completely, thin layer of separating agent was used to coat the plastic model, silicon mould as well as the stone surface then left to dry, then the upper half of the flask were fitted over the lower half, and pouring the mixture of dental stone with the use of vibrator to prevent air bubbles incorporation. Finally, when the second layer of the stone has fully set, the flask has been opened and the plastic patterns were expelled out of the molds [21].

Proportioning, Mixing Ratio and Incorporation of Chitosan Nps with Heat Cured Soft Liner Powder

The specimens were prepared according to manufacturer's directions (P/L ratio: for each 1ml of the liquid monomer /1.2g of powder), soft liner powder was weighed for each group and mixed with the monomer in a clean, dry glass container. The mixing of CHs NPs powder and soft liner powder with the liquid monomer was done by taking into consideration that the weight of CHs NPs powder subtracted from the total weight of soft liner powder to get accurate P/L ratio [22]. For incorporating chitosan nanoparticles powder with liner powder by utilizing an amalgamator device for 1 minute with a mixing frequency 3000 vibrations to ensure obtaining a homogeneous distribution of the nanoparticles within the polymer particles [23].

Packing, Curing and De-Flasking

The packing was done at the late of dough stage, the dough manipulated by hands and packed into the mould and covered by polyethylene sheet then the two halves of the flask were re-assembled and putted under the hydraulic press with continuous application of pressure for equal flow of the soft liner material through the mould space, then the pressure was removed and the flask opened, the polyethylene sheet removed and with the using of the sharp knife the flashes of the soft lining material were cut off. At last, both pieces of the flask were assembled together and placed under pressure until complete contact was established and left at of 100 Kg/cm^2 for 5 minutes to be ready for curing clamping was done [24].

As directed by manufacturer instructions the packed flask was cured by using thermostatically controlled water bath for 90 minutes, heating up the temperature to 70 °C and then raised up and hold up to 100 °C for 30 minutes. Then the cured flask was removed from the water bath and left to cool down to room temperature for 30 minutes followed by placing the flask in tap water for 15 minutes, after cooling the flask opened and all the samples were removed out of the moulds [22].

Finishing, Polishing and Conditioning

The excess flashes of soft lining material of all specimens were removed from the specimens by using the sharp blade then finished by using fine grit silicone burs followed by fine grit sandpaper under continuous water cooling, then the specimens rinsed with distilled water and sterilized by the autoclave (121 °C /15 Pci) [21].

Antifungal activity test

Isolation and Identification of Candida Albicans:

Candida albicans was isolated from five patients' mouth with symptoms of denture sore mouth who's visit the college of dentistry/Baghdad, and this was made by direct sampling technique which including gently touching a sterilized cotton swab over the lesion site then inoculated the isolation medium then aerobically incubation was done at 37 °C for (24-48 hours), finally, it saved in cool place (4 °C) to be used later [25]. After that, identification was made according to:

A- Microscopic examination

With the using inoculation loop, take the single isolated colony then on glass slide, emulsified the colony in a drop of normal saline to form suspension material, then

spread it on the slide and left to dry to room temperature, passing the slide several times over the flame of the burner for fixation, finally the slide was stained following the instructions of Gram's method by using staining kit [26].

B- Germ tube formation

This test was done by taking an isolated colony then suspended in 0.5 ml of human serum for stimulation of the growth of pseudo-filaments after it has been incubated at 37 °C for 2-4 hours [27].

C- Biochemical Identification

VITEK 2 YST System (bioMérieux, France) is completely automated device with a sensitive fluorescence technique, based on enzymes detection. Before testing, a rod applicator was used to sterile saline polystyrene in clear test tube then turbidity was adjusted according to a McFarland standard [28]. Then automatically the VITEK card filled with prepared yeast suspension, then closed, incubated at 35.5 °C for 18 hours. Finally, the results were compared with the database and unknown microorganisms have been recognized and identified [15].

Antifungal activity test of CHs NPs / PEMA using disk diffusion test

Following the directions of **CLSI 2016**, Mueller-Hinton agar prepared. After that with inoculating loop took a pure and unattached colony from freshly cultured of ***C.*** *albicans* and diluted in 0.85% sterile normal saline solution to form candidal suspension then with the using sterile cotton swab submerged in the candidal suspension, the swabbing was done in 3 directions to obtain equal development of ***C.*** *albicans* on Mueller-Hinton agar surface plate [29], then left 5 minutes soft lining discs (with and without CHs NPs) were fitted on the agar surface after that for 2 hours, all the plates were left at room temperature then aerobically incubated at 37 ° C for 24 hours then by utilizing digital caliper ruler, for measuring the inhibition zone that appeared [11].

Antifungal activity test of CHs NPs/PEMA using viable count of *C. albicans*

Following the directions of manufacturer's, Sabouraud dextrose broth and sabouraud dextrose agar were prepared, both of them were autoclaved for 15 minutes at 121° C/15 psi and stored. After that took a pure and unattached colony from freshly cultured of *C. albicans* and diluted it in 0.9% NaCl to form candidal suspension approximately 10^7 CFU/ml (0.5 McFarland standards) and checked the result with McFarland densitometer [11]. Thirty specimens were prepared, the specimen with dimensions (10 x 10 x 2.3 mm) width, length and thickness respectively [19].

All soft lining specimens were autoclaved for 15 minutes before used in test procedure. Each sample placed in the sterilized tubes containing 9.9 ml of previously prepared SDB and 100 µL of the candidal suspension then incubated for one day at 37 °C. After incubation all samples were taken out, removed from the suspension and rinsed five times by submersion it, in autoclaved deionized water to get rid of loosely attached cells [30]. The viable cells were spread in SDA plates then calculated and analyzed statistically.

The data analyzed statistically using software computer program (SPSS) version #26 to maintain the descriptive statistics and the inferential statistics including analysis of variations (ANOVA) test with Bonferroni test to accept or reject the statistical hypothesis. At significant differences at level $p < 0.05$.

Results

The results of disk diffusion test showed the highest mean value of inhibition zones for 3.5% CHs NPs group (36.333) followed by 4.5% CHs NPs group (8.000), while there was no inhibition zone for control and other experimental groups Fig. (1). By using **One-way ANOVA test**, comparison of the means of inhibition zones for all

groups showed there was highly significant difference and listed in Table (1). For further analysis, Bonferroni test was conducted and showed there was non-significant difference among all groups except there were highly significant differences between 3.5% CHs NPs group and other groups Table (2).
The viable count test results showed the highest mean value for both control and experimental groups (0.5 %, 1.5%, 2.5% CHs NPs respectively) while there was no colony forming unit for both 3.5% CHs NPs and 4.5% CHs NPs groups Fig. (2).
In addition, **One-way ANOVA test** was used for comparison the mean values of the viability count of *Candida albicans* for all groups that was showed there was a significant difference and listed within Table (3).

For further analysis, Bonferroni multiple comparisons test was conducted there was a significant difference for all groups except there were no significant differences between 3.5% and 4.5% CHs NPs groups Table (4).

Discussion

The most serious problems associated with the using of denture lining material is colonization and penetration of the soft liner surface by *Candida albicans* and related Candida species [8], and this is reinforced by environmental oral conditions under the denture, as well as the physical structure of the material itself [9]. As a soft denture lining material with antifungal effectiveness is still not developed yet, at the same time it is very easily to be colonized in oral cavity environments by both fungi and dental plaque [20]. This can lead to clinical problems, and material degradation and deterioration [32]. For this reason, many tries have been done to impregnate the antifungal agents within the soft liner material [29].

Chitosan, considered as a natural derivative from chitin, which has superior properties: antimicrobial, biocompatibility, and low toxicity, it composed from β-(1-4)-linked d-glucosamine and N-acetyl-d-glucosamine. Many studies have been proved that chitosan nanoparticles are a broad spectrum of antimicrobial activity against fungi, yeast and bacteria [33,34].

The results of this study revealed there were decreasing in the *Candida albicans* with increase of Chitosan Nano particles concentration so the null hypothesis was rejected. In the present study, an attempt was done to develop the antifungal properties of soft lining materials against *C. albicans*, by incorporating Chitosan Nanoparticles into soft liner. From the study's statistical findings, for viable count test revealed a highly significant decrease in colony forming units/ml of *C. albicans* after incorporating soft lining material with chitosan Nanoparticles and this indicated to formation of a composite with antifungal activity this in accordance to the previous finding that was indicated to the decrease of antifungal activity with the increase of Chitosan Nano powder [15], while the Kirby-Bauer disk diffusion susceptibility test revealed highly significant increase in the inhibition zone measurement as the chitosan concentrations increase with highest result for 3.5% CHs NPs, this result in agreement with [16] .

Multiple hypotheses have been suggested to explain the chitosan antimicrobial activity but the exact ones are unknown. It was suggested that the interactions between the negatively charged cell membrane and positive charged chitosan, leading to changes its permeability, permitting leakage of the intracellular material to the media, thus preventing RNA and DNA reproduction finally direct cell death [35].

Another suggested mechanism is that chitosan has the ability to penetrate the fungal cell membrane and its nuclei, bonding to its DNA, inhibits mRNA reproduction finally inhibits the development of protein and enzyme production in the cells, this will prevent the radial growth of fungus [36]. In addition, chitosan acts as a chelating agent

and limits access of the fungi to nutritional components present in the environment [37].

Conclusions

From the presented research, it was concluded that the impregnation of chitosan Nano particles into soft denture lining material help in producing a soft lining material with antifungal activity, decreasing the candidal colonization and thus decreasing candida associated infection, also 3.5 % CHs NPs experimental group showed a better activity against *Candida Albicans* comparing to control and other experimental groups.

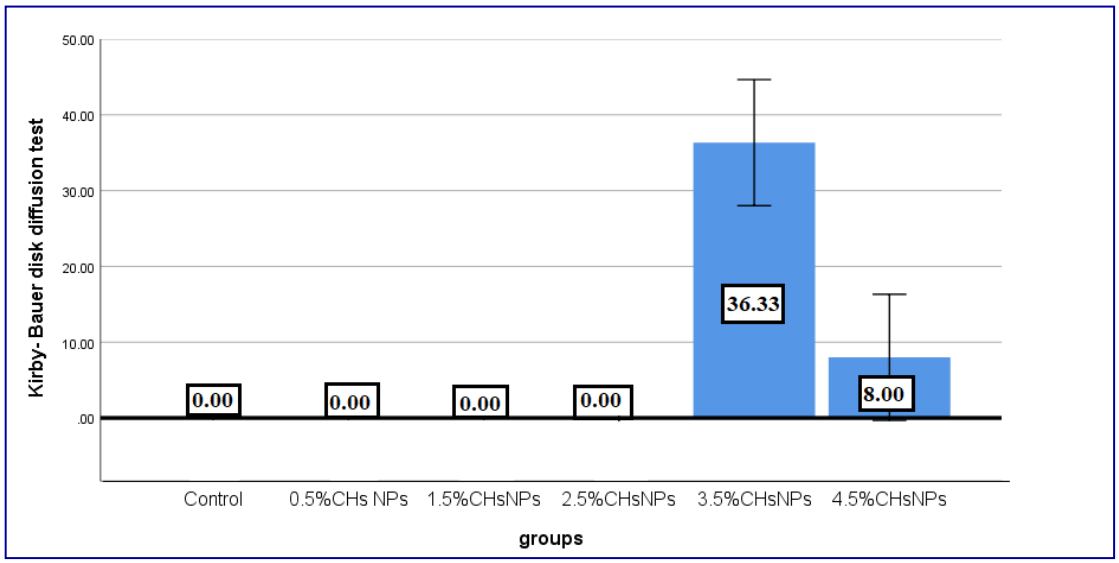

Fig. (1): Bar charts of the mean values and standard deviation of disk diffusion test.

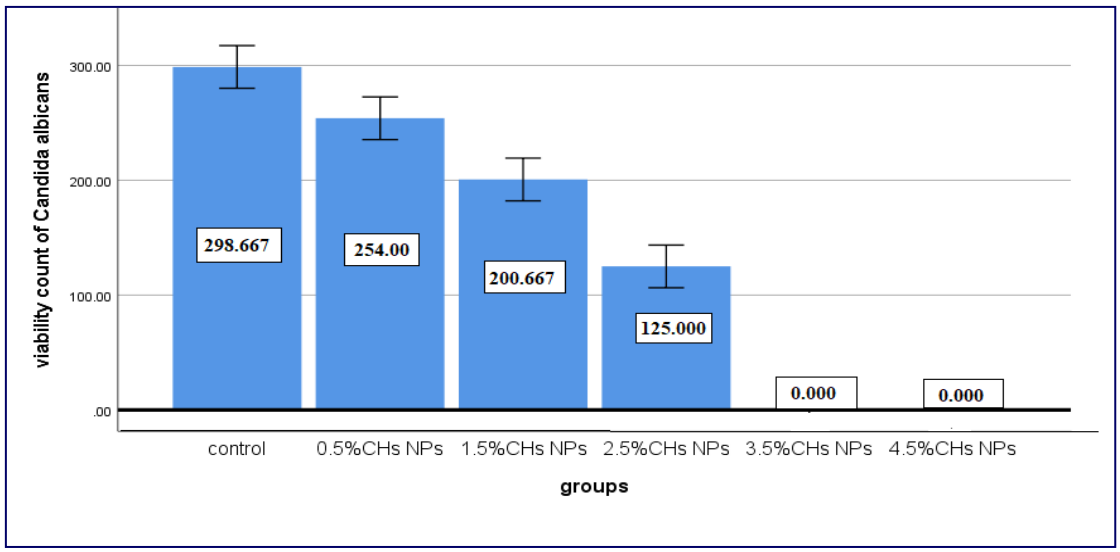

Fig. (2): Bar charts of the mean values and standard deviation showed the distribution of the viability count of *Candida albicans.*

Table (1): Comparison the means of disk diffusion test-using one-way ANOVA for all groups

	Sum of squares	**Df**	**Mean Square**	**F**	**Sig**
Between groups	3169.611	5	633.922	14.499	0.000*
Within groups	524.667	12	43.722		
Total	3694.278	17			

*S: significant at P-Value *P*<0.05

Table (2): Bonferroni multiple comparisons test.

Tested groups		Mean difference	Sig.
Control	0.5% CHs NPs	0.000	1.000**
	1.5% CHs NPs	0.000	1.000**
	2.5% CHs NPs	0.000	1.000**
	3.5% CHs NPs	36.333	0.000*
	4.5% CHs NPs	8.000	1.000**
0.5% CHs NPs	1.5% CHs NPs	0.000	1.000**
	2.5% CHs NPs	0.000	1.000**
	3.5% CHs NPs	36.333	0.000*
	4.5% CHs NPs	8.000	1.000**
1.5% CHs NPs	2.5% CHs NPs	0.000	1.000**
	3.5% CHs NPs	36.333	0.000*
	4.5% CHs NPs	8.000	1.000**
2.5% CHs NPs	3.5% CHs NPs	36.333	0.000*
	4.5% CHs NPs	8.000	1.000**
3.5% CHs NPs	4.5% CHs NPs	28.333	0.003**

*S: significant at P-Value *P*<0.05
*NS: non-significant at P-Value *P* > 0.05.

Table (3): Comparison the means of viable count test-using one-way ANOVA for all groups.

	Sum of squares	**Df**	**Mean Square**	**F**	**Sig**
Between groups	243094.944	5	48618.989	222.569	0.000*
Within groups	2621.333	12	218.444		
Total	245716.278	17			

*S: significant at P-Value *P*<0.05

Table 4: Bonferroni multiple comparisons test

Tested groups		Mean difference	Sig.
Control	0.5% CHs NPs	44.667*	0.045
	1.5% CHs NPs	98.000*	0.000*
	2.5% CHs NPs	173.667*	0.000*
	3.5% CHs NPs	298.667*	0.000*
	4.5% CHs NPs	298.667*	0.000*
0.5% CHs NPs	1.5% CHs NPs	53.333*	0.013*
	2.5% CHs NPs	129.000*	0.000*
	3.5% CHs NPs	254.000*	0.000*
	4.5% CHs NPs	254.000*	0.000*
1.5% CHs NPs	2.5% CHs NPs	75.667*	0.001*
	3.5% CHs NPs	200.667*	0.000*
	4.5% CHs NPs	200.667*	0.000*
2.5% CHs NPs	3.5% CHs NPs	125.000*	0.000*
	4.5% CHs NPs	125.000*	0.000*
3.5% CHs NPs	4.5% CHs NPs	0.000	1.000**

*S: significant at P-Value $P<0.05$
*NS: non-significant at P-Value $P > 0.05$

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