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은-키토산 나노복합체를 투여한 의치 조직조절제의 항균 특성

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Antibacterial activity and characterization of tissue conditioner containing silver-chitosan nanocomplex

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본 연구의 목적은 은-키토산 나노복합체를 함유하는 의치 조직조절제의 항균성 및 특성을 연구함에 있다. 나노복합체의 합성은 질산은 및 키토산을 수용액에서 화학적으로 환원처리 후 자외선 가시광분석으로 확인하였고, 건조된 복합체 분말을 조직조절제 분말에 1.0, 3.0, 5.0 및 7.0%의 중량 백분율로 각각 첨가하여 단량체와 중합하였다(대조군; 0%). 항균성은 시편 상에 접종된 2종의 연쇄상구균 현탁액을 각각 24시간 배양 후 세균집락형성단위 백분율로 평가하였고 세포독성은 시편 추출액을 인간섬유아세포에 24, 72시간 배양한 후 세포생존율로 평가하였으며 특성연구로 미세표면관찰, 은 이온 용출 및 미세인장강도 실험을 시행하였다. 은-키토산 나노복합체가 5.0% 이상 투여된 조직조절제에서 대조군과 비교하여 통계적으로 유의한 세균부착억제가 관찰되었고($P<0.01$) 유의한 세포독성은 관찰되지 않았다. 은 이온 용출은 복합체 투여 용량에 의존적으로 검출되었으나 계류시간 경과에 따라 비례적으로 감소하였으며 복합체 투여에 따른 조절제의 유의한 인장강도 및 표면 물성 변화는 관찰되지 않았다($P>0.01$). 일부 실험적 한계에도 불구하고 본 연구의 5.0% 은-키토산 나노복합체가 함유된 조직조절제는 내재적 세균부착억제와 생체적합성 그리고 안정된 물성을 가진 의치생체재료로의 가능성을 도출하였고 명확한 임상 적용을 위한 생체 실험, 다중 분석을 포함한 향후 연구는 필요하다.

색인단어 : 은, 키토산. 나노복합체, 조직조절제, 항균

Introduction

Tissue conditioner (TC) has widely been used to treat abused mucosal tissues underlying ill-fitting acrylic dentures as temporary expedients. TC is one of short-term used denture soft liners approximately for 2 weeks and formed in situ from the mixture of polyethylmethacrylate (PEMA) and ester-based liquid plasticizer in ethyl alcohol solution without an acrylic monomer. However, loss of plasticizer can lead to gradual hardening with the

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roughened surface with time-elapsed after the onset of curing. Therefore, there can be major complications due to subsequent microbial colonization or inevitable biofilm formation on TC surface (1). Colonization of micro-organisms as streptococci, candida or staphylococci has been reported in patients wearing removal prostheses. Such pathogens are capable of initiating infectious diseases as denture stomatitis, or even pharyngeal and respiratory infections particularly in elderly and medically compromised individuals (2). Chemical or mechanical cleansing could cause physical damages to the liners (3) and a combination of antiseptic drugs into TC was associated with problems such as; the short-term durations, harmful reactions in the elderly patients, drug resistance and costs (4). With the advancement in nanocomposite technology, numerous trials have been conducted by blending silver nanoparticles (AgNP) into PEMA to assign an intrinsic antimicrobial function (5-7). Though AgNP are as considered a promising alternative to antibiotics for multidrug-resistant pathogens, their toxicology remain largely unknown or controversial (8, 9) and metallic nanoparticles such as AgNP are technically difficult to disperse, as nanosized particles tend to aggregate. Recently, AgNP-doped chitosan, a polysaccharide biopolymer, was introduced to exert little cytotoxicity with slow silver release from the matrix (10, 11). Several pieces of research have focused on the synergistic effect between silver ions and chitosan; silver ions improve antibacterial effect and chitosan stabilizes AgNP or prevents their agglomerations (12, 13). The unique catalytic and surface characteristics of chitosan play a pivotal role in enhancing the antibacterial effect of silver (14, 15) and compared to uncoated AgNP, the nanosilver-chitosan coupling is known to be less toxic to gingival fibroblasts (16). The development of intrinsic antimicrobial denture soft liner is mandatory to prevent soft liner-related inflammations. Present study demonstrated the synthesis of silver-chitosan nanocomplex and antibacterial effect of modified PEMA liner incorporated with Ag-chi. In addition, biocompatibility and mechanical validities were characterized for a challenge to clinical use.

Materials and Methods

1. Synthesis of silver-chitosan nanocomplex

Silver-chitosan nanocomplex (Ag-chi) was synthesized by the chemical reduction method and its schematic diagram of the process is as follows (Figure 1). Typically, 8.0 g of chitosan (Low molecular type, 448869, Sigma-Aldrich Co. St. Louis, MO, USA) with 75% deacetylation on molecular weights was mixed with 1.0 g $AgNO_3$ (Junsei Chemical Co., Tokyo, Japan) in 100 mL deionized water at 50℃ under stirring at 2,000 rpm using homomixer (TK homomixer II, Tokushu Kika Co., Hyogo, Japan) overnight. Then, 0.05 g of NaBH4 was added to the chitosan-$AgNO_3$ solution then centrifuged at 2,000 rpm for 12 hours until the solution became cloudy. The supernatants were treated with 200 mL methanol (100%) and transferred to a new tube, followed by drying by vacuum at 65℃ for 2 hours. The colour of dried powder changed from colourless to light yellow and finally to yellowish-brown indicating the formation of nanosilver. Completely desiccated Ag-chi powder was passed through a sieve (60 mesh) and homogenized in a ball mill for 1 hour. The resultant chitosan-coated AgNP was determined by UV-Vis spectrophotometer (UV3600, Shimadzu Corporation, Kyoto, Japan).

2. Preparation of tissue conditioner containing Ag-chi (Ag-chi-TC)

A tissue conditioner, Coe-Soft (GC America, Alsip, IL, USA) supplied in the form of powder and liquid, was selected for the preparation of tested samples. Ag-chi was preliminarily combined with pristine PEMA powder

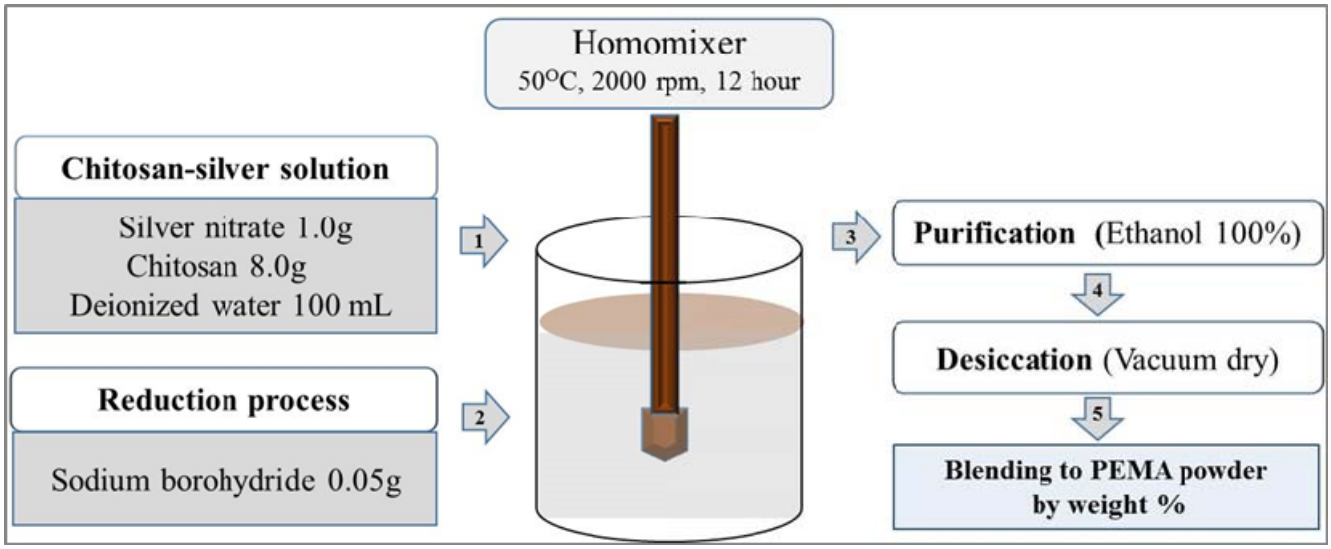

Figure 1. Schematic diagram for Ag-chi synthesis and sample making process.

at 5 different weight ratios; 1.0, 3.0, 5.0, and 7.0% respectively, and unmodified (0%) TC was designated as the control. Secondarily, each blended powder was mixed with monomer liquid at a powder/liquid ratio of 11/8 (g/mL) according to the manufacturer's instruction. The dough-staged mixtures were packed into two custom molds, a disc shape (20.0 mm diameter×3.0 mm depth) and a dumbbell-shape with a central cross-sectional area of 33.0×6.0×3.0 mm by ASTM (American society for testing and materials) D412. Cured samples were trimmed and immersed for 2 hours in sterilized distilled water for leaching of excess residual monomer. The morphological surface of Ag-chi-TC was examined by SEM (Scanning Electron Microscopy; S-4200 FESEM, Hitachi, Tokyo, Japan) under magnification of 100.

3. Antibacterial assay

Streptococcus mutans (*S. mutans*, ATCC 25175) and *streptococcus sobrinus* (*S. sobrinus*, ATCC 27607) were obtained from department of oral microbiology, school of dentistry, Kyungpook National University, Daegu, Korea and maintained in BHI (brain heart infusion) under aerobic conditions. Disc specimens (n=15) were infiltrated by ethylene oxide gas for 8 hours to secure the sample's sterility before the assay. Disc specimens were placed in 12-well cell culture plates (Costa, Corning, Corning, NY, USA) and coated with synthetic saliva (Taliva, Hanlim Pharm. Co., Seoul, Korea), subsequently, bacterial suspensions (100 μL) were inoculated onto each specimen. After overnight incubation with sabouraud broth (1.0 mL), suspensions were withdrawn and colony-forming units (CFU) were determined using the spread plate method at a level of detection within 500 CFU per plate through serial dilutions. Assays were independently performed with three repetitive tests.

4. Cytotoxicity

Cytotoxicity was measured by MTS (2,5-diphenyltetrazolium bromide) assay to human gingival fibroblasts (HGF-1, ATCC 2014, Department of Molecular Medicine of Keimyung Medical College, Daegu, Korea). Disc specimens (n=7) were immersed in phosphate buffered saline (PBS) overnight at 37℃. After removal of sample, the supernatant (10 μL) from centrifuged PBS were filtered then diluted with 90 μL of cell culture medium. Extracts treated with HGF-1 for 24 and 72 hours and cell viabilities

were rated as severely, moderate and non- cytotoxic based on the activity of control negative containing cell culture medium without sample.; 〈 30, 30-60 and 〉 60% (17) respectively.

5. Silver release

Each disc specimen (n=7) was put into 50 mL of sterile distilled water and stored at 37℃ under agitation in polypropylene tubes. The released silver ions were measured after 24 hours, 7 days and 14 days with daily replacement of distilled water using atomic absorption spectrophotometer (Analyst 100, Perkin-Elmer, Waltham, MA, USA) and shaking incubator (SI-600R, JEIO TECH, Seoul, Korea). The quantity of elution was scored as the amounts of silver ion present in the solution per unit of the surface area of the disc (cm^2).

6. Ultimate tensile strength (UTS)

Dumbbell-shaped specimen (n=7) was stored for either 24 hours or 7 days in 200 ml of distilled water, subsequently, specimens were subjected to tension in the universal testing machine (MTS Model 4,200, Instron Inc., Eden Prairie, MN, USA) at a rate of 40 mm/min. A specific claw (20.0 mm 20.0 mm) was made so the central cross-sectional area could stand exposed, while both ends would stay confined by the claw. The values of UTS were calculated in MPa unit.

7. Statistics

The data were analyzed using the statistical software (SPSS v.24.0, SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by Student's t-test were performed with Tukey's test for post-Hoc at a significance level of 0.01.

Results

Stable formation of Ag-chi nanocomplex was observed by a characteristic plasmon band of AgNP indicated at 412 nm wavelength in UV–vis absorption spectra (Figure 2). Compared to control, the modified TC with 5.0% and

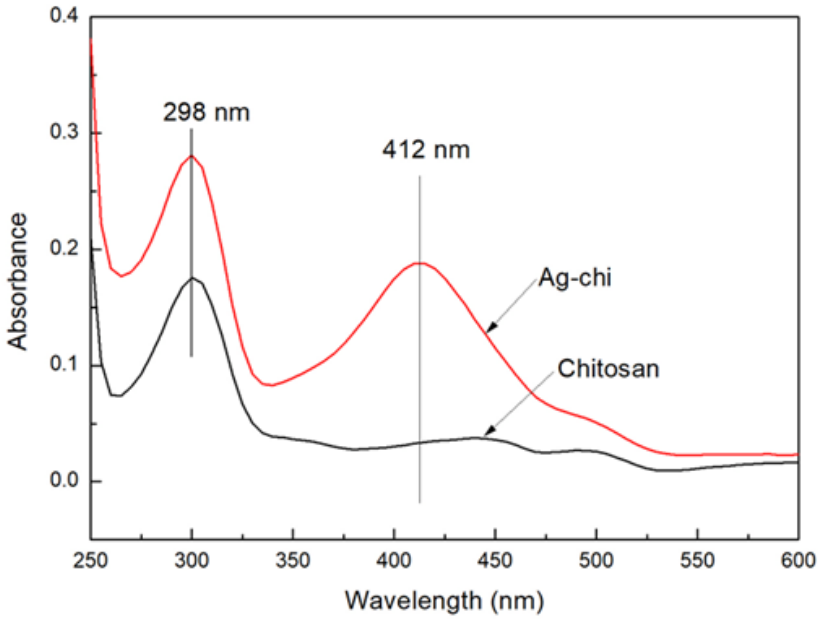

Figure 2. UV-vis spectra of chitosan with and without AgNP. The peak at 298 nm indicates nitrogen transition of noncovalent electron pair in O_2 of chitosan, the peak at 412 nm indicates the surface plasmon band of AgNP.

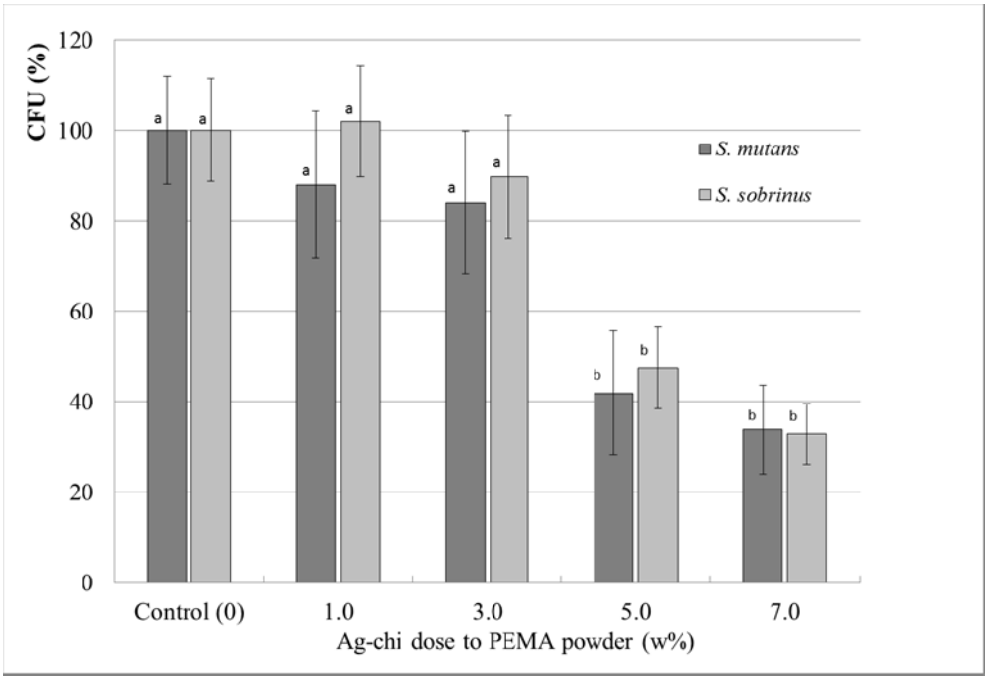

Figure 3. Anti-adherent effect of Ag-chi-TC against two streptococci after 24-hour incubation. As compared to control, 5.0 and 7.0% groups significantly reduced bacterial adhesion ($p<0.01$). Different letter indicates a statistical difference ($p<0.01$).

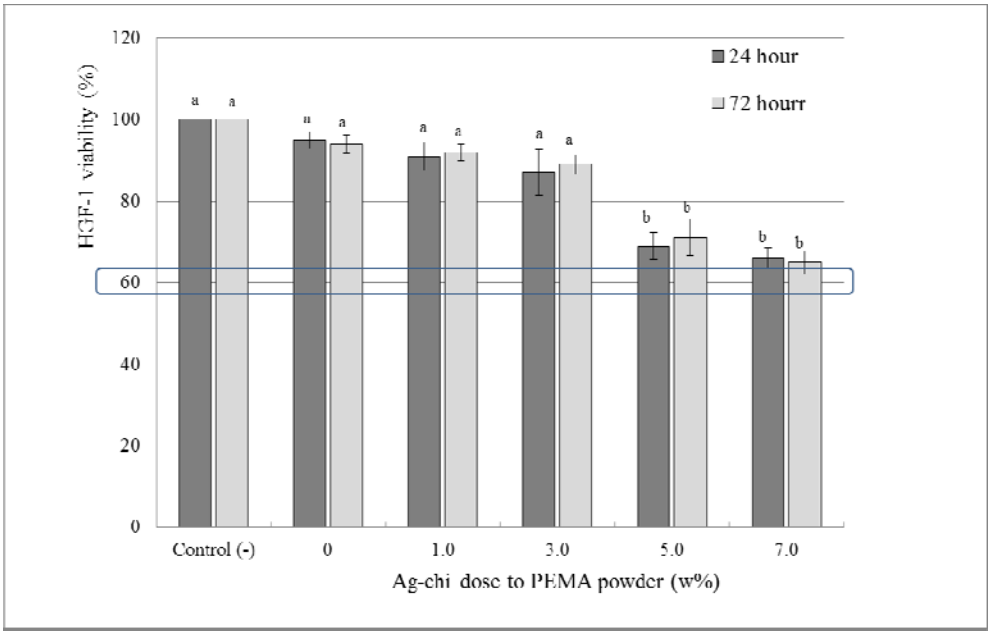

Figure 4. Cell viability (MTS assay) of HGF-1 cells cultured with extracts from of Ag-chi-TC for 24 and 72 hour. HGF-1 viabilities above 60% were rated as non-cytotoxic based on the activity of control negative containing cell culture medium only. Different letter indicates a statistical difference ($p<0.01$).

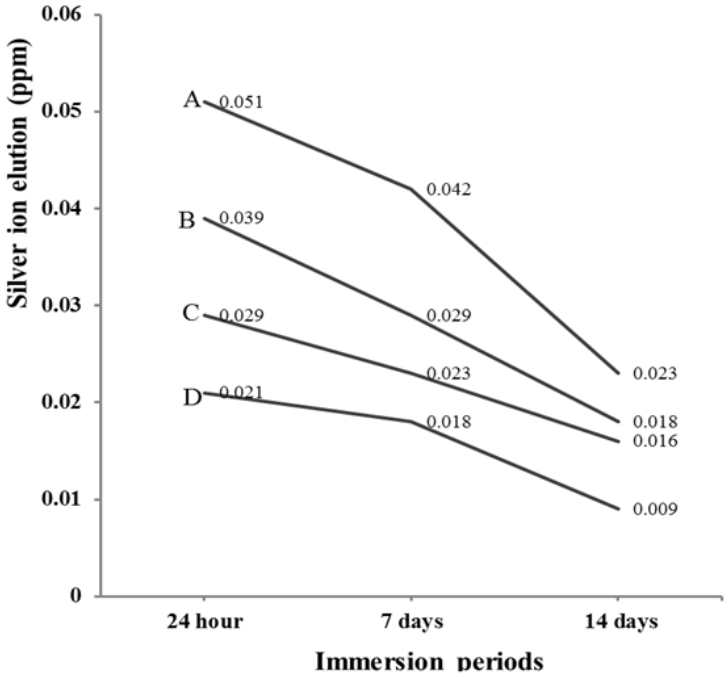

Figure 5. Average concentration of released silver ion from (A) 7.0, (B) 5.0, (C) 3.0 and (D) 1.0% of Ag-chi-TC as a function of immersion time elapsed in water from 24 hours to 14 days.

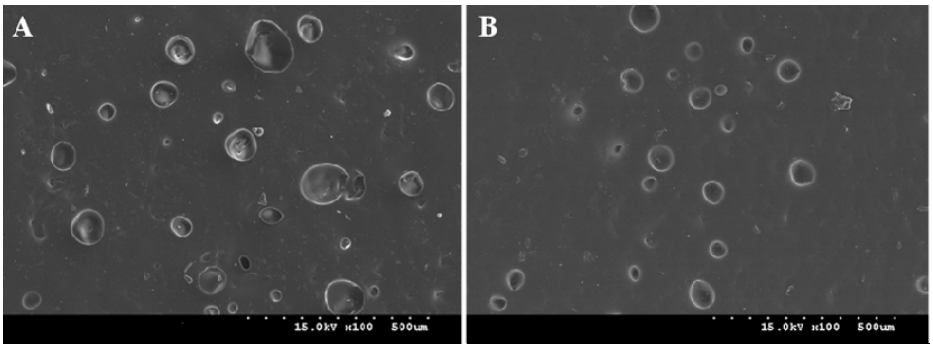

Figure 6. SEM topography (×100) on 7.0% Ag-chi-TC (A) and pristine TC (B). Similar surface texture is seen in gross, some enlarged blisters may be related to Ag-chi additives.

Table 1. Mean UTS values (MPa±SD) of Ag-chi-TC on two storage periods.

	Control (0%)	1.0%	3.0%	5.0%	7.0%
24 hour	5.24 ± 0.34[a]	5.17 ± 0.29[a]	5.04 ± 0.15[a]	4.98 ± 0.43[a]	4.92 ± 0.23[a]
7 days	3.27 ± 0.17[b]	3.31 ± 0.27[b]	3.19 ± 0.18[b]	3.21 ± 0.16[b]	3.18 ± 0.13[b]

Different letter indicates a statistical difference ($p<0.01$), SD: standard deviation

7.0% Ag-chi significantly reduced bacterial adhesion by 59.1 (±13.7)% and 66.1 (±9.8)% in *S. mutans*, and by 52.6 (±9.1)% and 67.2 (±6.7)% in *S. sobrinus* respectively after 24 hour incubation ($p<0.01$) (Figure 3). There was no statistical difference in antibacterial effect between 5.0% and 7.0% groups and no significant bacterial susceptibility between the two streptococci was observed ($p>0.01$). MTS result showed none of Ag-chi-TC induced cytotoxicity to HGF-1 (>60% of cell viability), and 5.0% and 7.0% groups exhibited the statistically lower cell viability from negative control in the two incubation periods (Figure 4). The extent of leaching of silver ion ranged 0.009 to 0.051 ppm with a dose-dependent of Ag-chi amounts and gradual decrease of silver ion were observed in all of groups as a function of immersion times from 24 hours to 14 days in water (Figure 5). SEM topography of Ag-chi-TC exhibited similar surface texture with some enlarged blisters or increased porosities as compared to control (Figure 6). Ag-chi did not affect the UTS of pristine TC regardless of its blending doses and storage intervals (Table 1), ($p>0.01$).

Discussion

The stable molecular structure of chitosan is presented in Fgure 7, and the lone-pair electrons in chitosan polysaccharide are known to be easily bound to a metal surface by nitrogen and oxygen atom. The aqueous chitosan in this study was protonated with positive charges and chelated onto the surfaces of AgNP due to presence of active amino ($-NH_2$) and hydroxyl (-OH) functional groups. The couplings may lead to the stabilization of the nanoparticles to be stabilized or the protection of nanoparticles from their aggregations (18). UV-Vis spectrum of synthesized Ag-chi is presented in Figure 2, the peak at 298 nm is nitrogen transition of noncovalent electron pair in O_2 of chitosan and the peak at 412 nm

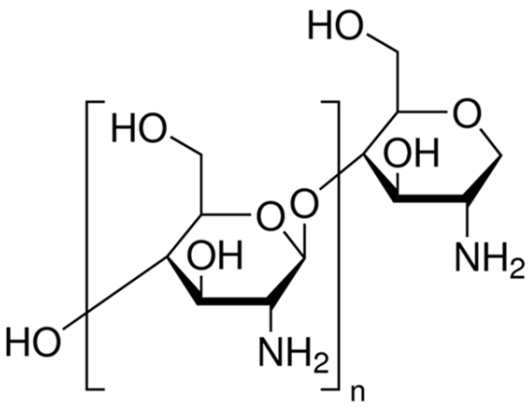

Figure 7. The most stable molecular structure of chitosan calculated by HyperChem PM3 method (HyperChem Professional 8.0, Hypercube Inc., Gainsville, FL, USA).

is a typical plasmon band of spherical AgNP, thereby implying a stable conversion of $AgNO_3$ to AgNP synthesized by in situ reduction with chitosan. Dental composites or PMMA containing nanosilver could act a role of the reservoir for silver ions and demonstrate a strong bactericidal activity with active elution of oxidized silver ion into the medium (19, 20). Within 24-hours of incubation, Ag-chi-TC did not exhibit any biocidal action rather provided a significant anti-adherent effect at above 5.0% Ag-chi compared to control (Figure 3). Ag-chi-TC showed evident silver ion release in water as a function of immersion in a dose-dependent manner to the concentration of additives with the maximal 0.051 ppm in 7.0%. Eluted values were proportionally decreased overtime and restricted ion elution were related to plasticizers in Coe soft, usually leached out in the saliva over a period of time and a hydrophobic character of PEMA. Reductions in softness of this material may entrap silver ion due to its insufficient water uptake for ion release from PEMA bulk. Unlike the elution test for Ag-chi-TC immersed in 50 cc of water, practical leaching within the oral cavity may be restricted due to lower saliva flow (21, 22). In the present antimicrobial assay, a small volume of broth (1 mL) and bacterial suspension (100 μL) were

inoculated on specimens with synthetic saliva-coated before incubation to mimic a clinical TC use. Denture stomatitis is commonly related to the Sjögren's syndrome where the salivary flow is absent or minimal and as most of *in vivo* dentures are closely engaged to fit the gingival mucosa. Generally, microbes in suspension (planktonic phase) are sensitive to lower antiseptic doses than colonized by a biofilm (23), thus, it is speculated that the anti-adherent effect was probably due to the direct contact (24) between streptococci and the surface of Ag-chi-TC. In present study, chitosan was utilized to control AgNP agglomeration and even distribution of agents on the specific surface area is critical to guarantee a stable role of contact inhibitor. Biologic and toxicological aspects of denture materials are important in relation to their clinical usage along with antimicrobial effect. A polysaccharide biopolymer chitosan was doped to silver to exert little cytotoxicity with slow silver release from the matrix (10, 11); however, minute silver leaching may cause adverse reactions such as allergies and local chemical irritation on oral mucosa. In MTS assay, control negative (culture medium only) group was designated to exclude the influence of plasticizers in the structure of TC. The significantly lower cell viabilities at 5.0 and 7.0% groups from control may be related to influence of silver, however, there was little influence to HGF-1 proliferation in maximal 0.051 ppm silver ion eluted. Other studies similarly reported that addition of 1.0% of AgNP to MTA (mineral trioxide aggregate) did not alter its biocompatibility in an animal study (25) and fibrin sponge dispersed by 47 ppm, 23 ppm of AgNP was non-toxic response to tissue, especially under low concentration (26).

Ag-chi particles, an extrinsic element, could act as heterogeneous nuclei or impurities and this might influence or jeopardize the PEMA matrix, thereby compromising the functionality of pristine TC. UTS provides information on the ultimate strength of rubber in tension, which is one of the fundamental properties of the elastomer. In all the tested samples, UTS were significantly decreased at 7-day storage due to the loss of plasticizer. As compared to control, UTS values were not influenced by Ag-chi incorporation at two storage periods (Table 1), though some enlarged blisters or porosities are detected in Ag-chi 7.0% (Figure 6). Present results corroborate with other studies for the incorporation of some functional agents into PEMA; the addition of nystatin into Dura conditioner® at concentrations below 1,000,000 units did not affect the tensile strength (27) and 10% silver-zeolite incorporation did not alter the dynamic viscoelastic properties of Shofu tissue conditioner® (28).

Within the limitations of this study, it is proposed that modified TC loaded Ag-chi can be a candidate as a denture biomaterial with mechanical validity. Nevertheless, evident conclusions on the antimicrobial mechanism of modified TC cannot be achieved because there is still no consensus in the literature for bacterial inhibition in silver-containing compounds. In addition, to guarantee the stable role of contact inhibitor, the even distribution of agents on the specific surface area should be emphasized. Herein, aqueous chitosan was utilized to control AgNP agglomeration and blending of Ag-chi to TC was performed by powder to powder protocol. A futuristic trial on the combination of Ag-chi into TC monomer is considered to an alternative protocol for improved its dispersion as well as homogenized distribution. Further studies including *in vivo*, multi-brand, multi-strain, and multifactor assays are also necessitated for clinical applications.

Conclusion

Damaged tissue conditioners can induce microorganism development that can threaten the health of the dentures wearer. Tissue conditioner with 5.0% silver-chitosan

nanocomplex is proposed as an appropriate carrier for the prevention of stomatitis and this composition did not influence the consistency of pristine PEMA. Based on the outcomes, modified tissue conditioner loading nanosilver-chitosan is suggested as a new denture biomaterial.

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Original Article

Antibacterial activity and characterization of tissue conditioner containing silver-chitosan nanocomplex

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The objective of this work was to verify the antimicrobial effect and the characteristics of the tissue conditioner containing silver-chitosan nanocomplex at *in vitro* level. Nanocomplex was synthesized by the chemical reduction of silver nitrate and chitosan in aqueous solution with sodium borohydride as a reducing agent. Silver-chitosan nanocomplex was incorporated to tissue conditioner as powder to powder ratio with different weight percentage (%) of 0 (control), 1.0, 3.0, 5.0 and 7.0. The antimicrobial effect was assessed by colony forming unit in retrieved two streptococcal suspensions inoculated on specimens and cytotoxicity was measured using human gingival fibroblasts by MTS assay. Surface topography, Ag ion release and ultimate tensile strength tests were conducted for mechanical validity. The significantly reduced bacterial adhesions were observed above 5.0% loading while all of tested samples did not show cytotoxicity when compared to the control. Ag ion releases were detected with the dose-dependent of initial silver loadings with gradual decreasing over time. Modified tissue conditioner revealed similar microscopic surface textures and expressed no significant tensile strength changes ($P > 0.01$) as compared to unmodified. Within some limitations in present study, the tissue conditioner loaded by 5.0% of silver-chitosan nanocomplex can be candidate as a novel denture biomaterial without mechanical hazards. For a clinical specification, future studies including *in vivo*, multi-strain or factor assays, and additional physical tests were still required.

Key Words : Antibacterial effects, Silver-chitosan nanocomplex, Tissue conditioner