



Original Article

Antifungal effect of tissue conditioners containing poly(acryloyloxyethyltrimethyl ammonium chloride)-grafted chitosan on *Candida albicans* growth *in vitro*



Hsin-Lin Lee ^a, Ren-Syue Wang ^b, Yung-Chuang Hsu ^a,
Chuan-Chung Chuang ^a, Hui-Rong Chan ^a, Hsien-Chung Chiu ^a,
Yi-Bing Wang ^a, Kuo-Yu Chen ^b, Earl Fu ^{a,c*}

^a Department of Dentistry, Tri-Service General Hospital and National Defense Medical Center, Taipei, Taiwan

^b Department of Chemical and Materials Engineering, National Yunlin University of Science and Technology, Yunlin, Taiwan

^c Department of Dentistry, Taipei Tzu Chi General Hospital, Xindian, Taipei, Taiwan

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KEYWORDS

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Abstract *Background/purpose:* Denture stomatitis is a pathological condition affecting the mucosa underneath ill-fitting dentures, and *Candida albicans* is considered its main etiologic factor. Tissue conditioners are temporary lining materials often applied to dentures to treat inflamed tissues. However, tissue conditioners do not exert antifungal activity, and the soft surface texture harbors *C. albicans* easily. The aim of this study was to examine the antifungal activity of tissue conditioners modified with chitosan (CS) or a quaternized chitosan (QCS), which was synthesized by grafting 2-[(acryloyloxy)ethyl] trimethyl ammonium chloride onto CS.

Materials and methods: Tissue conditioners containing varying weight percentages of CS or QCS were prepared as experimental discs 10 mm in diameter and 1 mm in thickness. Samples were co-cultured with *C. albicans* and the number of colony forming units was recorded. Other evaluations included cell toxicity and tensile bond strength to the resin denture base.

Results: It was found significantly fewer fungal colonies in tissue conditioners modified with CS or QCS, and none when the weight percentage of QCS exceeded 5%. CS and QCS did not affect the viability of human gingival epithelium cells or fibroblasts, and tensile bond strength did not differ between control and modified tissue conditioners.

* Corresponding author. School of Dentistry, Tri-Service General Hospital and National Defense Medical Center, Taipei 11490, Taiwan, ROC. Fax: +886 2 87927147.

E-mail address: dentalab@tpts5.seed.net.tw (E. Fu).

Conclusion: This study provides a foundation for the development of QCS as a novel and safe antifungal agent applied to tissue conditioners in clinical practice.

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Introduction

Denture stomatitis (DS) is the manifestation of inflammation underneath denture-bearing mucosa, often observed in patients who wear ill-fitting dentures for prolonged durations.¹ In a rapidly aging society, an increasing number of elders are wearing removable dentures,² and epidemiological studies have reported that DS can affect up to 72% of denture wearers.³ The etiology of DS is multifactorial, encompassing systemic factors such as diabetes,⁴ malnutrition,⁵ and immune suppression,⁶ as well as local factors such as denture-related trauma,⁷ poor denture hygiene, and continuous denture wearing.⁸ However, *Candida albicans* infection is considered the main etiologic factor. A randomized clinical trial in Brazil reported that *C. albicans* was found in 93% of patients with DS⁹; thus DS is also known as *Candida*-associated denture stomatitis.¹⁰

A wide range of complex treatments are available for DS, and tissue conditioner (TC) application and antifungal agent delivery are commonly used. TCs are temporary soft lining materials routinely used to condition mucosal inflammation and decrease the force of mastication by partially absorbing impact.^{11,12} However, the soft-surface texture of TCs, coupled with their lack of antifungal properties, allow *C. albicans* to accumulate easily, further aggravating DS. Topically applied antifungal agents may be washed away by saliva or diet,¹³ rendering them ineffective, whereas the effective dose for systemic administration may cause side effects.¹⁴

To overcome these limitations, antifungal agents have been incorporated into TCs. Douglas and Walker reported incorporating the antifungal agent nystatin into TCs in 1973¹⁵; similar studies have been published since, classifying antifungal agents based on natural and synthetic origins.¹⁶ Natural agents, such as oils may possess effective antifungal properties¹⁷; however, few studies have investigated the incorporation of natural agents into TCs and the subsequent alterations in their mechanical properties. Synthetic agents containing drugs such as antibiotics may induce side effects such as microbial resistance and drug allergies.¹⁴

Chitosan (CS) is a biodegradable, non-toxic polysaccharide derived from chitin and found in abundance in natural sources.¹⁸ Due to its antibacterial and antifungal properties, CS has been applied in various industrial and medical settings.¹⁹ However, CS exhibits poor solubility in environments where the pH exceeds 6.5.²⁰ Quaternization is a common modification which converts CS into a quaternary ammonium salt, improving its water solubility by increasing its positive charge, a modification which may also enhance its antifungal properties.²¹ In this study, quaternized CS (QCS), a polycationic compound capable of

carrying a higher positive charge than CS, was synthesized by grafting 2-[(acryloyloxy)ethyl] trimethyl ammonium chloride (AETMAC) onto CS. This quaternary ammonium group was selected for CS modification as it exhibited promising characteristics in our previous study.²² Results displayed significantly reduced numbers of microorganisms and no cytotoxicity, indicating AETMAC's potential in CS quaternization. In the present study, we evaluated the antifungal properties, cytotoxicity, and tensile bond strength of TCs after incorporation with CS and QCS.

Materials and methods

Chitosan quaternary ammonium salt synthesis

QCS was obtained by grafting AETMAC monomers (Sigma-Aldrich, St. Louis, MO, USA) onto CS (MW: 50–190 kDa, 75–85% deacetylated, Sigma-Aldrich) using a grafting copolymerization method. The chemical structure and Fourier transform infrared spectrophotometry (FTIR) spectra are shown in Figs. 1 and 2. Briefly, a 1 wt% CS solution was prepared by dissolving CS powder in 2% aqueous acetic acid (Showa, Tokyo, Japan) at 60 °C in a four-neck flask equipped with a mechanical stirrer, nitrogen inlet tube, dropping funnel, and condenser. As the CS solution was heated to 80 °C, 0.012 M AETMAC monomers and 0.03 M ammonium sulfate initiator (Showa) were added successively dropwise to create the graft copolymerization. After 3 h of reaction at 80 °C, the polymer solution was precipitated in acetone. The precipitated product was then washed thoroughly with methanol to remove unreacted monomers and homopolymers. Finally, the purified products (QCS) were dried under vacuum overnight at 60 °C. The dried QCS was stored in a desiccator until needed. The grafting percentage is 21% (G%) and grafting efficiency is 81% (GE%) were estimated based on the difference in weights before and after the grafting reactions and were calculated as follows:

$$G(\%) = \frac{W_1 - W_0}{W_0} \times 100\%$$

$$GE(\%) = \frac{W_1 - W_0}{W_2} \times 100\%$$

where, W_0 and W_2 are the initial weight of CS and AETMAC, respectively, and W_1 is the weight of dried product.

Preparation of TCs

Three types of TCs were evaluated in this study: A commercially available TC (GC Soft Liner, GC Corp., Tokyo, Japan), GC Soft Liner with CS, and GC Soft Liner with QCS.

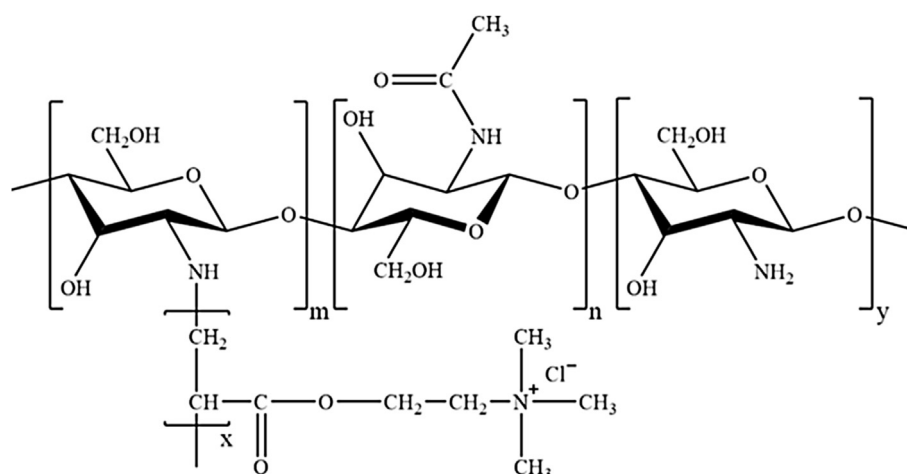


Figure 1 Chemical structure of 2-[(acryloyloxy)ethyl] trimethyl ammonium chloride (AETMAC)-grafted chitosan.

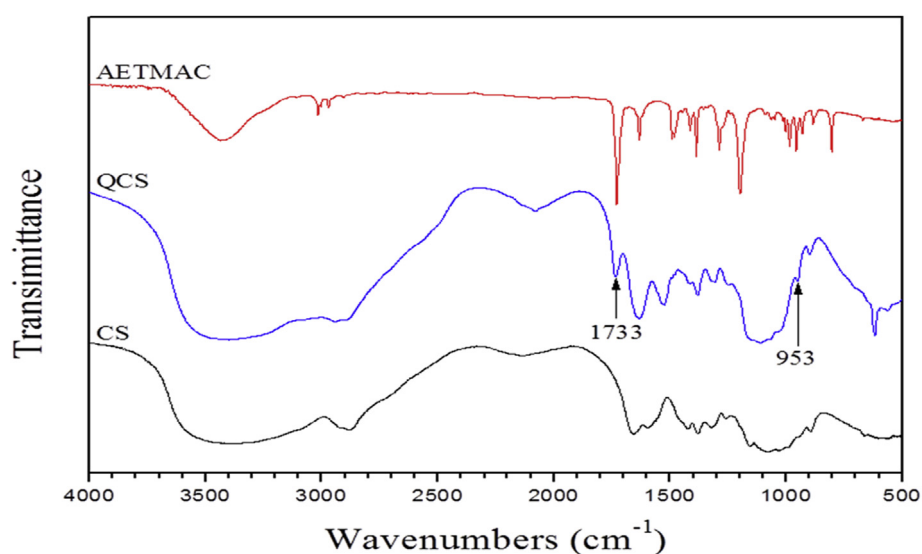


Figure 2 Fourier transform infrared spectrophotometry (FTIR) spectrum for AETMAC, chitosan (CS) and quaternized chitosan (QCS). The FTIR spectrum of CS and QCS were largely similar, however, QCS exhibits two distinct absorption peak at 1733 cm^{-1} and 953 cm^{-1} , these correspond with specific functional groups in AETMAC: the C=O stretching vibrations of the ester groups and the C-N stretching vibration of quaternary ammonium groups. These two absorption peaks demonstrate the successful grafting of AETMAC onto chitosan.

Briefly, various quantities of CS or QCS powders (5.0, 7.5, and 10.0 wt%) were blended with the poly(ethyl methacrylate) (PEMA) soft liner powder (Table 1). Next, the soft liner solution was mixed uniformly with the blended powders at the manufacturer's recommended ratio (1.8:2.2) for 30 s. The mixed solution was poured into a glass mold and heated to $40\text{ }^{\circ}\text{C}$. Finally, the film was removed and cut into a disc shape (10 mm in diameter and 1 mm in thickness).

Preparation of denture base resin

Lucitone 199 (Dentsply Sirona, York, PA, USA), a heat-polymerized poly(methyl methacrylate) (PMMA) resin, was selected for denture base material in this study. Rectangular denture base resin blocks with a length of 40 mm and cross-sectional area of $10 \times 10\text{ mm}^2$ were fabricated

Table 1 Weight percent (wt%) of poly(ethyl methacrylate) (PEMA), chitosan (CS), and quaternized chitosan (QCS) powders in tissue conditioners (TCs).

Material	PEMA (wt%)	CS (wt%)	QCS (wt%)
TC (control)	100	—	—
TC-CS			
CS 5	95	5	—
CS 7.5	92.5	7.5	—
CS 10	90	10	—
TC-QCS			
QCS 5	95	—	5
QCS 7.5	92.5	—	7.5
QCS 10	90	—	10

according to the manufacturer's instructions. Briefly, polymer powders was mixed with monomer at the manufacturer's recommended ratio (2.1:1.0) for 1 min. After pouring the viscous solution into a Teflon mold, the mold was immersed in warm water at 73 °C for 90 min, followed by heating to 100 °C for 30 min to create polymerization. After polymerization, the PMMA samples were removed from the mold, and the surfaces to be bonded with TCs were smoothed with 240-grit silicon carbide paper (Prawn Brand®, Seoul, Korea), cleaned, and dried. The dried PMMA blocks were used for tensile bond strength testing.

Evaluation of antifungal activity

Sabouraud dextrose broth (SDB broth; HiMedia, Mumbai, India) and nutrient agar (High Standard Enterprise Co., Ltd., Taichung, Taiwan) were mixed with deionized water and sterilized in an autoclave at 121 °C for 20 min. The sterilized agar medium was transferred into Petri dishes and allowed to solidify in a laminar air flow chamber. *C. albicans* [American Type Culture Collection (ATCC) 24433] obtained from the Bioresource Collection and Research Center in Hsinchu, Taiwan, was used as the test organism to evaluate the antifungal activity of TCs. Briefly, *C. albicans* was suspended in SDB broth. One loopful of microorganism suspension was plated on agar to obtain single colonies and incubated at 37 °C for 24 h. Single colonies were then transferred to 50 mL of SDB broth and cultured in a shaking incubator (100 rpm) at 37 °C. After culturing for 24 h, the optical density of the fungal suspension was measured at 600 nm in a Metertech SP-830+ spectrophotometer (Metertech Inc., Taipei, Taiwan), and the value was adjusted to 0.1 by adding SDB broth for use in experiments. The cell concentration of the suspension was 2×10^6 colony-forming unit (CFU)/mL, which was determined by plating serial dilutions on agar plates.

The *in vitro* antifungal activity of TCs with various quantities of CS and QCS against *C. albicans* was assayed according to the procedure described previously by Lee et al.²³ Sterilized samples with a diameter of 10 mm and thickness of 1 mm were placed into 24-well tissue culture plates and immersed in 500 µL of SDB broth at room temperature for 5 min. After immersion, 500 µL of fungal suspension were added to each well. The mixtures were cultured at 37 °C with shaking at 100 rpm. After 1, 2, and 7 days of incubation, 100 µL of fungal culture were withdrawn, and serial dilutions were carried out to determine the number of CFUs in the cell suspension. The agar plates were incubated at 37 °C for 24 h, and the subsequent CFUs were counted visually. Each experiment was repeated for three replicates.

Evaluation of cytotoxicity in human gingival epithelium and fibroblast cells

Human gingival fibroblasts (hGF) and gingival epithelium cells (hGE), obtained from the junctional epithelium of teeth that required extraction from patients for orthodontic treatment. The Ethics Committee at Tri-Service General Hospital reviewed and approved the study protocol (the TSGHIRB approval number is 1-106-05-017) and all

patients provided written, informed consent to the extraction of their teeth and the use of their periodontal tissues for research. In brief, the primary culture of cells of hGF and hGE was obtained from surgical procedures of non-inflamed periodontal soft tissues. Specimens were immersed in a medium containing 2 mg/mL protease and 10% fetal bovine serum (FBS) for 2 days at 4 °C. After being separated from the epithelium, Cells of hGF and hGE were seeded in 96-well plates at a density 1×10^3 cells/cm² in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Seven treated discs (3 mm in diameter and 1 mm in thickness) were added to each well. Cultures were further incubated at 37 °C, after which cells were assayed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) at time intervals of 1–3 days. Cell proliferation was measured by optical density at 490 nm using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Cell viability was calculated using the following formula:

$$\text{Cell viability} = \frac{\text{material MTS value}}{\text{corresponding control MTS value}} \times 100\%$$

Measurement of tensile bond strength to acrylic resin denture base

A tensile bond test method was used to determine the bond strength between denture base resin and TCs. Briefly, two PMMA blocks with a 3-mm-thick spacer between them were placed into a Teflon mold. TC solution was then poured into the space. After 30 min, the sample was removed from the mold and trimmed with a sharp blade. Each specimen was immersed in deionized water at 37 °C for 1 or 7 days before testing. The tensile bond test was conducted immediately using a universal testing machine (HT-2402, Hung Ta Instrument Co., Ltd., Taichung, Taiwan) at a crosshead speed of 20 mm/min until failure. The strength of the tensile bond was calculated as the maximum load at failure divided by the cross-sectional area of the interface and expressed in MPa.

Statistical analysis

All quantitative data are expressed as mean \pm standard derivation. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by *post hoc* Fisher's LSD multiple comparison test at a significance level of $p < 0.05$.

Results

Antifungal activity

Compared with the TC control group, significantly fewer colonies were observed in the CS or QCS groups (Fig. 3). In the 7.5 wt% or 10.0 wt% groups, significantly fewer colonies were observed for the QCS modification than for the CS modification. In contrast, a higher number of colonies in QCS groups was noted in the 5 wt% treatment.

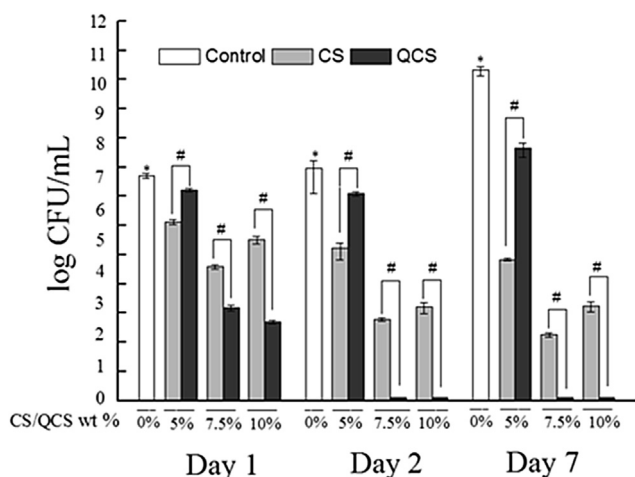


Figure 3 Comparison of *Candida albicans* growth in culture media with tissue conditioners, tissue conditioners with chitosan (CS), and tissue conditioners with quaternized chitosan (QCS). Data are presented as mean \pm SD; *: $p < 0.05$ vs. control; #: $p < 0.05$ for CS vs. QCS.

Cytotoxicity to hGF and hGE

hGF and hGE cell viability did not differ between controls and the CS- or QCS-treated groups at 24 h, 48 h, or 72 h, despite increases in concentration of 5.0, 7.5 and 10.0 wt % (Fig. 4).

Tensile bond strength to acrylic resin denture base

Calculations of the strength of the tensile bond between the control, CS, and QCS groups and the heat-polymerized acrylic resin denture base material are displayed in Fig. 5. Strength increased over time (day 7 compared with day 1) in all groups; however, tensile bond strength did not differ between the groups at either time point.

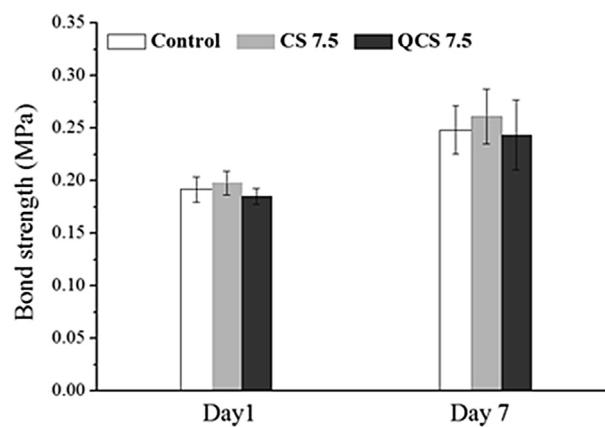


Figure 5 Comparison of tensile bond strength to the acrylic resin denture base between control tissue conditioners and chitosan (CS)- or quaternized chitosan (QCS)-modified tissue conditioners. Data are presented as mean and standard deviation.

Discussion

This study investigated the antifungal properties, cytotoxicity, and tensile bond strength of TCs modified with CS or QCS. It was found that TCs with CS were effective against *C. albicans*, while TCs modified with QCS demonstrated notable antifungal effects, no toxicity to hGE or hGF cells, and no alternation in tensile bond strength. To our knowledge, this is the first study to examine the antifungal effects and properties of TCs modified with CS or QCS.

TCs are temporary soft lining materials applied to the underside of a resin denture base, used to alleviate DS complications in elderly patients, many of whom have limited mobility, cognitive impairment, and a declining memory.²⁴ TCs can reduce the number of visits to the dentist and require minimal work on the part of the patient. However, patients with DS often exhibit poor denture

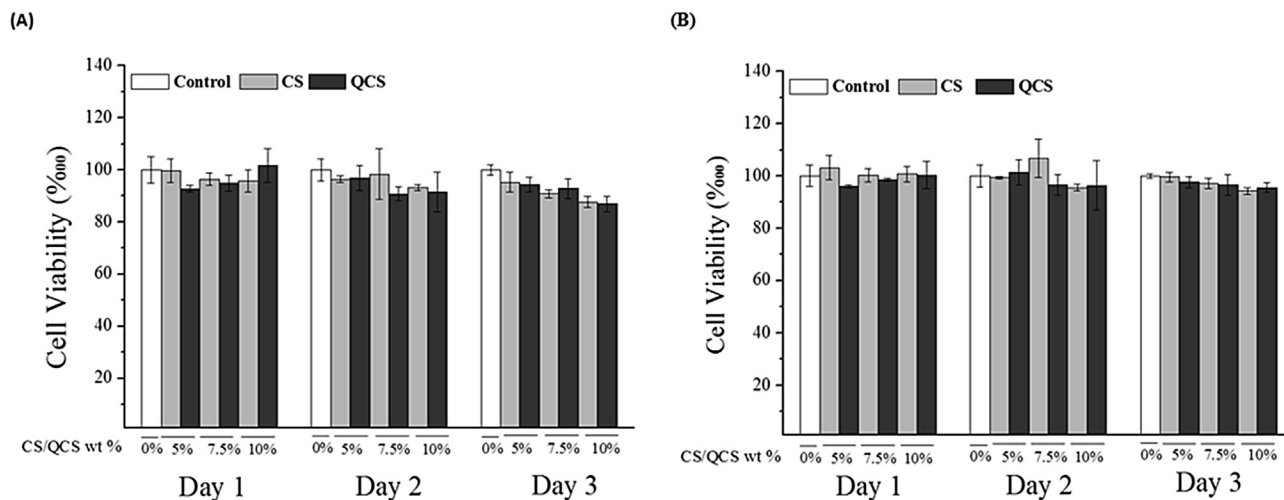


Figure 4 Cytotoxicity of chitosan (CS)/quaternized chitosan (QCS)-modified tissue conditioner discs to hGF and hGE cells. Cell viability after incubation with CS and QCS was calculated. (A) hGF cell viability. (B) hGE cell viability. Data are presented as mean and standard deviation.

hygiene and wear the dentures for prolonged periods.²⁵ Consequently, low pH and relatively anaerobic conditions often develop between the denture base and the gingival mucosa. This acidic oral environment allows *C. albicans* to thrive, and also promotes proteinase and lipase enzymatic activities.¹³ Studies have shown that the soft surface texture of TCs can harbor *C. albicans* and aggravate DS.^{26,27} The incorporation of antifungal agents into TCs provides a barrier, in the form of an effective antimicrobial agent, between the infection site and healthy tissues.²⁸

CS, an N-deacetylated derivative of chitin and natural polysaccharide, is effective against a broad spectrum of microorganisms; it is biodegradable, biocompatible, and displays no cellular toxicity.²⁹ However, because of its dense crystal structure, CS is insoluble in most solvents,³⁰ and thus modification through quaternization is often required to increase its usability.^{31,32} Quaternization of CS increases its positive charge, correlating with an enhanced antifungal ability compared with that of unmodified CS. The antifungal effect of CS and QCS may be derived from the interaction of CS cationic chains and the negatively charged macromolecule residues on the fungal cell membrane, causing apoptosis by leakage of intracellular electrolytes and other constituents.^{33,34}

Antifungal properties in TCs modified with CS or QCS was present from day 1, with no loss in efficacy on days 2 or 7. In TCs with QCS exceeding 5%, exceptional antifungal efficacy was observed on day 1, and no bacterial colonies were detected on days 2 and 7 (Fig. 3), indicating that AETMAC grafting enhances the antifungal properties of CS. Our data are consistent with a previous study that found that QCS was more antifungal than CS.²¹

Some studies have reported CS to be nontoxic,³⁵ but none have investigated the properties of CS grafted with AETMAC. We selected hGF and hGE cells for testing as *C. albicans* infection is often initiated through hyphae invasion of a host epithelium, leading to interaction between the cellular ligand of *C. albicans* and the cellular receptor of the host epithelium. No cytotoxicity was observed in hGE and hGF cells following exposure to TCs modified with CS or QCS (Fig. 4).

Adequate tensile bond strength between TCs and the resin denture base is necessary, as interfacial separation could allow *C. albicans* to adhere and proliferate. No significant difference in tensile bond strength was found between control and modified TCs. However, tensile bond strength increased in all groups on day 7 compared with the bond strength on day 1, possibly because of polymer hardening and water storage (Fig. 5).³⁶

Certain limitations should be considered in our current study. First, the assays were tested with samples immersed in a large volume of microbial suspension, which does not mimic TC use in the oral cavity.³⁷ Second, microorganisms in suspension are often sensitive to lower antiseptic concentrations than intraoral biofilm microbe colonies.³⁸

Within the limitation of this study, this is the first study to demonstrate that CS and its quaternized modification, when incorporated into TCs in small doses, are safe and effective in inhibiting *C. albicans* in this *in vitro* model. CS and QCS reduced *C. albicans* growth, which may be mediated, at least in part, by DS. The findings support a continued investigation in clinical trials using CS or QCS as

potential therapeutic agents. Our findings may be leveraged for clinical evaluation and treatment of DS.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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