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# In vitro evaluation of the biomedical properties of chitosan and quaternized chitosan for dental applications

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ABSTRACT

The aim of this study was to evaluate the potential dental applications of chitosan (CS) and *N*-[1-hydroxy-3-(trimethylammonium)propyl]chitosan chloride (HTCC). HTCC was prepared by reacting CS with glycidyltrimethylammonium chloride (GTMAC). CS and HTCC were characterized by infrared (FITR) and $^1$H NMR spectroscopy. The antibacterial activity of CS and HTCC against oral pathogens, their proliferation activity and effects on the ultrastructure of human periodontal ligament cells (HPDLCs) were investigated. The results indicated that four oral strains were susceptible to CS and HTCC with minimum inhibitory concentrations (MICs) ranging from 0.25 to 2.5 mg/mL. The in vitro 3-(4,5-dimethyl-2-thizolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay determined that CS at 2000, 1000, 100, and 50 μg/mL could stimulate the proliferation of HPDLCs. Instead, HTCC inhibited the proliferation at the same concentrations but accelerated the proliferation of HPDLCs at relatively low concentrations (10, 3, 1.5, 1, and 0.3 μg/mL). Transmission electron microscopy (TEM) observations revealed that the ultra-architecture of HPDLC was seriously destroyed by HTCC treatment at 1000 μg/mL. Taken together, these results contribute information necessary to enhance our understanding of CS and HTCC in the dental field.



## 1. Introduction

Dentistry is one of the first fields where artificial biomaterials were used for the treatment of diseased tissues.[1,2] Recent developments in the areas of biomaterials and biomedical devices have resulted in a number of advances in dentistry. In particular, research has focused on novel biomacromolecules and biocompatible materials for use in dentistry and clinical applications. Because different biomacromolecules possess a variety of properties, exploring ways of how to weigh the respective advantages of different components on one type of biomaterial has become increasingly important in the field of biomaterials.[3]

Chitosan (CS) is a naturally occurring polysaccharide that is prepared by the deacetylation of chitin, which is mainly obtained from crab and shrimp shells.[4] CS is generally regarded as non-toxic, biocompatible, biodegradable, and is intrinsically antibacterial in nature.[5,6] In addition, CS has shown in different studies to prolong the residence time of drug-delivery systems at the site of drug absorption.[7] In recent years, chitosan has been utilized widely in drug delivery[8,9] and tissue engineering[10] in the dental field. However, chitosan can only be dissolved when the pH is less than 6.5, which limits its antibacterial activity.[11] Quaternized chitosan, which has quaternary amino groups introduced into the chitosan chain, is both a facile and effective method to render it soluble in water. Moreover, quaternized chitosan has cationic activity, bioadhesive properties, permeation enhancing effects, and high efficacy against bacteria and fungi even under neutral conditions.[12] However, little research is available on the inhibition of quaternized chitosan against oral pathogens.

In addition, ongoing concerns exist about the human safety of biomaterials used for oral medicine. Accordingly, only after the biomedical material is thoroughly tested in vitro and proven to be biocompatible and provide further benefits for in vivo applications, can it be used in the human body. In this report, an agar minimum inhibitory concentration (MIC) method and inhibitory zone measurement were used to assess the antimicrobial property of CS and HTCC. The in vitro cytocompatibility assessment was measured using the MTT assay and TEM observation of HPDLCs.

## 2. Materials and methods

### 2.1. Materials

Chitosan (MW 3800 kDa, DD 86%) was provided by Putian Zhongsheng Weiye Co., Ltd (Fujian, China, medicine grade). The

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chitosan sample (MW 1080 kDa and DD 86%) used in this experiment was obtained by an acetic acid degradation method, and viscosity molecular mass was calculated by using an Ubbelohde viscosimeter as previously described.[13] GTMAC was obtained from Dongying Guofeng Fine Chemical Co., Ltd. (Shandong, China). DMEM (Dulbecco's Modified Eagle's Medium) and fetal bovine serum (FBS) were obtained from Hyclone (Greiner, The Netherlands). HBSS (Hanks Balanced Salt Solution) was purchased from Gibco BRL (Life Technologies BV, Breda, The Netherlands). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) was purchased from Sigma Chemical Company (USA). The four bacterial strains were provided by Beijing Dental Institution, Affiliated Hospital of Capital Medical University (Beijing, China). All other reagents were of analytical grade.

### 2.2. HTCC synthesis[14]

*N*-[1-Hydroxy-3-(trimethylammonium)propyl]chitosan chloride (HTCC) was synthesized by reacting CS with glycidyltrimethylammonium chloride (GTMAC). Briefly, 6 g of CS was mixed and dispersed in 225 mL of 2-PrOH. The reaction was carried out with stirring at 80–90 °C for 1 h. GTMAC was dissolved in deionized water (30% w/v) to form a solution. The GTMAC solution was added to the CS suspension slowly under continuous stirring. The molar ratio of GTMAC to the amino groups of CS was 4:1. After 4 h of reaction at 80 °C, any precipitate was filtered. The product was then poured into EtOH and washed three times. Quaternized CS was obtained by drying at 80 °C for 48 h.

The degree of quaternization (DQ) was determined by titrating the amount of $Cl^-$ ions on the HTCC with a 0.1 M aq $AgNO_3$.[15]

### 2.3. FITR of CS and HTCC

The infrared spectra (FITR) of CS and HTCC were measured using KBr pellets on an FTIR-430 Fourier-transform infrared spectrometer (Jasco Co., Tokyo, Japan) to confirm the existence of characteristic groups on CS and HTCC at room temperature.

### 2.4. $^1$H NMR spectra

The $^1$H NMR spectra of chitosan and quaternized chitosan were acquired at 353 K by using a 200-MHz spectrometer (Bruker AC200). For this analysis, samples of CS and HTCC were dissolved in 100:1 $D_2O$–HCl. The parameters for the acquisition of the $^1$H NMR spectra were as follows: a pulse of 90°, corresponding to a pulse width of 8.2 μm; LB 0.55 Hz and 16 transients were acquired.

### 2.5. Bacterial strains and growth conditions

The reference bacterial strains which represented the oral microbiota included two strict anaerobes and two facultative anaerobic bacteria as follows: *Porphyromonas gingivalis* (*P. gingivalis ATCC33277*), *Prevotella intermedia* (*P. intermedia ATCC 25611*), *Actinobacillus actinomycetemcomitans* ((*A. actinomycetemcomitans Y4*), and *Streptococcus mutans* (*S. mutans Ingbritt C*). Bacterial cells were grown in BHI culture media supplemented with hemin (5 g/mL), menadione (1%), and defibrinated goat blood (5%) before the medium was transferred into sterilized petri dishes at about 50 °C. The agar plates were incubated in an anaerobic chamber (USA) with an atmosphere of 80% $N_2$, 10% $H_2$, and 10% $CO_2$ with deoxidized palladium for 72 h (*P. gingivalis*, *P. intermedia)* or 48 h (*A. actinomycetemcomitans*, *S. mutans*). A few singular colonies of each organism were picked from the blood agar plate and diluted into sterile physiological saline. The suspension was adjusted spectrophotometrically at 800 nm ($OD_{800}$) to match a turbidity of $1.5 \times 10^8$ CFU $mL^{-1}$ (equivalent to 0.5 McFarland standard), and used for further antibacterial activity testing.

### 2.6. Test of MIC

The MIC method was carried out in vitro using a twofold dilution technique approved by CLSI (Clinical and Laboratory Standard Institute).[16] The concentrations of the CS and HTCC standard samples ranged from 5 mg/mL to 0.00122 mg/mL. A serial sample was obtained by mixing 1 mL of the standard sample with 9 mL of anaerobic medium. A total of 100 μL of 0.5 McFarland standard organism suspension was dropped onto the surface of the blood agar medium plate. The blood plates were incubated in the anaerobic chamber (Thermo Life Science, USA) under an atmosphere of 80% $N_2$, 10% $H_2$, and 10% $CO_2$ with deoxidized palladium for 72 h.

Control tests were simultaneously run to ensure reliable results. Each assessment was performed three times to ensure the reproducibility of the experiments. The MIC was defined as the lowest concentration of the tested sample at which the bacterial colonies were not visible to the naked eye.

### 2.7. Determination of inhibitory zone diameters

A total of 150 μL of 0.5 McFarland standard bacterial suspensions was spread onto the agar plate. Sterilized stainless steel tubes of 8.0 × 1.0 × 10 mm (inner diameter, 6 mm) were added to the surfaces of the media and filled with 100 μL of sterilized antimicrobial solution. The blood plates were incubated in an anaerobic chamber under an atmosphere of 80% $N_2$, 10% $H_2$, and 10% $CO_2$.

The inhibitory zone was considered to be the shortest distance (mm) between the outer margin of the cylinder and the initial point of the microbial growth. Six replicates were made for each microorganism. Each assessment was performed three times to ensure the reproducibility of the results.

### 2.8. Cell culture and inoculation

Primary HPDLCs were obtained from premolars that had been extracted from periodontal healthy patients for orthodontic reasons with a good oral health status. Briefly, the human periodontal ligament tissues were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin solution (5000 units/mL penicillin and 50 μg/mL streptomycin) at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$ with the medium changed every 2 or 3 d. Passage number 3–5 was used for the study.

### 2.9. MTT assay

Cytotoxicity was estimated using the MTT assay in vitro in accordance with ISO10993-1:1997[17] and GB/T 16886.1-2001.[18] Briefly, cells were plated in 96-well, flat-bottomed microplates (Costar, USA) at a density of approximately $3 \times 10^4$ cells/well, in medium containing 10% FBS. The medium was replaced after 24 h, and the cells were divided into nine different groups that were diluted with DMEM medium with varying concentrations of CS or HTCC. CS and HTCC were tested at the following standard concentrations: 2000, 1000, 100, 50, 10, 3, 1.5, 1, 0.5, and 0.2 μg/mL. There was a negative control in every group, in which the cells were cultured in medium containing 10% FBS alone. After culturing for 1 d, 3 d, and 5 d, 200 μL of MTT solution was added to each well and incubated for 4 h. Subsequently, 200 μL of dimethyl sulfoxide (DMSO) was added to each well. The plates were then shaken until the crystals dissolved. Absorbance was determined at 490 nm with a microplate reader (Bio-Rad Model 550, Hercules, CA, USA). All reported values are the means of triplicate samples.

### 2.10. TEM of HPDLCs

A morphological study of the effects of CS and HTCC on HPDLCs was carried out using TEM at concentrations of 1000 μg/mL CS and 3 μg/mL HTCC. The density of HPDLCs was adjusted to $5 \times 10^4$/mL in culture medium containing 10% FBS. After incubation at 37 °C for 72 h, HPDLCs were centrifuged for 15–20 min after adding 10 mL of ice-cold Hank's solution. The pellets were immediately fixed in 4% cold glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, for 1 h at room temperature, and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 1 h at 4 °C. The pellets were then dehydrated in graded acetone, passed through propylene oxide, and embedded in Epon 812. Semi-thin sections, 2 μm thick, were obtained from the same specimens stained with uranyl acetate and alkaline bismite subnitrate and then examined using transmission electron microscopy at 80 kV (Japan, JEM-1200EX).

### 2.11. Statistical analysis

Statistical data were analyzed using SPSS 13.0, and differences were considered to be significant at a level of $P < 0.05$, using a two-tailed paired *t*-test.

## 3. Results and discussion

### 3.1. Characteristics of synthesized CS and HTCC

The chemical structures of CS and HTCC are shown in Figure 1. The HTCC as prepared was a white powder that dissolved in water at acidic, neutral, or basic pH because of the presence of the positively charged quaternary ammonium groups. The degree of CS quaternization was calculated to be 74.5%.

FITR measurement (Fig. 2) showed that there were three characteristic peaks for CS at 3363 $cm^{-1}$ for OH, 1382 $cm^{-1}$ for C–O–C, and 1603 $cm^{-1}$ for $NH_2$. The saccharide oxygen bridge peaks of the skeletal vibrations involving the C–O stretching appeared between 1146 $cm^{-1}$ and 1083 $cm^{-1}$. Compared with CS, the characteristic peak (1600 $cm^{-1}$) representing $NH_2$ deformation was weakened, and the appearance of a new band at 1482 $cm^{-1}$, which is attributed to the methyl groups of the ammonium groups. Thus the IR spectrum provided evidence for the existence of the quaternary amino groups on the CS chains.

The $^1$H NMR spectrum of chitosan (Fig. 3A) indicated the following characteristic signals: (a) $\delta$ 4.76 is attributed to hydrogen bonded to the chitosan bone skeleton carbon 1; (b) $\delta$ 3.5–4.0 is due to hydrogen bonded to the carbon atoms 3, 4, 5, and 6 of the glycopyranose unit; (c) $\delta$ 3.05 is attributed to the hydrogen atom bonded to the carbon 2 of the glycopyranose ring and (d) $\delta$ 1.9–2.0 corresponds to the hydrogen atoms of the methyl moieties of the acetamido groups.

The $^1$H NMR spectra of HTCC (Fig. 3B) were similar to those of the corresponding chitosan except a characteristic signal. The chemical shift at $\delta$ 3.19–3.27 was not found in the spectrum of chitosan and was assigned to *N*,*N*,*N*-trimethyl protons on benzyl substituent. These data are similar to those in the literature of Wu[19] and Britto.[20]

### 3.2. Antibacterial activity

*P. gingivalis, P. intermedia,* and *A. actinomycetemcomitans* are Gram-negative strains, and *S. mutans* is a Gram-positive strain.

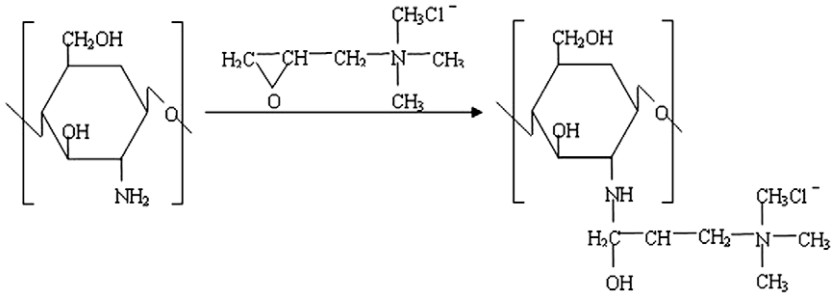


**Figure 1.** The chemical structure of CS and HTCC.

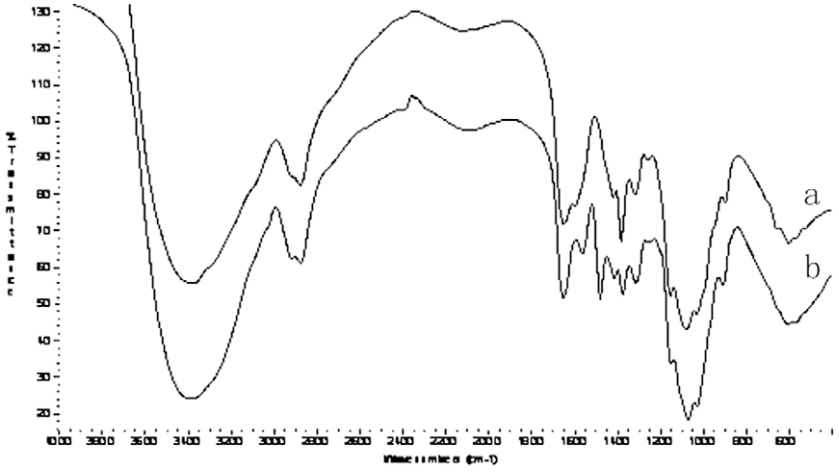


**Figure 2.** FITR spectra of CS (a) and HTCC (b).

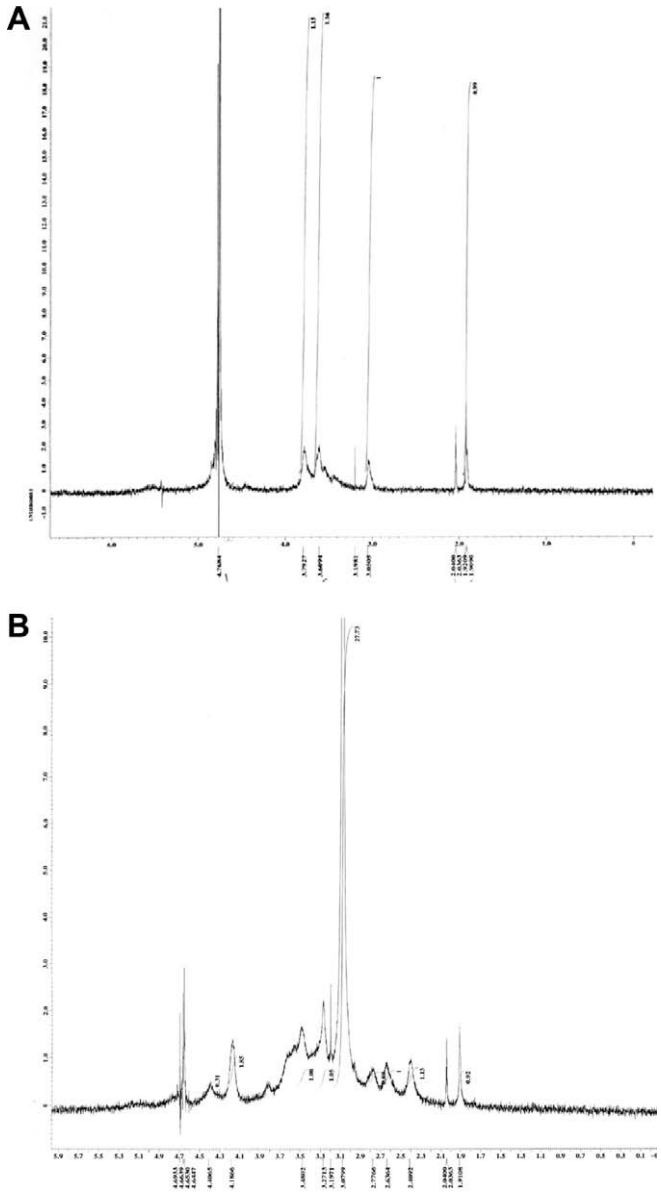


**Figure 3.** (A) $^1$H NMR spectrum of chitosan dissolved in 100:1 $D_2O$–HCl. (B) $^1$H NMR spectrum of quaternized chitosan dissolved in 100:1 $D_2O$–HCl.

The MIC was quantified for all the standard bacterial strains selected, and the results are shown in Table 1. MIC values for each bacterial strain ranged from 0.25 to 2.5 mg/mL. The lactic acid (LA) solution of HTCC exerted higher antibacterial activities against *P. intermedia, A. actinomycetemcomitans,* and *S. mutans* (MICs were 1, 1, 0.5 mg/mL, respectively) than the LA solution of CS (MIC 2.5 mg/mL). However, the LA solution of CS and HTCC had the same MIC value against *P. gingivalis* (MIC 0.5 mg/mL). The aqueous solution of HTCC exhibited relatively lower antibacterial activity against *P. gingivalis* and *S. mutans* than the LA solution of HTCC. In addition, Gram-negative and Gram-positive strains were all susceptible to CS and HTCC and there was no significant difference between them.

**Table 1**
MIC values of CS and HTCC for four oral bacterial strains (mg/mL)[a]

| Antibacterial samples | MIC ($n$ = 3) | | | |
|---|---|---|---|---|
| | *P. gingivalis* | *P. intermedia* | *A. actinomycetemcomitans* | *S. mutans* |
| HTCC (LA) | 0.5 | 1 | 1 | 0.5 |
| HTCC ($H_2O$) | 1 | 1 | 1 | 1 |
| CS (LA) | 0.5 | 2.5 | 2.5 | 2.5 |
| CS + HTCC ($W_{CS}$/$W_{HTCC}$ = 5/1 LA) | 0.25 | 0.25 | 0.25 | 0.25 |

[a] (LA): lactic acid solution, ($H_2O$): aqueous solution.

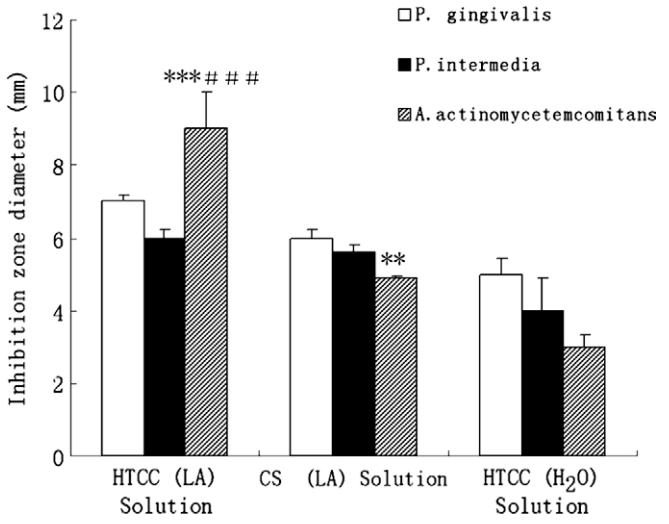


**Figure 4.** Average (in mm) zone of inhibition produced by HTCC (LA), HTCC ($H_2O$), CS (LA), and CS-HTCC ($W_{CS}/W_{HTCC}$ = 5:1 LA) in the indicated solutions against *P. gingivalis, P. intermedia,* and *A. actinomycetemcomitans*. (LA) is the lactic acid solution, ($H_2O$) is the aqueous solution. $^{**}P < 0.01$, $^{***}P < 0.001$: significant difference compared with CS (LA) solution. $^{###}P < 0.001$: significant difference compared with HTCC ($H_2O$) solution.

The inhibitory zones are depicted in Figure 4. HTCC (LA) solution showed the most potent inhibitor against *P. gingivalis, P. intermedia,* and *A. actinomycetemcomitans* with a larger inhibitory zone than that created by a solution of either HTCC ($H_2O$) or CS alone.

To date, the mechanism of inhibition of CS on bacteria is not clear.[21] One possibility is that, due to the interaction between the two charges, the bacterial cell wall breaks, leading to cytoplasmic leakage, which eventually causes cell death.[22–24] In addition, stacking of CS molecules over the microbial cell surface may block the transport of nutrients[25] or binding to DNA, thus inhibiting transcription or permeabilization of the microbial cell wall/membrane. Helander et al.[23] showed that the binding of CS to the outer membrane of a Gram-negative bacteria resulted in a vesicular structure, causing disruption and extensive alteration to the outer membrane surface, resulting in the loss of its barrier properties.

No and co-workers found that the antibacterial activities of chitosan are strain-dependent.[26] Other investigators have shown that an increase in the molecular weight (MW) and degree of deacetylation (DD) of chitosan enhances antibacterial activity.[27] A higher DQ of HTCC should enhance the antibacterial activity. In our experiments, HTCC exerted more potent antibacterial activities than CS in the same solution against *P. intermedia, A. actinomycetemcomitans,* and *S. mutans*. This was likely due to the introduction of the quaternary ammonium salts onto the chitosan backbone which enhanced the water solubility of chitosan as well as increased the antimicrobial activity of HTCC over the entire pH range. However, it is difficult to directly compare our results with those from other investigations since many factors such as the assay conditions or strains of bacteria are different.

### 3.3. MTT assay

The periodontal ligament (PDL) is a soft connective tissue located between the cementum (a thin layer of mineralized tissue covering the roots of the teeth) and the alveolar bone, which acts to sustain and help constrain teeth within the jaw. PDL not only has an important role in supporting teeth, but also contributes to tooth nutrition, homoeostasis, and repair of damaged tissue.[28,29] PDL contains heterogeneous cell populations that can differentiate into either cementum-forming cells (cementoblasts) or bone-forming cells (osteoblasts).[30–32] Accordingly, evaluating the cytocompatibility to HPDLCs is a necessary step for assessing the usefulness of biomaterials or biomacromolecules.

The MTT assay was performed in order to examine the cytocompatibility of CS and HTCC. The MTT reagent is a yellow tetrazolium salt that produces a dark-blue formazan crystal when incubated with viable cells. Therefore, the level of the reduction of MTT into formazan will reflect the level of live cell metabolism.

Figure 5 shows the results from MTT assays for evaluation of the proliferation of HPDLCs cultured with CS and HTCC at concentrations of 2000, 1000, 100, 50, 10, 3, 1.5, 1, 0.5, and 0.2 μg/mL for 1 d, 3 d, and 5 d. Compared with the negative control, CS can stimulate the proliferation of HPDLCs, particularly at concentrations of 2000, 1000, 100, and 50 μg/mL after 5 d of incubation ($P < 0.001$) (see Fig. 5A). In contrast to CS, the absorbance OD values were significantly lower for HTCC at the higher concentrations of 2000, 1000, 100, and 50 μg/mL than the values for the negative control. Moreover, with an increase in concentration from 50 μg/mL to 2000 μg/mL, the absorbance values remarkably decreased. The results obtained by the MTT assay revealed a concentration-dependent relative cytotoxicity for HTCC at a relatively higher concentration. Because HTCC was prepared by introducing quaternary ammonium groups on the amino groups of chitosan, the quaternary group of HTCC may have a strong electrostatic attraction with the negative charges on the surface of HPDLCs, which might result in cell death, thus inhibiting the growth of HPDLCs.

However, HTCC improved the proliferation of HPDLCs at relatively low concentrations (10, 3, 1.5, 1, 0.5, and 0.2 μg/mL). Among them, 3 μg/mL of HTCC resulted in a considerable increase in cell proliferation. This was particularly evident after 5 d of culture compared with that of the normal control group ($P < 0.001$) (see Fig. 5B).

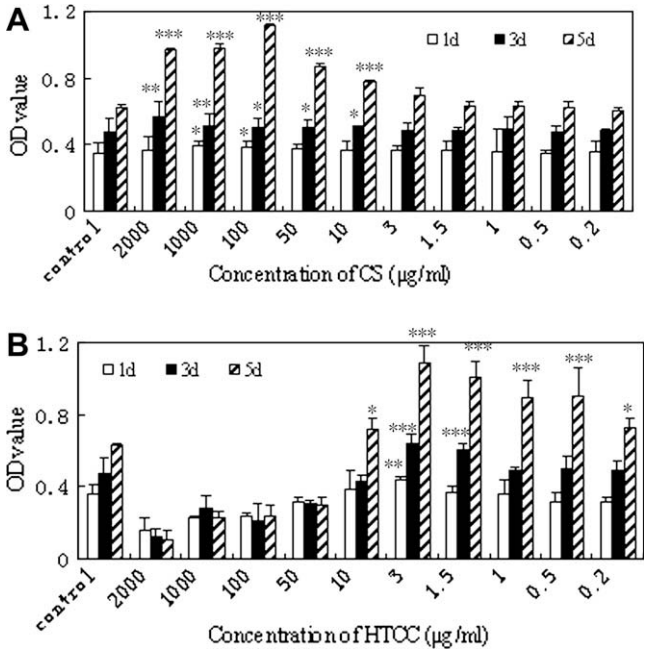


**Figure 5.** Results from the MTT assay of CS (A) and HTCC (B) incubated with HPDLCs at concentrations of 2000, 1000, 100, 50, 10, 3, 1.5, 1, 0.5, and 0.2 μg/mL.

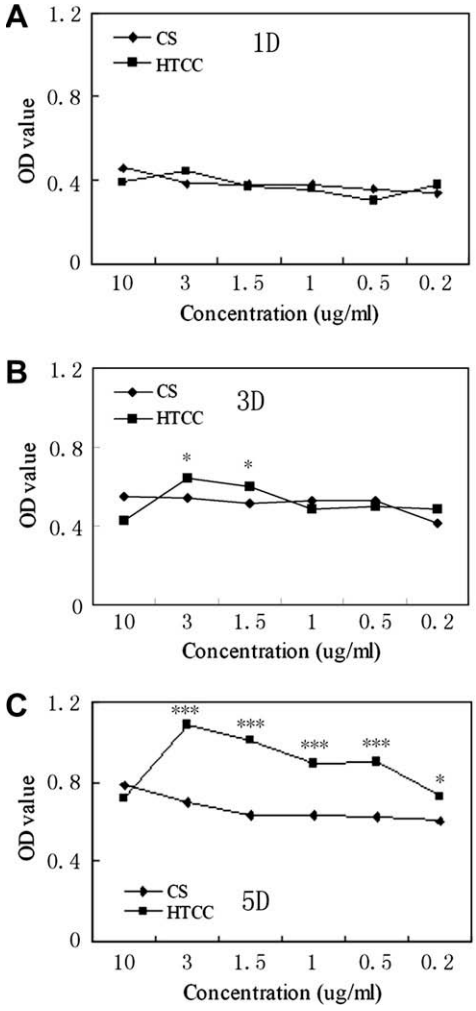


**Figure 6.** Results from the MTT assay of CS and HTCC incubated with HPDLCs at concentrations of 10, 3, 1.5, 1, 0.5, and 0.2 μg/mL for 1 d (A), 3 d (B), and 5 d (C).

Figure 6 illustrates the ability of CS and HTCC to stimulate proliferation of HPDLCs at concentrations of 10, 3, 1.5, 1, 0.5, and 0.2 μg/mL after 1 d, 3 d, and 5 d in culture. After 1 d of culture (Fig. 6A), the cell growth in the presence of CS was similar to HTCC and no significant difference was observed. Compared with CS, the proliferation of HPDLCs in the presence of HTCC at concentrations of 3 and 1.5 μg/mL was significantly increased after 3 d ($P < 0.05$) as depicted in Figure 6B. After 5 d of culture, HTCC, obviously stimulated the growth of HPDLCs as shown in Figure 6C. The difference was statistically significant at concentrations of 3, 1.5, 1, and 0.5 μg/mL HTCC ($P < 0.001$), and 0.2 μg/mL HTCC ($P < 0.05$).

### 3.4. TEM of HPDLCs

TEM observations revealed cytomorphology changes after HPDLCs were incubated with different concentrations of CS and HTCC (Fig. 7). Figure 7A represents the normal ultrastructure of HPDLCs. The cell membrane was intact, and all kinds of cell organelles such as chondriosome, rough-surface endoplasmic reticulum, ribosomes, the Golgi complex, and cellular microfilaments could be observed. The ultrastructure of HPDLCs cultured in 3 μg/mL CS and HTCC medium as shown in Figure 7B and C were quite similar to

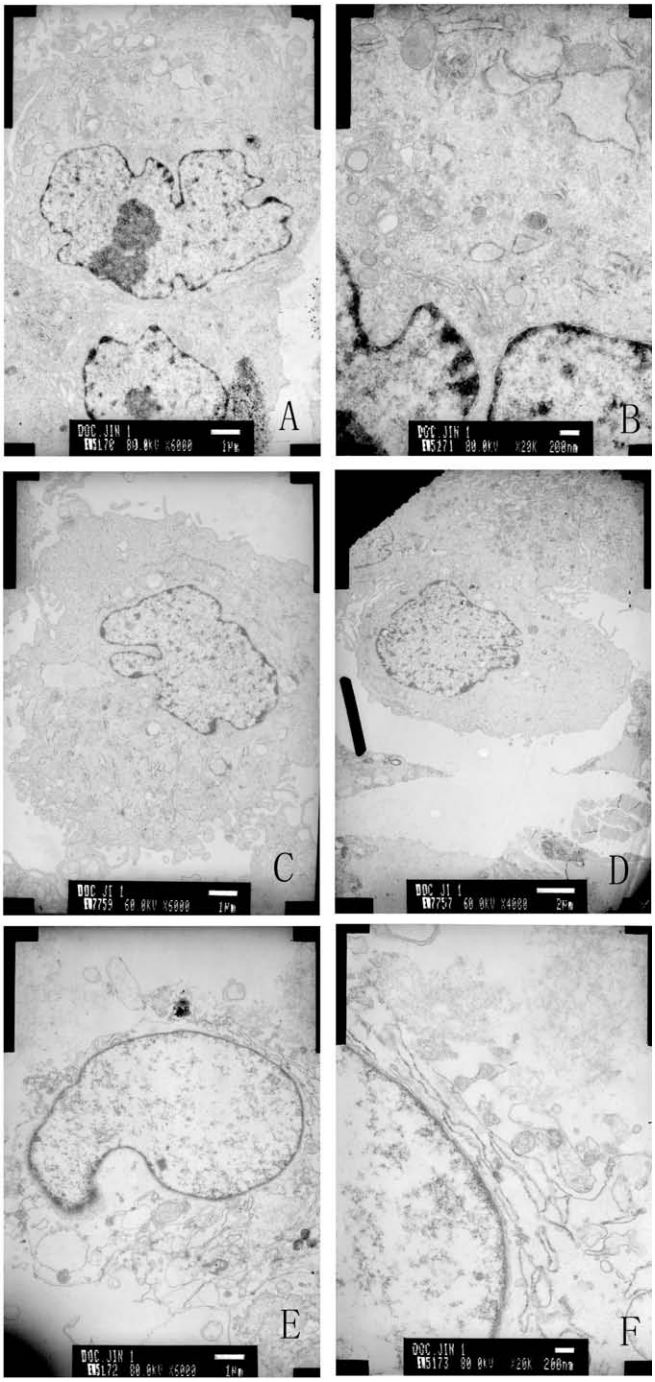


**Figure 7.** The ultrastructure of HPDLCs was examined by transmission electron microscopy (TEM). (A, B, C, E, F × 6000, D × 4000).

that of the normal control cell structure. No apoptotic signs such as condensed nuclear chromatin and extensive cytoplasmic vacuolization were observed. A similar result for HPDLCs incubated with 1000 μg/mL CS medium is shown in Figure 7D. In contrast to what is described above, the architecture of HPDLCs at a concentration of 1000 μg/mL was seriously destroyed. As shown in Figure 7E and F, cell membranes were not intact, and even lysis of the cell was observed. The organelles, such as rough-surface endoplasmic reticulum and the Golgi complex disappeared. Accordingly, severe cytotoxicity was observed for HPDLCs in the presence of HTCC at this higher concentration.

All the results from TEM observations further confirmed the conclusions from the MTT assay. The quaternary groups on the HTCC at the higher concentrations provided too strong of a cationic environment and a strong electrostatic attraction with the negative-charged groups on the surface of HPDLCs, which resulted in the destruction of the cells, thus causing a serious cytotoxic effect.

In summary, the information provided by this research was necessary to enhance our understanding of the biomedical properties of CS and HTCC for inhibiting oral pathogens and their cytocompatibility with HPDLCs. We can partly deduce that the better antibacterial activity of HTCC is related to the cytotoxicity, although there must be more research carried out in the future on this point. Also, when HTCC is associated with other composites and forms a new structure such as a film, gel, microcapsule, microsphere or nanoparticle, re-evaluation of biocompatibility will be absolutely necessary.

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