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Modulation of pro-inflammatory mediators in LPS-stimulated human periodontal ligament cells by chitosan and quaternized chitosan

Qiuxia Ji*, Jing Deng, Xinbo Yu, Quanchen Xu, Hong Wu, Jianfeng Pan

The Affiliated Hospital of Medical College, Qingdao University, Qingdao, Shandong 266001, PR China

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ABSTRACT

The aim of this study was to evaluate the effects of chitosan and quaternized chitosan (HTCC) modulate IL-1β and TNF-α in LPS-stimulated human periodontal ligament cells (HPDLCs). MTT assay revealed that chitosan stimulated the proliferation of HPDLCs. However, HTCC inhibited the proliferation of HPDLCs at concentrations of 1000 and 100 μg/mL more than the control, especially after 5 d ($P < 0.001$). ELISA assay exhibited that chitosan inhibited the production of IL-1β and TNF-α at 24, 48 and 72 h. IL-1β and TNF-α secreted by HPDLCs with LPS and treated with 1000 μg/mL of HTCC significantly increased compared to both the control and the chitosan group ($P < 0.001$). The bioactive role for bFGF in modulating the responses of HPDLCs cells to LPS *via* inhibiting IL-1β and TNF-α production was demonstrated. All results were necessary to enhance our understanding of the biomedical properties of chitosan and HTCC for modulation of pro-inflammatory mediators.

1. Introduction

Periodontal disease is a chronic infection caused by accumulation of bacteria in dental plaque which produces localized inflammation of the periodontium (Loe, Theilade, & Jensen, 1965). These are generally destructive, non-reversible conditions which can result in the formation of pockets and tooth loss ultimately (Tatakis & Kumar, 2005).

Although periodontal diseases are initiated by bacteria that colonize the tooth surface and gingival sulcus, the host response in most cases resulting from periodontopathogens in subgingival sites is believed to play an essential role in the breakdown of connective tissue and bone (Graves, 2008). The continuous challenge to host immune and resident cells by periodontal pathogens and their virulence factors result in enhanced and uncontrolled secretion of cytokines. Host mediators including interleukin (IL)-1β, IL-6, IL-8, IL-12 and tumor necrosis factor alpha (TNF-α) participate in tissue destruction and bone resorption directly or indirectly (Ebersole & Taubman, 1994; Kornman, Page, & Tonetti, 1997; Okada & Murakami, 1998). Therefore, elimination or control of periodontopathogens prevents the progression of periodontal diseases and related inflammatory processes, and are essential for periodontal treatment.

Chitosan, isolated from chitin, is the linear and partly acetylated (1-4)-2-amino-2-deoxy-β-D-glucan (Muzzarelli, 1977, 2012), a well-known functional aid for the ordered regeneration of human tissues (Muzzarelli, 2009; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012). Many studies have shown that chitosan has beneficial effects on various physiological functions including wound healing acceleration, intestinal bile acid metabolism (Fukada, Kimura, & Ayaki, 1991), reduction in fat storage (Han, Kimura, & Okuda, 1999) and atherosclerosis (Muzzarelli, 1999). Chitosan decreased the production of TNF-α and it can balance between pro- and anti-inflammatory signals associated with implant biomaterials (Oliveira, Santos, Oliveira, Torres, & Barbosa, 2012). Other studies showed chitosan can enhance the function of different cell types, such as polymorphonuclear leukocytes, macrophages and fibroblasts (Mori et al., 1997; Porporatto, Bianco, Riera, & Correa, 2003).

Chitosan has been proposed in the field of periodontal therapy either with a tissue engineering scaffold (Ji, Deng, et al., 2010) or with local biodegradable sustained-release agents (Ji, Zhao, Deng, & Lu, 2010). Research reported that chitosan exhibits a positive effect on bacterial, fungal infection and anti-inflammatory properties (Ji et al., 2009). However, there are few studies examining the effect of chitosan and quaternized chitosan (HTCC) on the level of cytokines related to the cellular immune system in human periodontal ligament cell (HPDLCs). In this study, the modulation of IL-1β and

* Corresponding author at: The Affiliated Hospital of Medical College, Qingdao University, 16# Jiangsu Road, Qingdao, Shandong 266001, PR China.
Tel.: +86 532 8291 1213; fax: +86 532 8291 1840.
E-mail address: jqx_1@163.com (Q. Ji).

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TNF-α in the LPS-stimulated human periodontal ligament cell (HPDLCs) by chitosan (chitosan) and quaternized chitosan (HTCC) will be evaluated in order to investigate the anti-inflammatory potential of chitosan and HTCC against periodontal inflammation as well as to understand the underlying mechanism.

2. Materials and methods

2.1. Materials

Chitosan (1080 kDa; deacetylation degree 86%) was prepared in our laboratory as previously described (Chen, Zheng, Wang, Lee, & Park, 2002). LPS (*Escherichia coli*, O_{55}:B_5), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-dipHenyltetrazolium bromide; thiazolyl blue), IL-1β and TNF-α ELISA kits were bought from Sigma Chemical Company (St. Louis, MO, USA). 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). Other chemicals and reagents used in this study were of analytical grade.

2.2. Synthesis of HTCC and characteristic chitosan

N-[2-hydroxy-3-(trimethylammonium)] chitosan chloride (HTCC) was synthesized by reacting chitosan with glycidyltrimethylammonium chloride (GTMAC) (Ji et al., 2009). Briefly, chitosan was mixed and dispersed in dimethylcarbinol. The reaction was carried out with stirring at 80–90 °C for 1 h. GTMAC solution (30% w/v) was added to the chitosan suspension slowly under continuous stirring. The molar ratio of GTMAC to the amino groups of chitosan was 4. The degree of quaternization (DQ) was determined by titrating the amount of Cl^- ions on the HTCC with a 0.1 M aqueous $AgNO_3$ solution (Xu, Kaar, Russell, & Wagner, 2006). And also, both the infrared spectrum (FITR) and ^1H NMR spectrum of chitosan and quaternized chitosan were acquired respectively.

2.3. Cell culture

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% CO2 with the medium changed every 2 or 3 d. Passage numbers 3–5 was used for the study.

2.4. MTT assay

For cell proliferation experiments the HPDLCs cultures were incubated with chitosan 100 and 1000 μg/mL at 37 °C in CO_2 for 1 d, 3 d and 5 d. At each time point, the cell numbers were determined using a colorimetric MTT assay based on the cytoplasmic enzyme activity present in the viable cells. 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.

2.5. Treatment of HPDLCs with E. coli LPS and chitosan or HTCC

A quantity of 0.5 mL of HPDLCs was seeded in forty-eight-well plates at a density of 4×10^4 cells per well and were allowed to attach for 24 h. They were then stimulated by *E. coli* LPS (50 μg/mL), with or without chitosan and HTCC for 1, 3 and 5 d. The untreated

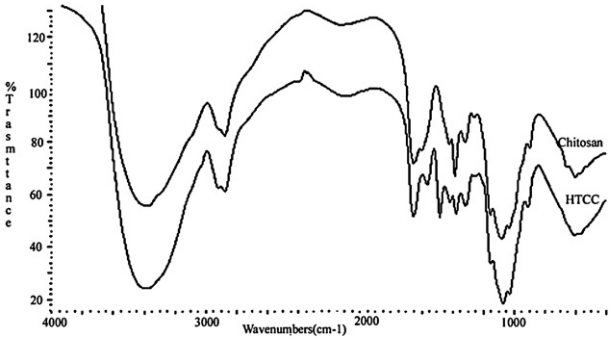

Fig. 1. IR spectrum of chitosan and quaternized chitosan (HTCC).

HPDLCs served as a normal control. At specific times, culture media were collected. The HPDLCs of all treatment groups underwent lysis in 0.2% Triton X-100, and the cell lysates were collected and subjected to centrifugation. The cell-free supernatants were stored at −20 °C prior to assay. The experimental groups are listed as follows:

control,
LPS (50 μg/mL),
chitosan (1000 μg/mL) + LPS (50 μg/mL),
chitosan (1000 μg/mL) + LPS (50 μg/mL) + bFGF (100 ng/mL),
HTCC (1000 μg/mL) + LPS (50 μg/mL),
HTCC (1000 μg/mL) + LPS (50 μg/mL) + bFGF (100 ng/mL),
bFGF (100 ng/mL) + LPS (50 μg/mL).

2.6. Inflammatory cytokine measurement by ELISA

The concentration of cytokines IL-1β and TNF-α in the cell supernatant was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction. All experiments were performed in triplicate. The concentration of cytokines in the cell supernatants was calculated by reference to the standard curve constructed with fixed concentrations of the cytokines provided in the kits.

2.7. Statistical analysis

Statistical data were analyzed using SPSS 13.0. The data were presented as mean ± SD ($n = 3$). Differences between the means of the individual groups were assessed by ANOVA (a one-way analysis of variance). Statistical significance was determined at $P < 0.05$.

3. Results

3.1. The characteristic chitosan of prepared HTCC

HTCC as prepared was 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 chitosan quaternization was calculated to be 75%. FT-IR (Fig. 1) 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 spectrum for HTCC shows the disappearance of the NH_2-associated band near 1600 cm^{-1} for the N—H bending in the primary amine; and the appearance of a new band at 1482 cm^{-1}, which is attributed to the methyl groups of the ammonium. Thus, the IR spectrum provided evidence for the existence of the quaternary amino groups on the CS chains. The ^1H NMR spectrum of chitosan (Fig. 2) indicated the following characteristic signals: (a) d 4.76 is attributed to hydrogen bonded to the chitosan

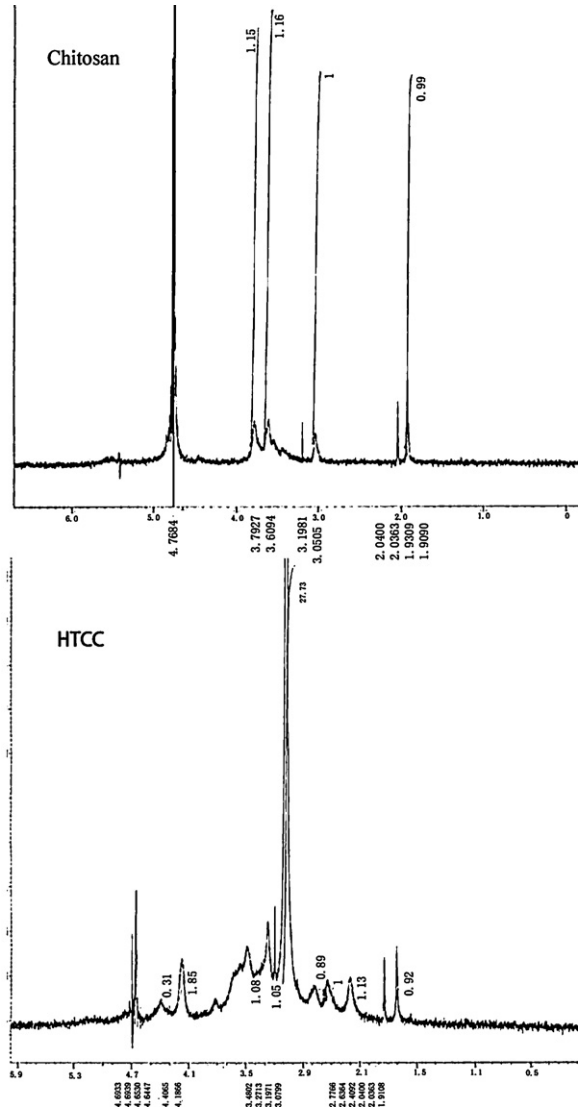

Fig. 2. ^{1}H NMR spectrum of chitosan and quaternized chitosan (HTCC).

bone skeleton carbon 1; (b) d 3.5–4.0 is due to hydrogen bonded to the carbon atoms 3, 4, 5 and 6 of the glycopyranose unit; (c) d 3.05 is attributed to the hydrogen atom bonded to the carbon 2 of the glycopyranose ring and (d) d 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. 2) were similar to those of the corresponding chitosan except for a characteristic signal. The chemical shift at d 3.19–3.27 was not found in the spectrum of chitosan and was assigned to N,N,N-trimethyl protons on benzyl substituent.

3.2. Determination of HPDLCs proliferation and viability in chitosan and HTCC treated cells

Fig. 3 shows the MTT assay of chitosan and HTCC on the proliferation of HPDLCs at concentrations of 1000, 100, 10 and 1 μg/mL. Chitosan increases the proliferation of HPDLCs at 1 d, 3 d and 5 d. Significant differences were shown at 1000 μg/mL ($P < 0.005$) and 100 μg/mL ($P < 0.001$) after 5 d of incubation compared with the control (Fig. 3A). And HTCC exhibited inhibiting activity on HPDLCs at 1 d and 3 d with tested concentrations. At 5 d, a decreasing effect was shown to be statistically significant at concentrations of 1000 and 100 μg/mL compared to the control ($P < 0.001$). However, HTCC

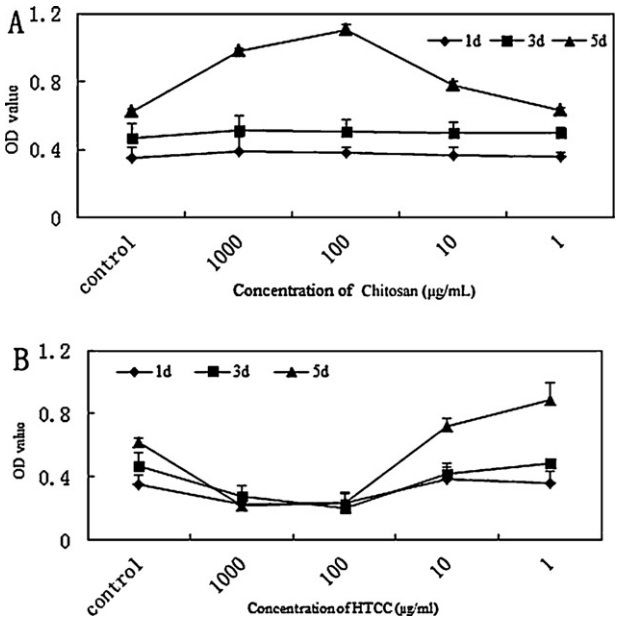

Fig. 3. MTT assay of chitosan (A) and HTCC (B) on the proliferation of HPDLCs at concentrations of 1000, 100, 10 and 1 μg/mL.

improved the proliferation of HPDLCs at 1 μg/mL concentration after 5 d of culture ($P < 0.05$) (Fig. 3B).

Compared with chitosan and HTCC, it is no significant difference for the growth of cells after 1 d ($P > 0.05$) (Fig. 4A). The decreased incline of HTCC with 1000 and 100 μg/mL was obvious at 3 d culture compared with chitosan. Statistical significance was shown at the concentration of 100 μg/mL at 3 d ($P < 0.05$) (Fig. 4B). Promoting activity and inhibiting activity of HPDLCs were significant at the concentration of 1000 and 100 μg/mL ($P < 0.001$) compared with chitosan and HTCC. However, HTCC improved the proliferation of HPDLCs at 1 μg/mL concentration after 5 d of culture. The difference was statistically different ($P < 0.05$) (Fig. 4C).

3.3. Effects of chitosan and HTCC on LPS-stimulated IL-1β and TNF-α production and expression

To investigate whether chitosan and HTCC could affect LPS-stimulated inflammatory cytokine expression in HPDLCs, we measured IL-1β and TNF-α expression at protein levels by ELISA assay. Operations of ELISA assay were done strictly according to the kits' operating instructions. Standard curves (Fig. 5) were established and showed good linear relationship at the concentrations of 0–100 and 0–250 pg/mL of IL-1β (Fig. 5A) and TNF-α (Fig. 5B), respectively.

Fig. 6 shows the levels of IL-1β of HPDLCs stimulated with LPS, chitosan, HTCC and bFGF at 24, 48 and 72 h. The secretions of IL-1β into the culture medium of HPDLCs were significantly increased by stimulation with 50 μg/mL LPS, compared to the unstimulated control ($P < 0.001$). When cells were stimulated with LPS and treated with 1000 μg/mL of chitosan, the IL-1β decreased significantly at 24 and 48 h (Fig. 6A). 1000 μg/mL chitosan also greatly increased the production of IL-1β at 72 h compared with the control ($P < 0.001$). Compared with chitosan, the IL-1β secreted by HPDLCs with LPS and treated with 1000 μg/mL of HTCC significantly increased at 24, 48 and 72 h whenever compared to control and chitosan groups (Fig. 6B and C) ($P < 0.001$). Meanwhile, the effect of bFGF on secretion of IL-1β was detected. When cells were stimulated with LPS and treated with bFGF, the secretion of IL-1β was decreased compared with the LPS group. The amount of IL-1β can be significantly decreased by adding bFGF into the culture medium ($P < 0.001$).

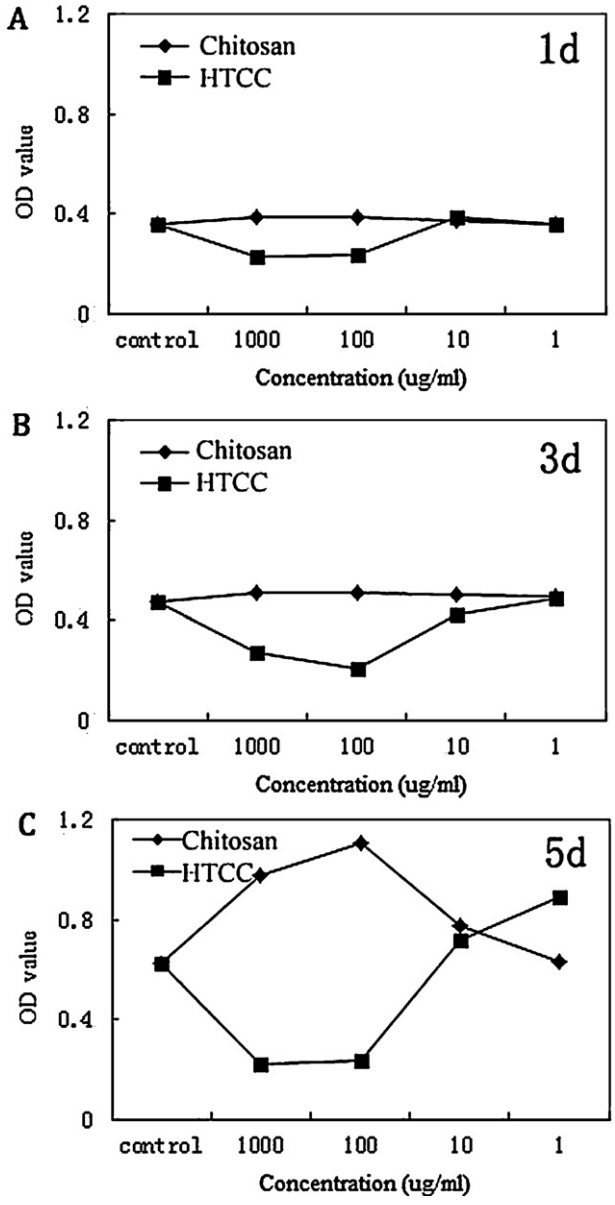

Fig. 4. The results of chitosan and HTCC on the proliferation of HPDLCs at concentrations of 1000, 100, 10 and 1 μg/mL for 1 d (A), 3 d (B) and 5 d (C).

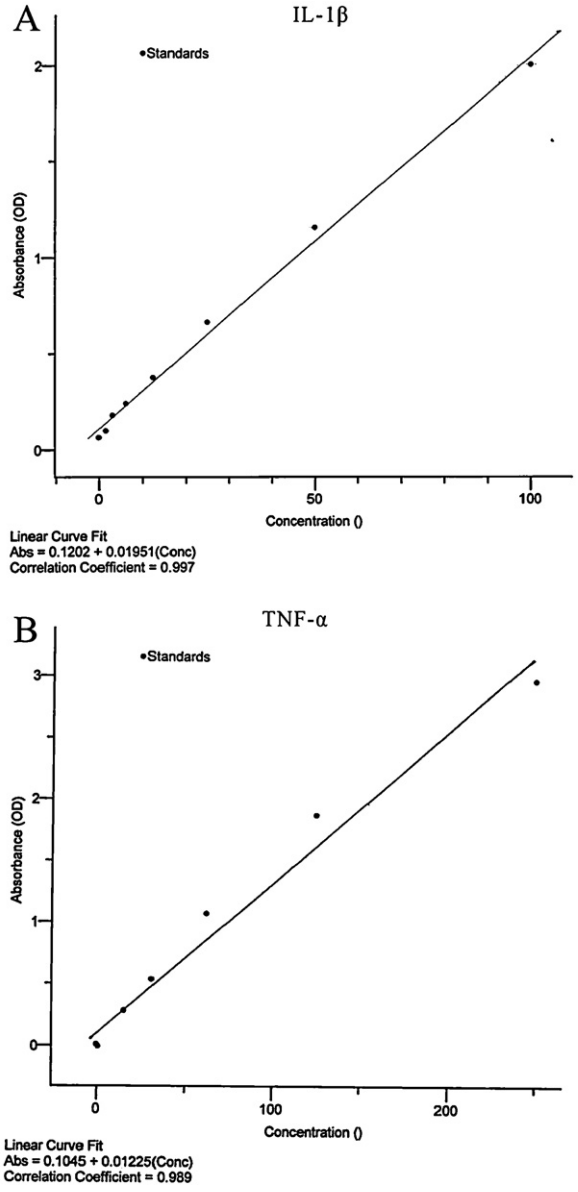

Fig. 5. Standard curve of IL-1β (A) and TNF-α (B).

LPS could up-regulate TNF-α within 72 h as shown in Fig. 7. The secretions of TNF-α in cultures of HPDLCs were increased by stimulation with LPS, compared to non-LPS controls. The highly statistically significance was shown at 72 h ($P < 0.001$). The amount of TNF-α was reduced when HPDLCs were stimulated by LPS and treated with chitosan compared with LPS, especially at 72 h ($P < 0.001$) (Fig. 7A). Compared with chitosan, TNF-α stimulated by LPS and treated with HTCC was significantly increased (Fig. 7B and C) ($P < 0.001$). The addition of bFGF at concentrations of 100 ng/mL to the culture media inhibited the stimulatory effects of LPS, chitosan and HTCC on inflammatory cytokine secretion (Fig. 7D). Therefore, a bioactive role for bFGF in modulating the responses of HPDLCs to LPS *via* inhibiting IL-1β and TNF-α production was demonstrated.

4. Discussion

With the research of the etiology of periodontal disease, the host response is believed to play an essential role in the breakdown of connective tissue and bone. Cytokines, the intermediate mechanism, lies between bacterial stimulation and tissue destruction and stimulates inflammatory events. Lipopolysaccharides (LPS) is one of the most important cell wall components for all subgingival Gram-negative organisms. LPS are known to induce polymorphonuclear leukocyte infiltration, edema, and vascular dilatation in inflamed periodontal tissues (Page, 1991). Furthermore, LPS plays a key role in the destruction of periodontal tissue, including the gingiva, periodontal ligament (PDL), and alveolar bone, through the production of pro-inflammatory mediators, such as interleukins-1 (IL-1), IL-6, and IL-8, tumor necrosis factor-α (TNF-α), and prostaglandin (PG) (Agarwal et al., 1995). Both interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α) are pro-inflammatory cytokines involved in systematic inflammation and cause apoptotic cell death, cellular proliferation, differentiation, and inflammation (Maury, 1989).

The Periodontal Ligament Cell (PDL) is a highly vascularized and cellularized connective tissue that attaches the tooth root to the surrounding alveolar bone (Lekic & McCulloch, 1996). PDL cells not only play function as support cells for periodontal tissues, but

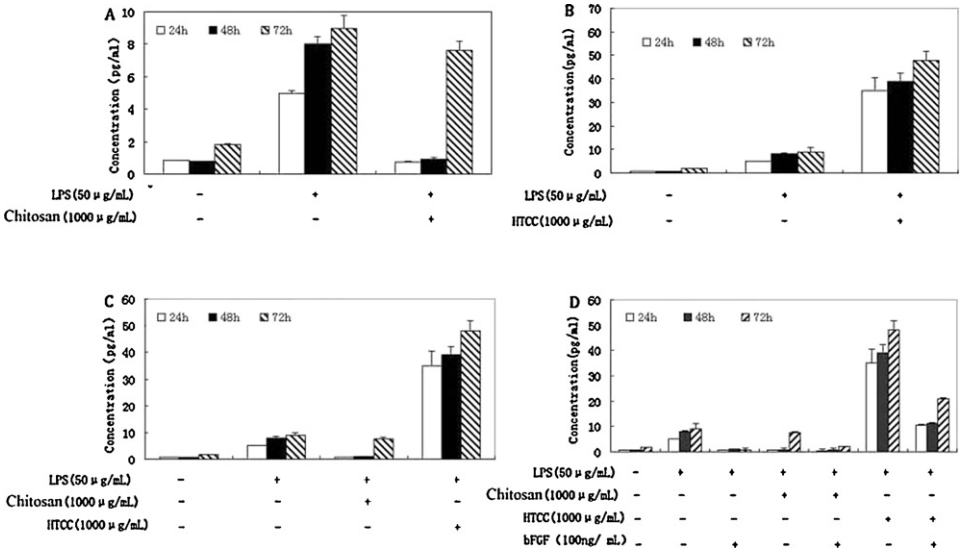

Fig. 6. (A) IL-1β secretion in HPDLC cultures stimulated with LPS and treated with chitosan (1000 and 100 μg/mL) for 1 d, 3 d and 5 d. IL-1β was measured by ELISA. (B) IL-1β secretion in HPDLC cultures stimulated with LPS and treated with HTCC (1000 μg/mL) for 1 d, 3 d and 5 d. IL-1β was measured by ELISA. (C) The comparison of chitosan and HTCC on IL-1β secretion in HPDLCs cultures for 1 d, 3 d and 5 d. (D) The effect of chitosan, HTCC and bFGF on production of IL-1β in HPDLCs stimulated by LPS for 1 d, 3 d and 5 d. ***$P < 0.001$: *versus* cells stimulated with negative group in the same culture time.

also produce various inflammatory mediators to recognize somatic components, including LPS (Hou & Yaeger, 1993; Lerner, 1994). Thus, HPDLCs have been used for the investigation of periodontal metabolism, which is essential to understanding the pathways related to pathological degeneration and tissue inflammation.

The MTT assay was performed in order to examine the cytocompatibility of CS and HTCC. The level of the reduction of MTT into formazan will reflect the level of live cell metabolism. We analyzed the effect of HTCC on the proliferative activity of HPDLCs. HTCC exhibited inhibiting activity on HPDLCs at 1 d and 3 d with tested concentrations. At 5 d, a decreasing effect was shown to be statistically significant at concentrations of 1000 and 100 μg/mL compared to the control ($P < 0.001$). However, HTCC improved the proliferation of HPDLCs at 1 μg/mL concentration after 5 d of culture ($P < 0.05$) (Fig. 3B). The results obtained by the MTT assay revealed a serious cytotoxicity for HTCC at concentrations of 100 and 1000 μg/mL. 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 to the negative charges on the surface of HPDLCs, which might result in cell death, thus inhibiting the growth of HPDLCs.

Compared with the negative control, chitosan increases the proliferation of HPDLCs at 1 d, 3 d and 5 d at concentrations of 1000, 100, 10 and 1 μg/mL. This study showed an important anti-inflammatory effect of chitosan in HPDLCs *in vitro*. We believe that, apart from the direct effect of chitosan on periodontal pathogens, chitosan may suppress IL-1β and TNF-α indirectly *via* modulating the synthesis of cytokines locally produced by HPDLCs, which may be one of the ways in which chitosan contributes to its antibacterial effect in periodontal tissue. Further studies are necessary to investigate the exact mechanisms of chitosan on inflammatory cytokines in HPDLCs at molecular levels.

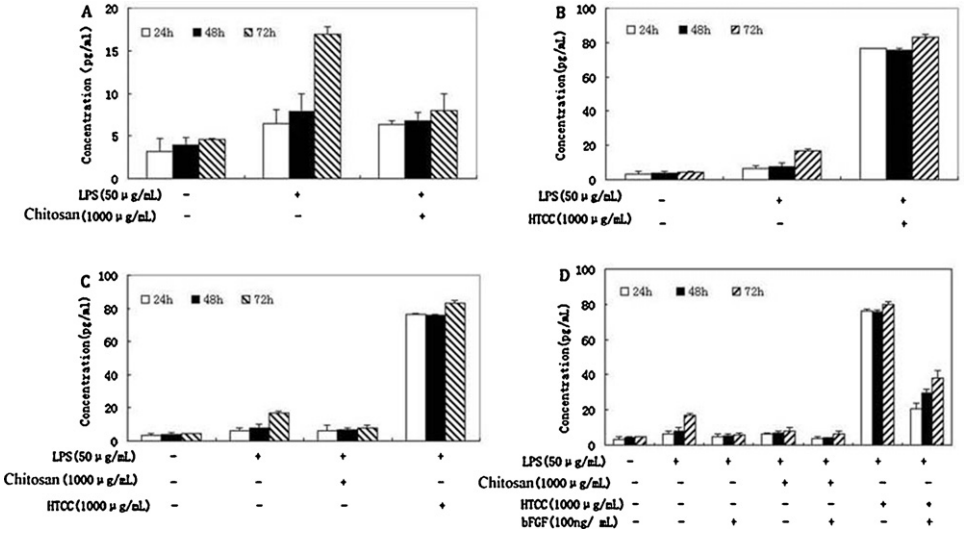

Fig. 7. (A) TNF-α secretion in HPDLC cultures stimulated with LPS and treated with chitosan (100 and 1000 μg/mL) for 1 d, 3 d and 5 d. IL-1β was measured by ELISA. (B) TNF-α secretion in HPDLC cultures stimulated with LPS and treated with HTCC (1000 μg/mL) for 1 d, 3 d and 5 d. TNF-α was measured by ELISA. (C) The comparison of chitosan and HTCC on TNF-α secretion in HPDLCs cultures for 1 d, 3 d and 5 d. (D) The effect of chitosan, HTCC and bFGF on production of TNF-α in HPDLCs stimulated by LPS for 1 d, 3 d and 5 d. ***$P < 0.001$: versus cells stimulated with negative group in the same culture time.

In summary, the information provided by this research was necessary to enhance our understanding of the biomedical properties of chitosan and HTCC for modulation of pro-inflammatory mediators and their cytocompatibility with HPDLCs. Furthermore, the investigation suggests further options for utilizing chitosan and HTCC in oral and biomedical applications in different formulations, such as in mouthwash, gel, vanish, and chip, although there is still more research to be done in the future.

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