

Inhibitory effects of oligochitosan on TNF- α , IL-1 β and nitric oxide production in lipopolysaccharide-induced RAW264.7 cells

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Abstract. Oligochitosan has been reported to possess anti-inflammatory properties; however, the mechanism of the anti-inflammatory effects of oligochitosan remains unknown. The present study aimed to investigate the expression levels of inflammatory cytokines and the production of nitric oxide (NO), in the nuclear factor (NF)- κ B pathway of lipopolysaccharide (LPS)-stimulated RAW264.7 murine macrophages. The results of the present study demonstrated that different concentrations of oligochitosan could significantly lower the levels of NO, tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , released from LPS-stimulated RAW264.7 cells. This was shown to be mediated through inhibiting the activation of the NF- κ B pathway. These results demonstrate that oligochitosan may efficiently inhibit inflammation and has the potential to be an effective anti-inflammatory agent.

Introduction

Inflammation has an important role in the progression of numerous pathologies, including cardiovascular disease, cancer and autoimmune diseases (1-3). The inhibition of the inflammatory response has become a therapeutic priority for the treatment of these diseases. Previous studies have demonstrated that macrophages are important in the pathological process of inflammation (4-5).

In the inflammatory response, macrophages have three main functions: Antigen-presentation, phagocytosis and the production of various cytokines and active substances that regulate immune functions, including, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and nitric oxide (NO) (6).

Chitin is a homopolymer of β -1,4-linked N-acetylglucosamine, and chitosan is the product of partial deacetylation of chitin. Chitin and chitosan are the second most abundant polysaccharides occurring in nature, and are the main components found in the exoskeletons of crabs, shrimp and insects, as well as fungi (7). It has previously been reported that both chitin and chitosan have various biological functions, including anti-inflammatory, immuno-enhancing, and antitumor effects (8-10). However, their insoluble and high viscous properties limit their use *in vivo*. Therefore, research has focused on low molecular weight oligochitosan, which is water-soluble, nontoxic, biocompatible and possesses versatile functional properties. However, the mechanism of the anti-inflammatory effects of oligochitosan remains to be elucidated. Macrophages produce TNF- α , IL-1 β and NO, which have important roles in the occurrence and development of inflammation. Therefore, the aims of the present study were to investigate the effects of oligochitosan on the production of inflammatory cytokines and the activation of NF- κ B in lipopolysaccharide-induced RAW264.7 cells in order to illuminate the initial mechanisms of the anti-inflammatory effects of oligochitosan.

Materials and methods

Preparation of oligochitosan. A total of 9 g chitosan (degree of N-acetylation <15%; Sigma-Aldrich, St Louis, MO, USA) was dissolved in 300 ml HCl (pH 5.0). The mixture was incubated with 1.5 g cellulase in a reaction vessel at 50°C for 48 h. The solution was then neutralized to pH 7.0 using 1 mol/l NaOH. Following centrifugation at 8,000 x g for 10 min, the crude oligochitosan in the supernatant was precipitated by the addition of ethanol. The precipitate was dissolved in distilled water and vacuum-dried, prior to collection of the oligochitosan sample. The components of the oligochitosan sample were detected using high performance liquid chromatography (HPLC). The analysis was conducted on a TSK-Gel Amide-80 (TOSOH, Tokyo, Japan) equipped with a differential detector. The mobile phase consisted of C₂H₃N:water in a ratio of 37:63, pH 4.0, 25°C, with a sample volume of 1 μ l.

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Cell culture. The RAW264.7 murine macrophage-like cells, were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai,

China). The cells were cultured in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin G and 100 μ g/ml streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

MTT assay. The RAW 264.7 macrophages (1x10⁶ cells/ml) were seeded into 6-well plates in 1 ml RPMI-1640 medium, containing 10% FBS. Following a 24 h incubation, the cells were washed and incubated with 1 ml RPMI-1640 medium, containing various doses of oligochitosan. Following a further 24 h incubation, MTT reagent (Sangon, Shanghai, China) was added to each well for 4 h and formazan crystals were solubilized in dimethyl sulfoxide. Subsequently, the absorbance was measured at 570 nm, using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). All of the experiments were performed in triplicate.

Macrophage stimulation. The RAW264.7 cells (1x10⁶ cells/ml) were stimulated with 1 μ g/ml LPS (Sigma-Aldrich), with or without different concentrations of oligochitosan (0, 50, 100, or 500 μ g/ml), for 24 h at 37°C in the presence of 5% CO₂. The cells were collected for western blot analysis, and the culture supernatants were used for ELISA and nitrite assays.

Nitrite assay. The concentration of nitrite in the culture medium, which correlated with the amount of NO secreted by macrophages, was measured as described by previous methods (11). Briefly, 100 μ l aliquots of the culture supernatants were placed in triplicate in a 96-well ELISA plate and incubated with an equal volume of Griess reagent [1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride, 2.5% H₃PO₄] at room temperature for 10 min. The absorbance was measured at 540 nm, using a microplate reader. The concentration of nitrite was determined using sodium nitrite as a standard. All of the experiments were performed in triplicate.

ELISA. TNF- α and IL-1 β assay kits (Diacclone, Besancon, France) were used according to the manufacturer's instructions. Pre-coated ELISA plates were incubated with 50 μ l culture supernatants for 2 h at room temperature. Subsequently 50 μ l aliquots of biotin-conjugated antibody were added and the plates were incubated at 37°C for 90 min. The plates were washed thoroughly using the washing solution from the kit and 100 μ l streptavidin-horseradish peroxidase (HRP) was added, followed by a further 30 min incubation at 37°C. The plates were washed thoroughly and 100 μ l substrate solution (freshly prepared tetramethylbenzidine with H₂O₂) was added. The plates were then incubated at 37°C for 20 min in a dark chamber, and the optical density was measured at 450 nm. Recombinant murine TNF- α and IL-1 β were diluted and used as standards. All of the experiments were performed in triplicate.

Western blot analysis. The culture medium was removed from the RAW264.7 macrophages, following which, a total of 1x10⁶ cells were suspended in 1 ml of ice-cold phosphate-buffered saline (pH 7.2). The cells were then centrifuged at 1,000 x g for 5 min, resuspended in 400 μ l of ice-cold hypotonic buffer (10 mM HEPES-KOH pH 7.9, 2 mM MgCl₂,

0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 1 μ g/ml Leupeptin, 1 mM PMSF), kept on ice for 10 min, vortexed, and centrifuged at 15,000 x g, for 30 sec at 4°C. The pelleted nuclear protein was resuspended in 50 μ l of ice-cold saline buffer (50 mM HEPES-KOH, pH 7.9, 10% glycerol, 300 mM NaCl, 1.5 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 μ l/ml Leupeptin, 1 mM PMSF), kept on ice for 20 min, vortexed, and centrifuged at 15,000 x g for 10 min at 4°C. The protein concentration was determined by the BCA method (Pierce, Rockford, IL, USA) and the aliquots were stored at -70°C, until further use. A total of 30 μ g nuclear protein was loaded onto 10% SDS-polyacrylamide gels, separated by electrophoresis, followed by transfer to a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was then blocked with 5% skim milk in tris-buffered saline containing Tween® (TBST) for 1 h at room temperature, and incubated with rabbit anti-murine NF- κ B p65 antibody (Rockland Immunochemical Inc., Gilbertsville, PA, USA) for 1 h. The membrane was washed three times in TBST, and incubated with HRP-conjugated goat anti-rabbit immunoglobulin G (Rockland Immunochemical Inc.) for 1 h. The antibody-specific protein was visualized using an Enhanced Chemiluminescence Detection system (Pierce, Rockford, IL, USA). The intensities of the protein bands were analyzed using the Gel-Pro® Analyzer software (Media Cybernetics, USA).

Statistical analysis. The results of the present study are expressed as the means \pm standard deviation of the indicated number of experiments. The statistical significance was estimated using a t-test for unpaired observations. A P<0.05 was considered to indicate a statistically significant difference.

Results

Preparation of oligochitosan. The components of the oligochitosan sample were determined by HPLC (Fig. 1). The main peak corresponded to that of standard chitohexaose (Fig. 2), which indicated that the main component of the prepared oligochitosan sample was chitohexaose.

The effects of oligochitosan on RAW264.7 cell viability. A MTT assay was used to determine the viability of the RAW264.7 cells, following treatment with different concentrations of oligochitosan (0, 50, 100 and 500 μ g/ml). No cellular toxicity was observed in the cells treated with 50-500 μ g/ml oligochitosan, for 24 h (Fig. 3). These results suggest that nonspecific cytotoxicity could be excluded as a factor from the results of the present study.

Oligochitosan has inhibitory effects on LPS-induced proinflammatory cytokine production in RAW264.7 cells. An ELISA was used to determine the inhibitory effects of oligochitosan on LPS-induced production of TNF- α and IL-1 β in RAW264.7 cells. Following a 24 h incubation with medium alone, the expression levels of TNF- α (n=3, P<0.01, Fig.4B) and IL-1 β (n=3, P <0.01, Fig.4A) were low. However, the expression levels of TNF- α (n=3; 50, 100 and 500 μ g/ml : all P<0.01; Fig.4B) and IL-1 β (n=3; 50, 100 and 500 μ g/ml: P<0.05, P<0.01 and P<0.01, respectively; Fig.4A) in the supernatant of LPS-stimulated RAW264.7 cells, were significantly increased, as compared with the control group. Different concentrations

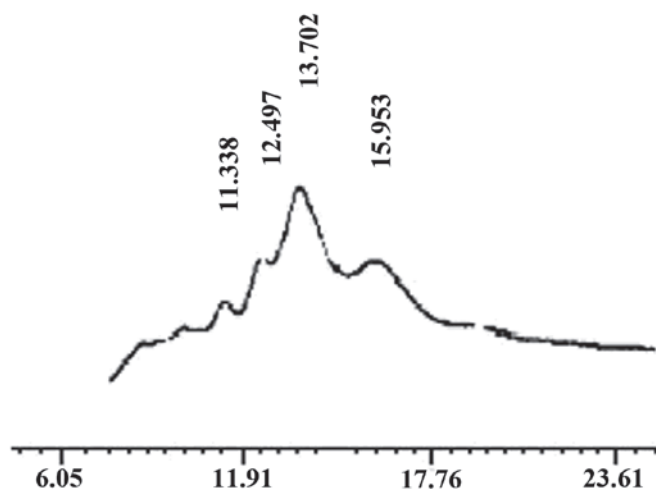


Figure 1. High performance liquid chromatography profile of oligochitosan sample. The retention time of the main peak was 13.702 min.

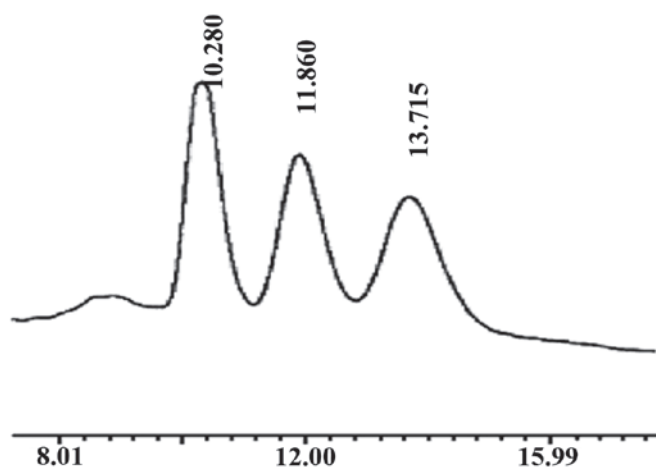


Figure 2. High performance liquid chromatography profile of standard chitotetraose, chitopentaose and chitohexaose. The retention time of chitotetraose, chitopentaose and chitohexaose was 10.280, 11.860 and 13.715 min, respectively.

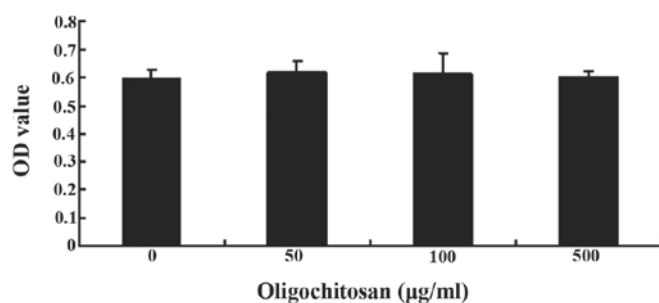


Figure 3. Effects of oligochitosan on RAW264.7 murine macrophage cell viability. The cell viability was determined by MTT assay, following treatment with different concentrations of oligochitosan (0, 50, 100 and 500 $\mu\text{g/ml}$). The data represent the means \pm standard deviation; $n=3$.

of oligochitosan (50, 100 and 500 $\mu\text{g/ml}$) significantly lowered the expression levels of TNF- α and IL-1 β , as compared with the LPS group (Fig. 4).

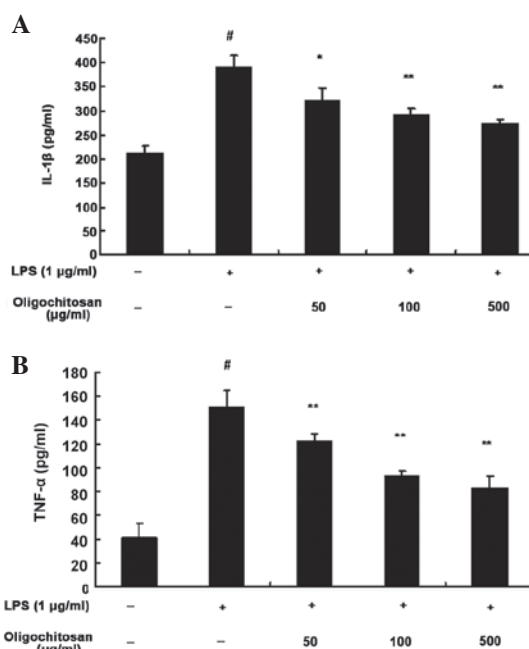


Figure 4. Effects of oligochitosan on lipopolysaccharide (LPS)-induced pro-inflammatory cytokine production in RAW264.7 murine macrophage cells. The culture supernatants were collected for detecting the level of (A) interleukin (IL)-1 β and (B) tumor necrosis factor (TNF)- α . The concentration of the cytokines were determined using a standard curve derived from known amounts of TNF- α and IL-1 β . The data represent the means \pm standard deviation; $n=3$; ^{*} $P<0.05$, ^{**} $P<0.01$, vs. the LPS group; [#] $P<0.01$, vs. the RPMI-1640 control group.

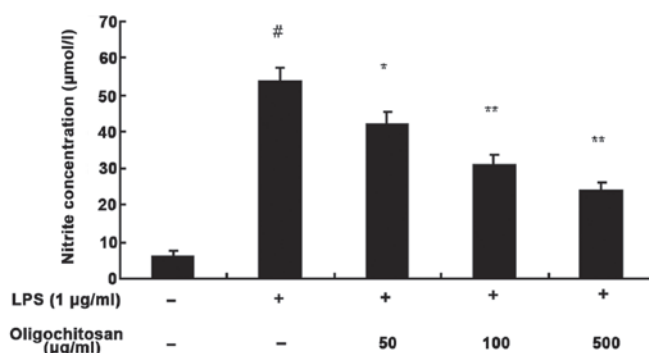


Figure 5. Effects of oligochitosan on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 murine macrophage cells. The concentration of nitrite in the culture medium was measured using the Griess method, to assess endogenous synthesis of NO upon stimulation of the macrophages. The data represent the means \pm standard deviation; $n=3$; ^{*} $P<0.05$, ^{**} $P<0.01$, vs. the LPS group; [#] $P<0.01$, vs. the RPMI-1640 control group.

Oligochitosan has inhibitory effects on LPS-induced NO production in RAW264.7 cells. The concentration of nitrite in the culture medium was measured using the Griess method, to assess endogenous synthesis of NO upon LPS-stimulation of the RAW264.7 macrophages. Following a 24 h incubation with medium alone, the production of NO was low; however, the production of NO in the culture medium of LPS-stimulated RAW264.7 cells was significantly increased, as compared with the control group ($n=3$, $P<0.01$, Fig. 5). Different concentrations of oligochitosan (50, 100 and 500 $\mu\text{g/ml}$) significantly reduced the production of NO, as compared with the LPS group ($n=3$;

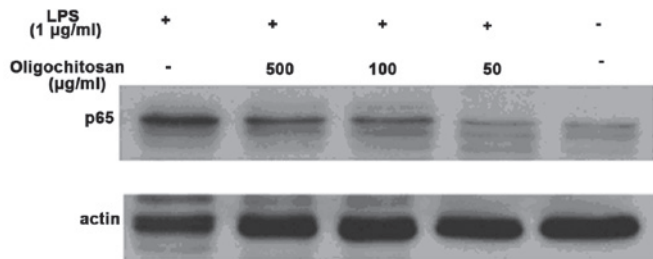


Figure 6 The effects of oligochitosan on lipopolysaccharide (LPS)-induced nuclear factor (NF)- κ B protein expression levels in RAW264.7 murine macrophage cells. The RAW264.7 cells (1×10^6) were treated with $1 \mu\text{g/ml}$ LPS and different concentrations of oligochitosan (0, 50, 100, $500 \mu\text{g/ml}$) for 24 h, the protein expression levels of NF- κ B (p65) were determined by western blot analysis.

50, 100 and $500 \mu\text{g/ml}$: $P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively) (Fig.5).

The effects of oligochitosan on NF- κ B activation in LPS-stimulated RAW264.7 cells. The NF- κ B signaling pathway is involved in the production of TNF- α , IL-1 β and NO in LPS-stimulated macrophages. To determine the mechanism of the anti-inflammatory effects of oligochitosan in macrophages, the effects of oligochitosan on the activation of NF- κ B activation in LPS-stimulated RAW264.7 cells was examined (Fig. 6). Western blot analysis showed that the protein expression levels of NF- κ B (p65) in the cells cultured in the absence of LPS, were very low. However, the protein expression levels of NF- κ B (p65) in the culture medium of LPS-stimulated RAW264.7 cells were significantly increased, as compared with the control group. Different concentrations of oligochitosan (50, 100 and $500 \mu\text{g/mL}$) significantly downregulated the expression levels of NF- κ B (p65), as compared with the LPS group.

Discussion

High molecular weight chitosan (300 kDa) with a high degree of N-acetylation (>30%) may activate macrophages, inhibit bacterial growth, induce apoptosis of tumor cells and promote wound healing (12,13). Chitosan has been used in various pharmaceutical products, however, its poor solubility in neutral aqueous solutions has restricted its technological application (12,13). In the present study, to facilitate its utilization, oligochitosan, with a lower molecular weight (1 kDa) and degree of N-acetylation (<15%), was prepared by enzymatic hydrolysis. The biochemical composition was analyzed using HPLC and the results showed that the main component of the oligochitosan was chitohexaose, which has good water solubility and low viscosity. In the present study, oligochitosan was shown to be capable of regulating the function of LPS-stimulated RAW264.7 cells, which were used as an *in vitro* macrophage model system. The results of the present study suggest that oligochitosan may inhibit the production of active molecules, including NO, IL-1 β and TNF- α in LPS-stimulated RAW264.7 cells through the NF- κ B pathway. This may provide an explanation in regards to the mechanism of the anti-inflammatory effects of oligochitosan.

Macrophages have an important role in the initiation, maintenance and resolution of inflammation. Activated macrophages

can directly counteract harmful pathogenic stimuli. In response to LPS, macrophages secrete numerous pro-inflammatory cytokines, including IL-1 β and TNF- α , in order to mediate the inflammatory response (14). Overproduction of these pro-inflammatory mediators results in excessive inflammation (15); therefore, inhibition of the release of pro-inflammatory mediators may be beneficial in attenuating the inflammatory response. During the process of inflammation and infection, activated macrophages are attracted to the site of inflammation and markedly increase the production of NO around the wounded tissue (16). NO is a highly reactive oxidant that is associated with numerous biological processes, including the regulation of inflammation, and is thought to be a major destructive factor in the wound healing process (17). Therefore, inhibition of the release of NO may be beneficial in attenuation of the inflammatory response.

NF- κ B is a major transcription factor that widely regulates the expression of genes responsible for both a series of immune responses and inflammatory reactions. It has been proven that NF- κ B has an important role in the inflammatory response (18). In the present study, western blot analysis showed that oligochitosan significantly downregulated the protein expression levels of NF- κ B in LPS-stimulated RAW264.7 macrophages. These results suggest that oligochitosan may have a role in suppressing the NF- κ B signaling pathway in the inflammatory response-mediated production of NO, IL-1 β and TNF- α .

In conclusion, the present study demonstrated that oligochitosan significantly inhibited the overproduction of NO, IL-1 β and TNF- α in LPS-stimulated RAW264.7 macrophages. This inhibition by oligochitosan may be mediated through the downregulation of NF- κ B. Due to its low toxicity and cost, oligochitosan may be a good macrophage targeting, anti-inflammatory drug.

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