

Chitooligosaccharides Attenuate Lipopolysaccharide-induced Inflammation and Apoptosis of Intestinal Epithelial Cells: Possible Involvement of TLR4/NF- κ B Pathway

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ABSTRACT

Marine-derived chitooligosaccharides (CHOS) are a new class of anti-inflammatory natural products characterized by its nontoxicity, low-cost and small-molecule. But whether CHOS exert a protective action against inflammatory bowel diseases (IBDs) is not yet fully understood. This paper studied the effect of CHOS on the inflammation and apoptosis of intestinal epithelial cells (IECs) using lipopolysaccharides (LPS)-stimulated Caco-2 cells as an *in vitro* model of IBD. Caco-2 cells were pre-incubated with various concentrations of CHOS (0.25, 0.5 and 1.0 mg/ml) for 2 h prior to being co-stimulated or not with LPS at a concentration of 1 μ g/mL for 48h. Cell apoptosis was measured with Annexin V-FITC and propidium iodide (PI) assay by flow cytometry. The levels of proinflammatory mediators including tumor necrosis factor (TNF)- α , interleukin-8 and prostaglandin (PG) E₂ were analyzed using Enzyme-linked Immunosorbent Assay. And the cell expression of toll-like receptor 4 (TLR4), nuclear factor kappa B (NF- κ B), caspase-3, bcl-2, and cyclooxygenase (COX)-2 was examined by western blot. We observed that CHOS significantly and concentration-dependently inhibited the LPS-induced inflammatory response and apoptosis of IECs, which was evidenced by the reduction in the release of proinflammatory mediators TNF- α , PGE₂, the expression of COX-2, apoptotic (Annexin V⁺/PI) cell populations, the pro-apoptotic caspase-3 expression, and the enhancement of the expression level of anti-apoptotic bcl-2. And CHOS treatment significantly reduced the protein expressions of TLR4 and NF- κ B in LPS-stimulated Caco-2 cells. Thus, the present study suggests the potential medical use of CHOS in the control of IBDs, which may be due to the downregulation of TLR4/NF- κ B pathway.

Key words: Chitooligosaccharides, Inflammatory bowel diseases, Lipopolysaccharides, Intestinal epithelial cells.

INTRODUCTION

Inflammatory bowel diseases (IBDs), mainly including Crohn's disease and ulcerative colitis, are defined as chronic and relapsing intestinal inflammatory conditions that are caused by the perturbation of intestinal homeostasis between commensal bacteria and mucosal immunity.¹ Murine and human studies^{2,3} have demonstrated that IBDs are characterized by an inflammation injury in response to intestinal microbiota, which develops in genetically susceptible individuals with a defect in intestinal epithelial barrier function. The overlying epithelial mucous

surface was found severely impaired in IBD patients.^{2,3} Essentially, the intestinal epithelial cell (IEC) is a major player in the maintenance of physical barrier integrity in intestinal mucosa. Under IBD conditions, IECs have the potential to trigger the over-production of cytokines (including tumor necrosis factor (TNF)- α , interleukin (IL) 1 β , IL-6 and IL-8) and other inflammatory mediators (for example, prostaglandin (PG) E₂) in response to the differential pathogenesis such as bacteria, pathogens and other antigens, thus leading to an

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uncontrolled intestinal inflammation.⁴ In addition, since the significantly increased apoptosis of IECs can also be found in mucosal tissue from inflamed intestine, excessive apoptosis has long been proposed as an important disease effector mechanism during IBDs.⁵

IEC impairment and concomitant barrier loss in the intestine increase the direct exposure of intestinal epithelium to large amounts of luminal bacteria. Mounting evidence^{6,7} suggested a pivotal role of the enteric bacterial flora in the induction and progression of intestinal inflammation in the course of IBD. In patients with active intestinal inflammation, adherent Gram-negative bacteria (for example, *Escherichia coli*) were found to accumulate at the inflamed tissue sites and potentiate the pathology by translocation *via* microlesions and ulcerations.⁶ Especially, the high numbers of *Escherichia coli* in the inflamed tissues point towards the important role of lipopolysaccharides (LPS), a glycolipid component of all pathogenic gram-negative bacteria outer membrane, in the induction and aggravation of intestinal inflammatory response of IBD.⁶ Known as one of the most potent activators of the inflammatory system in intestinal epithelium, LPS induce the inflammatory signal transduction through the cell surface Toll-like receptor 4 (TLR4), which specifically binds the lipid A portion of LPS.⁸ Recognition of LPS by TLR4 leads to cellular activation and pro-inflammatory gene expression, the latter of which is thought to be responsible for the increase in apoptosis and damage in IECs observed in IBD.⁸ Indeed, LPS was believed represent a robust, rapid and consistent stimulus for inducing pathological IEC apoptosis and inflammation,^{5,9} thus would exacerbate the dysfunction of the epithelial barrier and further weaken the frontline defence against pathogenic bacteria. It is remarkable that IECs rather than macrophages or other lamina propria populations are found as the predominant cells expressing TLR4 in human intestinal mucosa.¹⁰ And the same study demonstrated that TLR4 expression in IECs was strongly upregulated in inflamed mucosa of IBD patients. Whereas, low level TLR4 expression in epithelial cells is believed to limit dysregulated LPS signaling and account for normal mucosal hyporesponsiveness to enteric bacteria.¹⁰

Corticosteroids drugs, for example prednisone, have long been the mainstay of currently used anti-inflammatory treatment for IBD that can significantly reduce the symptoms of the disease and help to maintain its remission. However, prolonged use of these drugs would lead to undesirable side effects. Thus, it is urgent to continue

developing new IBD therapeutic agents with greater safety.² Remarkably, marine- derived chitoooligosaccharides (CHOS) are a new class of anti-inflammatory natural products characterized by its nontoxicity, low-cost and small-molecule. As the degraded oligomers prepared from chitin (which is distributed in the shell of crustaceans and marine zoo-plankton thus believed as the most abundant biopolymer in marine ecosystems) or its N-deacetylated derivative chitosan, CHOS attracted more interest than their starting materials because of higher water-soluble characteristics, nontoxicity and enhanced functional properties.¹¹ *In vivo* administration of CHOS significantly decreased the levels of inflammatory cytokines in human serum.¹² Animal studies also showed that and CHOS were suitable to treat acute inflammation cases following carrageenan-induced paw edema method¹³ and chronic inflammation- associated osteoporosis.¹¹ *In vitro* studies have also reported that CHOS inhibited the production and expression of proinflammatory mediators in TNF α -induced endothelial cells,¹⁴ LPS-stimulated microglia¹⁵ and macrophage cells.¹⁶ Nevertheless, the effect of CHOS on IECs challenged with LPS in the setting of IBD remains unclear. In the present study, using LPS-stimulated Caco-2, a transformed human intestinal cell line, as an *in vitro* inflammatory model of the human intestinal epithelium, we evaluated the effect of CHOS and investigated its possible mechanisms.

MATERIALS AND METHODS

Chemical

CHOS (degree of deacetylation of 90% and average molecular weight of 1500 Da) provided by the company of Qingdao BZ Oligo Biotech Co. Ltd, China, were prepared from chitosan isolated from crustaceans shell through enzymatic hydrolysis. The purity of CHOS is determined by HPLC and has achieved 90% at least.

Cell culture and treatments

Caco-2 transformed human intestinal cells (American Type Culture Collection, USA) were grown at 37°C in DMEM (Invitrogen, UK) with 20% fetal bovine serum, 4.5 g/L glucose and 100 units/ml antibiotics (penicillin/ streptomycin) under a humidified, 5% CO₂ environment. Cells were harvested at 60-70% confluency following treatments. And the serum concentration was reduced to 1% for overnight before treatments and also during the treatments.

Cells were inoculated into 24-well plates, and then divided into 5 groups of 4 well each: the control group, the LPS group, the low-, middle- and high-concentration CHOS groups. Cells in the LPS group were stimulated with LPS (from *Escherichia coli* 026:B6, Sigma-Aldrich, USA) at a concentration of 1 µg/mL for 48 h. And cells in CHOS groups were pre-incubated with various concentrations of CHOS (0.25, 0.5 and 1.0 mg/ml) for 2 h prior to being co-stimulated with 1µg/mL LPS for 48 h. As a negative control, a parallel group received phosphate-buffered saline (PBS) alone. The experiment was repeated five times.

Enzyme-linked immunosorbent assay (ELISA) for TNF- α , IL-8 and PGE₂

Concentrations of TNF- α and IL-8 in cell culture supernatants were measured using ELISA kits (eBioscience, USA). And the levels of PGE₂ released from cells were detected by specific ELISA kit (R&D Systems, Inc., USA) according to the instructions of the manufacturer.

Cell apoptosis assay

In order to evaluate the cell apoptosis, Caco-2 cells were collected for Annexin V-FITC and propidium iodide (PI) assay by flow cytometry. Cells were washed with cold PBS and re-suspended in 100 µl binding buffer. Then they were stained with Annexin V-FITC Apoptosis Detection Kit (Bestbio Co, Shanghai, China) and detected according the manufacture's protocol.

Detection of cell viability

MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method was used to detect cell viability. After adding 0.5 mg/L MTT, Caco-2 cells were sequentially incubated for 4 h. The supernatant was then removed, and dimethylsulfoxide was added with shaking for 5 min, until the crystals were fully dissolved. The resulting absorbance was measured at 570 nm.

Western blot

Proteins in cells were separated by SDS-PAGE and proteins were transferred onto a polyvinylidene difluoride membrane through electroblotting. Membrane was then blocked overnight and incubated for 2 hours with a 1:1000 dilution of rabbit polyclonal Caspase-3 antibody (Cell Signaling, USA) or bcl-2 antibody (Abcam, UK), a goat polyclonal Cyclooxygenase(COX)-2 (Santa Cruz, CA, USA), a mouse monoclonal TLR4 antibody(Santa Cruz, CA, USA), a rabbit monoclonal NF- κ B p65

antibody (ABGENT, USA) or β -actin antibody (Santa Cruz, CA, USA). After incubation with the secondary antibody, proteins were detected with an ECL chemiluminescence detection kit (Advansta, USA), and scanned. The amount of protein expression was corrected by the amount of β -actin in the same sample.

Statistical analysis

Data are reported as mean \pm S.D, and analyzed by One-way analysis of variance (ANOVA) followed by a Bonferroni test using SPSS 15.0 software. Differences were considered as statistically significant at P<0.05.

RESULTS

CHOS inhibited LPS-induced inflammatory response in IECs

The effect of CHOS on LPS-induced inflammation in Caco-2 cells was evaluated by measuring the secretion levels of pro-inflammatory cytokines TNF- α and IL-8 along with a proinflammatory mediator PGE₂, and the expression of COX-2 in IECs. TNF- α ¹⁷ and IL-8¹⁸ were known as key pro-inflammatory immunological mediators that contribute to tissue injury in the pathogenesis of IBD. Therein, the reduction of TNF- α in patients through anti-TNF- α therapy has been closely associated with the amelioration of IBD.¹⁷ Similar to cytokines, PGE₂ as a crucial mediator of inflammatory injury has also been shown to contribute to the development of IBD.¹⁹ As shown in Figure 1, LPS stimulation significantly induced the release of TNF- α , IL-8 and PGE₂ in Caco-2 cells (P<0.01), compared to the control cells. And CHOS treatment significantly and concentration-dependently reduced the LPS-induced secretion of TNF- α and PGE₂, indicating that CHOS may inhibit the inflammatory response in IECs (P<0.05; P<0.01, vs treatment with LPS). However, we observed no significant effect of CHOS treatment on IL-8 secretion of LPS-stimulated Caco-2 cells.

COX-2 accounts for the PGE₂ production in IECs.²⁰ The anti-inflammatory effects of CHOS have been reported related with their inhibition of COX-2 expression.^{11,15-16} And we found the protein expression of COX-2 in IECs were significantly reduced in middle and high concentration CHOS groups, compared with LPS group (P<0.05; P<0.01, Figure 1).

CHOS diminished LPS-induced apoptosis of IECs

Apoptosis of Caco-2 IEC cells was detected by Annexin V-FITC/PI assay. Increased levels of apoptosis were demonstrated upon exposure to LPS (P<0.01, vs the control without treatment, Figure 2a). Moreover,

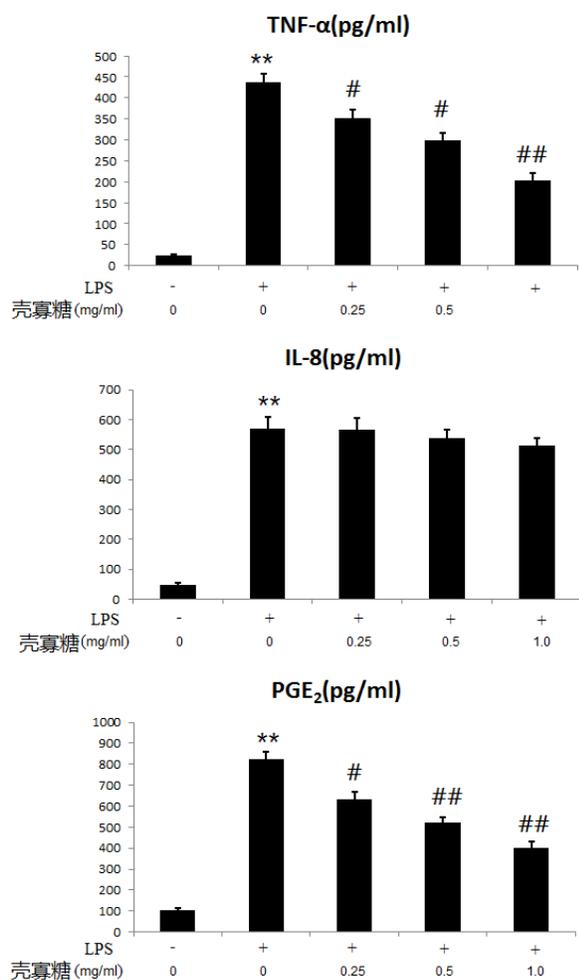


Figure 1: Effect of CHOS on LPS-induced secretion of TNF- α , IL-8, PGE₂, and COX-2 protein expression in intestinal cells. Concentrations of TNF- α and IL-8 in cell culture supernatants were measured by ELISA. Protein expression levels of COX-2 were determined by Western Blot.

The data represent the mean \pm S.D. of five separate experiments. ** $P < 0.01$, vs control cells; # $P < 0.05$, ## $P < 0.01$, vs cells treated with LPS but without CHOS. coexposure of cells to CHOS (especially at the concentrations of 0.5 and 1.0 mg/ml) and LPS resulted in the reduction in apoptosis ($P < 0.05$, vs treatment with LPS only, Figure 2a). And CHOS diminished LPS-induced apoptosis accompanied by the reduced expression levels of pro-apoptotic protein caspase-3 and the elevated levels of anti-apoptotic bcl-2 (Figure 2b).

Cytotoxicity of CHOS in Caco-2 cells

To exclude the possibility that the anti-inflammatory and anti-apoptosis activities of CHOS were due to cytotoxicity, we then determined CHOS' effect on Caco-2 cell viability using the MTT assay. As shown in Figure 3a, the cell viability was not affected by treatment with CHOS and/or LPS, suggesting that the inhibitory effect of CHOS on LPS-induced inflammation and apoptosis in IECs did not result from a cytotoxic action.

Potential involvement of TLR4/NF- κ B pathway in the preventive effect of CHOS on LPS-induced IEC inflammation and apoptosis

Based on the above finding about the preventive effect of CHOS on LPS-induced IEC inflammation and apoptosis, we further explored the potential molecular mechanism. It was believed that IECs require TLR4 to respond to LPS.¹⁰ Importantly, previous study²⁰ has demonstrated that TLR4/nuclear factor kappa B (NF- κ B) signal plays key role in regulating the expression of COX-2 in IECs. In addition, TLR4-NF- κ B pathway was also reported to be involved in the apoptosis process.^{21,22} So the effect of CHOS on TLR4 and NF- κ B expression of IECs was focused on (Figure 3b). In presence of CHOS, the protein expressions of TLR4 and NF- κ B in LPS-stimulated Caco-2 cells were reduced than those of the only LPS-treated group ($P < 0.05$; $P < 0.01$), these effects of CHOS being presented in a concentration-dependent manner.

DISCUSSION

At present, many people with IBD turn to alternative medicine including natural product-based remedies or the functional food, because most of current treatments are inadequate and drug-induced severe side effects occur. As a kind of marine natural products derived from the exoskeletons of crustaceans, CHOS have been proved to be potential in the treatment of many inflammatory disorders, such as asthma and osteoporosis.^{11,23} Although relatively little is known about the exact effects of CHOS on IBD, chitin, which is the starting material for CHOS production, has been reported to have the anti-inflammatory effect via suppressing NF- κ B activation in dextran sulfate sodium- induced IBD mice model,²⁴ thus has a potency as a new functional food for IBD patients. In the present study, using LPS-stimulated Caco-2 cells as an *in vitro* model of IBD, we observed that CHOS significantly and concentration-dependently inhibited the LPS-induced inflammatory response and apoptosis of IECs, suggesting the potential medical use of CHOS in the control of IBD. In fact, as highly water-soluble, absorbable and non-toxic derivatives of chitin, CHOS were believed to have enhanced functional properties as bioactive molecules, thus more applicable for the clinical practice.¹¹ Accordingly, our data from MTT assay also showed CHOS cause no direct cytotoxicity on IECs.

It has been found that CHOS significantly inhibited the adhesion of certain enteropathogenic *Escherichia coli* strains to human colon adenocarcinoma epithelial (HT29) cells in tissue culture,²⁵ indicating the protec-

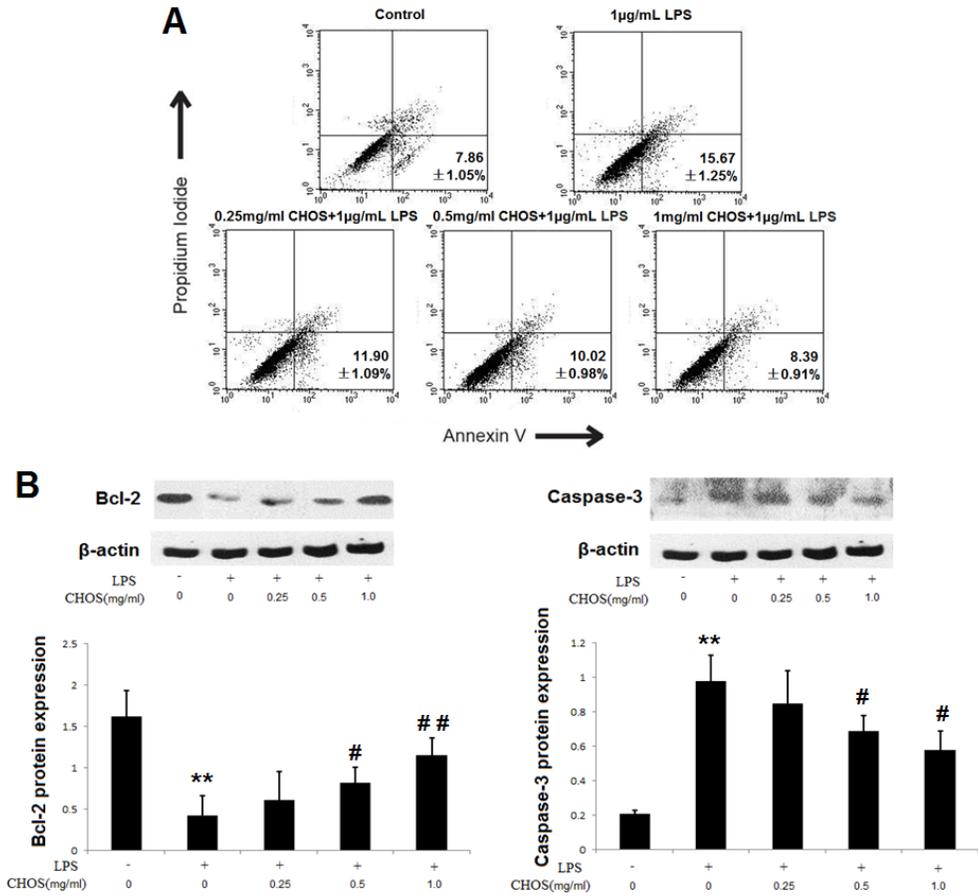


Figure 2: Effect of CHOS on LPS-induced apoptosis of intestinal cells. (A) Proportion of cells undergoing apoptosis was determined by FACS analysis of cells stained with Annexin V and propidium iodide (PI). Representative dot-plots showing apoptotic (Annexin V+ PI-) cell populations. (B) Protein expression levels of the anti-apoptotic protein bcl-2 and the pro-apoptotic caspase-3 were determined by Western Blot.

The data represent the mean ± S.D. of five separate experiments. **P < 0.01, vs control cells; # P < 0.05, ## P < 0.01, vs cells treated with LPS but without CHOS.

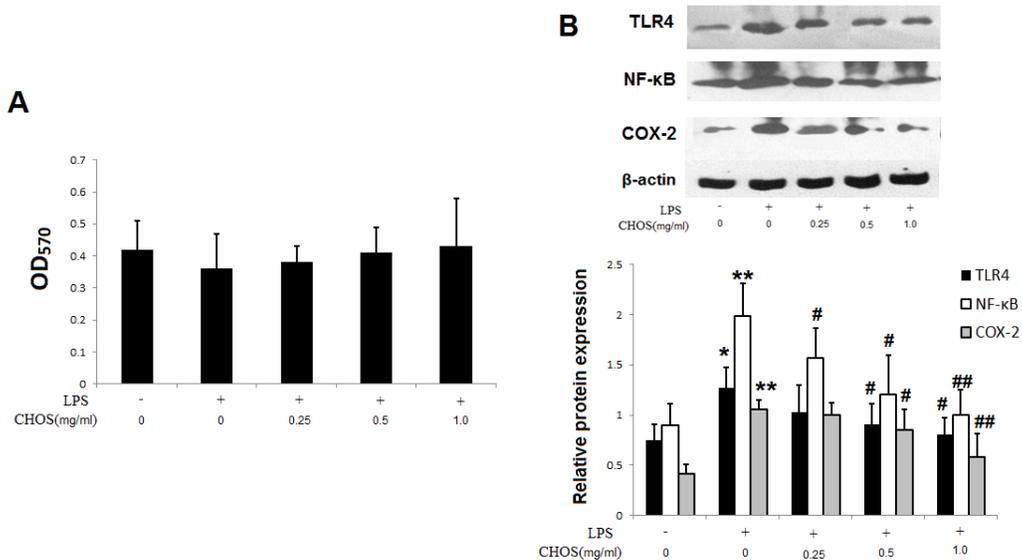


Figure 3: Effect of CHOS on cell viability, TLR4 and NF-κB protein expression in LPS-induced Caco-2 cells. (A) Caco-2 cell viability was studied using the MTT assay. (B) Protein expression levels of TLR4 and NF-κB were determined by Western Blot.

The data represent the mean ± S.D. of five separate experiments. * P < 0.05, ** P < 0.01, vs control cells; # P < 0.05, ## P < 0.01, vs cells treated with LPS but without CHOS.

tive action of CHOS in intestinal injury with pathogens. Consistent with this previous study, we found that exposure of IECs to LPS potentially initiated intestinal inflammation and cell apoptosis. Of importance, these LPS-induced IECs damages were effectively suppressed by CHOS treatment, evidenced by the decreased release of TNF- α , PGE₂, apoptotic (Annexin V⁺/PI-) cell populations, pro-apoptotic caspase-3 expression, and the enhancement of the expression level of anti-apoptotic bcl-2.

An important downstream-target of TLR4/NF- κ B signaling is the inducible COX-2, a key enzyme expressed during inflammation that is responsible for the production of mediators (such as PGE₂) involved in inflammation. The activation of COX-2 plays an important role in initiation and progression of IBD.²⁰ In addition, TLR4/NF- κ B signaling pathway is also considered to be a major signal transduction pathway involved in apoptosis.^{21, 26-27} It has been found that LPS caused IEC apoptosis through a TLR4-dependent mechanism.²⁶ TLR4 activation induced by LPS triggered the downstream signaling and modulates the expression of apoptosis-related protein such as bcl-2 and caspase-3.^{21-22, 27} At present, TLR4/NF- κ B pathway has been believed as a target for IBD control.²⁸ The results of this study demonstrated that CHOS treatment significantly down-regulated the enhanced expression of TLR4 and NF- κ B induced by LPS, suggesting the possible involvement of this signal pathway in the protective role of CHOS against IBD.

Interestingly, although IL-8 was also a pro-inflammatory cytokine involved in IBD,¹⁸ CHOS treatment appeared to have no obvious effect on LPS-induced IL-8 production of IECs in this study. The possible explanation

is that, compared with that of TNF- α or PGE₂, the transcriptional control of human IL-8 gene was more complicated. The indispensable role of NF- κ B binding for inducible IL-8 gene expression has been shown in pulmonary epithelial cells by the complete blockade of inducible promoter activity when NF- κ B contact sites were mutated.²⁹ Besides, the transcription factors NF-IL-6, AP-1 and so on are all necessary for IL-8 expression.³⁰ Therefore, it can be speculated that CHOS could exert some different effects on other signal pathways controlling IL-8 expression in Caco-2 cells.

CONCLUSION

In the present study, we observed that CHOS as a marine-derived anti-inflammatory natural product significantly and concentration-dependently inhibited the LPS-induced inflammatory response and apoptosis of IECs, which was evidenced by the reduction in the release of proinflammatory mediators TNF- α , PGE₂, the expression of COX-2, apoptotic (Annexin V⁺/PI-) cell populations, the pro-apoptotic caspase-3 expression, and the enhancement of the expression level of anti-apoptotic bcl-2. And CHOS treatment significantly reduced the protein expressions of TLR4 and NF- κ B in LPS-stimulated Caco-2 cells. Thus, the present study suggests the potential medical use of CHOS in the control of IBDs, which may be due to the downregulation of TLR4/NF- κ B pathway.

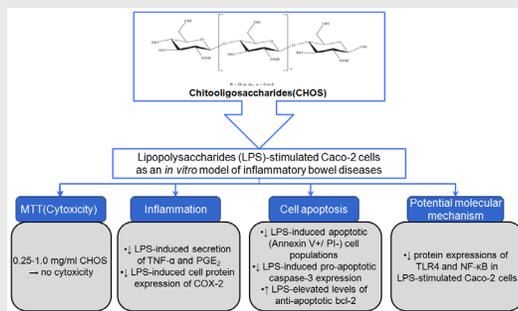
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SUMMARY

- Marine-derived chitooligosaccharides significantly and concentration-dependently inhibited the LPS-induced inflammatory response and apoptosis of intestinal epithelial cells, suggesting the potential medical use of chitooligosaccharides in the control of inflammatory bowel diseases.
- Chitooligosaccharides treatment significantly reduced the protein expressions of TLR4 and NF- κ B in LPS-stimulated intestinal epithelial cells, suggesting the possible involvement of TLR4/NF- κ B pathway.
- Chitooligosaccharides showed no significant cytotoxic effect.

Pictorial Abstract



About Authors



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ABBREVIATION USED

CHOS: Chitooligosaccharides; IBDs: Inflammatory bowel diseases; IECs: Intestinal epithelial cells; LPS : Lipopolysaccharides; PI: Propidium iodide; TNF- α : tumor necrosis factor alpha; IL: Interleukin; PG: Prostaglandin; TLR4: Toll-like receptor 4; NF- κ B: Nuclear factor kappa B; COX: Cyclooxygenase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AnnexinV-FITC: Annexin V conjugated with fluorescein isothiocyanate; ELISA: Enzyme-linked immunosorbent assay.

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