Chitosan Nanoparticles Attenuate Intestinal Damage and Inflammatory Responses in LPS-challenged Weaned Piglets via Inhibition of the NF- κ B Pathway

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Abstract

Background Chitosan nanoparticles (CNP), an extensively administered oral drug carrier, were found to have anti-inflammatory properties. In this study, the effects of CNP on lipopolysaccharide (LPS)-induced intestinal damage in weaned piglets and the related mechanisms were investigated. A total of 24 piglets (21 ± 2 days, Duroc $\times$ Landrace $\times$ Yorkshire, initial mass: 8.58 ± 0.59 kg) were randomly assigned into four groups: control, LPS, CNP, and CNP+LPS. The control and LPS groups were fed a corn-soybean meal-based control diet, whereas the CNP and CNP+LPS groups were fed a control diet supplemented with 400 mg/kg CNP. After 28 days of feeding, the LPS and CNP+LPS groups were injected with LPS at 100 μ g/kg, meanwhile the control and CNP groups were injected with equal volumes of sterile saline solution. After 4 hours from the LPS challenge, all pigs were sacrificed for analysis.

Results: CNP ameliorated the structural and developmental damages to the small intestine caused by LPS treatment. LPS induced invasion of neutrophils into the intestinal mucosa of the jejunum ($P < 0.01$), which resulted in marked increased secretion of marks of inflammation ($p < 0.01$) compared with the control group. Whereas, CNP administration obviously inhibited LPS induced neutrophils invasion ($P < 0.01$) and marks of inflammation, such as interleukin-8 ($p < 0.05$), intercellular adhesion molecule-1 ($p < 0.05$), and interferon- γ ($p = 0.7694$), secretion in jejunum mucosa compared with LPS group. Moreover, CNP was shown to reduce cytoplasmic I κ B- α degradation accompanied by reduced nuclear NF- κ B p65 levels in LPS-inflamed piglets.

Conclusion: CNP attenuates intestinal damage and inflammatory responses in LPS-challenged weaned piglets by impairing the NF- κ B signaling pathway. These findings provide a theoretical basis for the application of CNP as a functional feed additive in piglets.

Introduction

An optimally functioning gastrointestinal tract (GIT) is important for the overall metabolism, physiology, disease status, and performance of animals. As the main site of transport and absorb nutrients, the GIT epithelium and underlying lamina propria are continually exposed to a harsh luminal environment, which includes massive amounts of toxins, antigens, and pathogens. Thus, the GIT has developed a barrier to prevent overwhelming immune activation and potentially sepsis, which is critical for host survival ^[1-2]. In weaning piglets, the abrupt change in diet from milk to cereal-based solid feed leads to not only marked structural and functional changes to the small intestine but also contributes to an intestinal inflammatory status that in turn compromises villous crypt architecture, GIT barrier function, and causes disruption of the microbiota ^[3-5]. Weaned piglets commonly suffer from gastroenteritis caused by enterotoxigenic *Escherichia coli*. Bacterial infections in weaned piglets are responsible for considerable economic losses and reduced animal welfare in the pork industry ^[6].

Lipopolysaccharide (LPS), a component of the membrane of gram-negative bacteria, is one of the most effective stimulators of the immune system and has been widely applied in pigs as an experimental

model for bacterial infection^[7]. Toll-like receptor 4 (TLR4) is the major recognition receptor for LPS which interacts with the extracellular co-receptors, CD14 and MD2^[8-9]. After stimulation, two known intracellular signaling pathways are activated^[10]: the MyD88-dependent and - independent pathways, which both end in the translocation of nuclear factor κ B (NF- κ B) into the nucleus, inducing transcription of multiple genes. This transcription results in the release of cytokines in a characteristic chronology^[11]

Chitosan is a natural polysaccharide and is considered the largest biomaterial after cellulose in terms of its application and distribution. It is produced from chitin, the most abundant natural amino polysaccharide obtained from the exoskeleton of crustaceans such as shrimps, lobsters, and crabs. Chitosan has an abundance of hydroxyl ($-OH$) and amine ($-NH_2$) functional groups, which can be employed to react with cross-linking agents for *in situ* chemical cross-linking. Because of their unique chemical structures with desired biocompatibility and biodegradability, chitosan-based biomaterials have attracted a significant attention in the medical, food, and agricultural sectors^[12]. Due to their small size and high zeta potential, chitosan-based particulate systems, particularly chitosan nanoparticles (CNP), exhibit higher oral absorption than chitosan, and have revealed great success as carriers for oral delivery of peptides, proteins, and nucleotides^[13]. Our recent research found that CNP could protect Caco-2 cells, a model of human enterocyte, from LPS-induced cell membrane damage^[14], suggesting that CNP may relieve weaning-associated intestinal inflammation in various animal species. Therefore, in this research, LPS was applied on weaned piglets to investigate the protective effects of feeding CNP on the intestinal damage and the mechanism underline.

Materials And Methods

The experimental design and procedures in this study were reviewed and approved by the Animal Ethics Committee of Zhejiang A&F University, Hangzhou, Zhejiang, China

Materials

CNPs with a mean particle size of approximately 50 nm (Fig. 1) were prepared in our laboratory using cationic chitosan (Chitosan Company of Pan'an, Zhejiang, China) with an average molecular weight of 220 kDa and a degree of deacetylation of 95%. LPS (*Escherichia coli* serotype O111:B4, Sigma Chemical, USA) was dissolved in sterile saline (0.9%).

Animals, housing, and treatments

A total of 24 piglets (21 ± 2 days, Duroc $\times$ Landrace $\times$ Yorkshire, initial mass: 8.58 ± 0.59 kg) were assigned to two dietary treatment groups, taking into consideration of principle of equal numbers of males and females and similar body weight in all groups. The piglets were acclimatized for 1 week before the study. The dietary treatments included a corn-soybean meal-based control diet^[15] and a control diet

supplemented with 400 mg/kg CNP. On day 28 of the feeding trial, half of the piglets (male: female = 1:1) in each treatment group were injected via intraperitoneal injection (*i.p.*) with LPS at 100 µg/kg. The other piglets were injected with a sterile saline solution at an equal volume. Each group contained six piglets (male: female = 1:1) and each piglet was regarded as repeat. No antibiotics were administered to the piglets prior to or during the experimental period. The dosage of LPS was selected based on the results of previous studies ^[16].

Sample collection

After 4 hours from the LPS challenge, the pigs were sacrificed by exsanguination. Samples of the duodenum, jejunum, and ileum (about 2 cm) were cut out and placed into prepared 4% formaldehyde for subsequent hematoxylin and eosin (H&E) staining, immunohistochemistry, and immunofluorescence staining. Jejunum mucosa was collected for enzyme linked immunosorbent assay (ELISA) and western blot analysis.

Intestinal morphology

Intestinal tissues were embedded in paraffin. Sections of 5µm thickness were deparaffinized in xylene, rehydrated in graded solutions of ethanol, and then stained with hematoxylin and eosin. Images were acquired using a Zeiss Axiovert microscope (Zeiss, Germany), and villus height and crypt depth were measured using Image Pro software (Media Cybernetics, MD, USA).

Immunohistochemistry staining to assess neutrophil infiltration

To analyze the infiltration status of neutrophilic granulocytes into the jejunum, Anti-CD177 antibody (Boster, China) was used. Briefly, the sections were deparaffinized in xylene and rehydrated in graded ethanol solutions. Antigen retrieval was performed at 95 °C for 40 min in 0.01M sodium citrate buffer (pH 6.0). All sections were then immersed in 3% hydrogen peroxide for 20 min. Primary antibody was added at a dilution of 1:100 and incubated overnight at 4 °C. After washing three times with phosphate buffered saline, samples were incubated with rabbit anti-goat IgG (Boster, China) at a ratio of 1:200 at 4 °C for 1 h. Then, DAB was added (DAKO, USA), and hematoxylin (H-3404; Vector, USA) was used to counter stain the slices. Finally, the samples were dehydrated with gradient alcohol, and xylene was used to increase the transparency of the slides, and a neutral balsam was applied for mounting. The integrated optical density from the immunohistochemistry stain was counted using the digital image software Image-Pro Plus 6.0 (Media Cybernetics company, MD, USA).

Markers of inflammation production in the jejunal mucosa

The levels of three cytokines, interleukin (IL)-8, interferon (IFN)- γ and intercellular adhesion molecule-1 (ICAM-1) in the jejunum mucosa were determined through the double antibody sandwich method by using commercially available ELISA kits (Jiancheng Bioengineering, Nanjing, China). The absorbance (OD value) was determined at corresponding wave length with an FLx800 fluorescence reader (BioTek, USA), and the concentrations of these cytokines in the samples were calculated using a standard curve.

I κ B- α degradation in the cytoplasm and p65 accumulation in the nucleus

Nuclear and cytoplasmic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Boster, China). Protein samples were quantified by the method of micro Bicinchoninic Acid protein assay (BCA), using a commercially available BCA kit (Boster, China). The absorbance (OD value) was determined at a 560 nm wavelength with an FLx800 fluorescence reader (BioTek, USA), and the concentrations of protein in the samples were calculated using a standard curve. The expression of I κ B- α (primary antibody from Abcam, USA) in the cytosol and NF- κ B p65 (primary antibody from GeneTex, USA) in the nucleus were evaluated by western blot analysis. In brief, proteins from each sample were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes, 0.45 μ m (Boster, China). After blocking with 5% skimmed milk for 1 h at room temperature, membranes were incubated with appropriately diluted primary antibodies at 4 °C overnight. The next day, the membranes were washed with Tris Buffered Saline Tween (TBST) and immunoblotted with horseradish peroxidase-conjugated secondary antibodies (Boster, China) for 1 h at room temperature, washed with TBST, and then cultured with ECL chemiluminescence reagent. Blots were visualized using an Odyssey CLx Infrared Imaging System (Li-Cor Biosciences, Nebraska, USA). β -actin (primary antibody from Boster, China) and Lamin-Bprotein (primary antibody from Boster, China) were used as the loading control for the cytoplasmic and nuclear fractions, respectively.

Nuclear translocation of the p65 subunit belonging to the NF- κ B complex

Sections of intestinal tissue were incubated with primary antibody NF- κ B p65 (1:50 dilution) at 4 °C and then stained with goat anti-rabbit IgG-FITC (Boster, China) for 1 h at room temperature (RT) in the dark. After washing, nuclei were stained with DAPI for 10 min at RT, and the cover slips were mounted on slides with ProLong®Dia-mond Anti fade Mountant (Life Technology, NY, USA). Fluorescence was observed using a Zeiss Axiovert microscope (Zeiss, Germany).

Statistical analysis

Data were expressed as means $\pm$ standard error of mean (SEM) and examined for their statistical significance of their differences in an ordinary one-way ANOVA analysis followed by Tukey's multiple comparisons test, using GraphPad Prism 8.2.1 software (USA). P-values < 0.05 were considered to be statistically significant. The individual pigs were considered as the experimental unit.

Results

Effects of chitosan nanoparticles on intestinal morphology

LPS induced the destruction of villus structure in the duodenum (Fig. 2D) and ileum (Fig. 2F) and atrophy in the jejunum (Fig. 2E). In contrast, administration of CNP alleviated the damage of LPS to intestinal villi (Fig. 2J-L). Villus height and crypt depth in each section of the intestine were also measured (Table 1). CNP administration tends to improve villus height and reduce crypt depth, which resulted in a significant improvement of V: C in the CNP group compared with the control ($p < 0.05$) in the jejunum. Whereas, LPS treatment decreased villus height and increased crypt depth, which led to a significant decrease of V: C in LPS group compared with the CNP group in the jejunum ($p < 0.01$) and ileum ($P < 0.05$).

Table 1
Effects of CNP on the structure of little bowel

	Control	LPS	CNP	CNP+LPS
Duodenum				
Villous height/μm	392.3 $\pm$ 10	377.8 $\pm$ 15.31	404.0 $\pm$ 10.30	393.0 $\pm$ 17.32
Crypt depth/μm	213.7 $\pm$ 8.87	210.1 $\pm$ 13.58	199.4 $\pm$ 9.58	224.2 $\pm$ 10.64
V:C	1.85 $\pm$ 0.08	1.67 $\pm$ 0.12	2.06 $\pm$ 0.12	1.77 $\pm$ 0.11
Jejunum				
Villous height/μm	354.7 $\pm$ 33.96	321.7 $\pm$ 16.33	387.4 $\pm$ 19.32	365.6 $\pm$ 26.14
Crypt depth/μm	179.4 $\pm$ 7.68	199.4 $\pm$ 15.30	154.6 $\pm$ 8.24 [#]	174.6 $\pm$ 10.43
V:C	1.92 $\pm$ 0.15	1.65 $\pm$ 0.12	2.54 $\pm$ 0.17 ^{*##}	2.12 $\pm$ 0.17
Ileum				
Villous height/μm	354.5 $\pm$ 31.67	281.0 $\pm$ 22.60	343.3 $\pm$ 31.03	329.8 $\pm$ 13.81
Crypt depth/μm	184.6 $\pm$ 17.52	194.4 $\pm$ 9.99	153.2 $\pm$ 11.02	182.0 $\pm$ 13.01
V:C	1.99 $\pm$ 0.22	1.47 $\pm$ 0.15	2.25 $\pm$ 0.17 [#]	1.84 $\pm$ 0.09

The average values of six repetitions of each group were calculated, and multiple comparison tested were carried out by one-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$ vs the control group. # $p < 0.05$, ## $p < 0.01$ vs the LPS group.

Effects of chitosan nanoparticles on the infiltration of neutrophils

A significant ($p < 0.01$) invasion of neutrophils in the jejunal mucosa was observed in the LPS group compared with the other groups, whereas the level of infiltration in the CNP+LPS group was attenuated ($P < 0.01$) (Fig. 3).

Effects of chitosan nanoparticles on the cytokine production

The ELISA (Fig. 4) indicated that LPS treatment resulted in marked increases ($p < 0.01$) in ICAM1, IL-8 and IFN- γ secretion in the jejunal mucosa, whereas CNP administration significantly reduced LPS-induced ICAM1 and IL-8 production ($p < 0.05$).

Effect of chitosan nanoparticles on I κ B- α degradation and p65 translocation

To further elucidate the protective mechanism of CNP on intestinal mucosa, the cytoplasmic level of I κ B- α and nuclear level of NF- κ B p65 subunit were analyzed by western blotting. Immunofluorescence assays were performed to examine the location of p65 in the jejunum sections. Compared with the control group, LPS induced I κ B- α degradation in the cytosol and translocation of NF- κ B p65 subunit into the nucleus ($p < 0.01$), whereas CNP administration inhibited the degradation of I κ B- α ($p < 0.01$) in the cytoplasm and p65 accumulation in the nucleus stimulated by LPS treatment ($p < 0.01$) (Fig. 5). Immunofluorescence results further confirmed the results of the western blot assay. LPS stimulation induced the nuclear translocation of p65, whereas CNP exerted negligible effects on the translocation of p65 (Fig. 6). These results suggest that CNP can inhibit LPS-induced nuclear translocation of p65 by suppressing the degradation of I κ B- α .

Discussion

In this study, LPS was used to simulate a bacterial infection in weaned piglets to investigate the potential anti-inflammatory effects of CNP and the underlying mechanism *in vivo*. Using H&E staining, we could clearly see that the integrity of the three parts of the small intestine was compromised in LPS treated animals. CNP administration improved the intestinal development in the piglets and alleviated the damage of the intestinal epithelium induced by LPS, which was indicated by the more intact intestinal epithelial structure and significantly higher V:C ratio, compared with the control and LPS group.

Along with disturbances in intestinal permeability induced by LPS, neutrophil infiltration of epithelial surfaces leads to injury and leakage of the mucosal barrier. Such barrier defects underlie the basis of a

number of inflammatory disorders. Accumulation of neutrophil in epithelial intestinal crypts has been shown to be directly correlated with the severity of inflammation^[17]. The current study showed that the level of neutrophil infiltration in the jejunum of LPS-treated piglets was significantly higher than that observed in the other three groups. This was accompanied by significantly increased secretion of markers of inflammation such as IL-8, ICAM-1, and IFN γ . IL-8 is mainly active on neutrophils, promoting their recruitment and strong activation, characterized by the activation of the leukotriene pathway^[18]. The level of IL-8 expression correlates with the severity of the disease, particularly with neutrophilic inflammation and mucosal destruction^[19-20]. ICAM-1 mainly mediates neutrophil adhesion. It was reported^[21] that engagement of ICAM-1 exclusively on the luminal (apical) membrane of the intestinal epithelium would increase the accumulation of epithelial-associated neutrophils, thus contributing to mucosal injury. IFN- γ is considered to be one of the major drivers of the excessive immune response to commensal microbiota, leading to massive leukocyte infiltration and mucosal damage^[22-23]. CNP administration significantly reduced the infiltration of neutrophils and secretion of IL-8, ICAM-1, and IFN γ , which in turn attenuated the damage of the intestinal epithelium as compared to the LPS group.

The activated NF- κ B pathway stimulates the synthesis of markers of inflammation, including IL-8, ICAM-1, and IFN γ ^[24]. Thus, it will be interesting to explore whether NF- κ B is involved in CNP-mediated regulation of the inflammatory response to LPS stimulation *in vivo*. In non-inflamed intestinal epithelial cells (IEC), NF- κ B is kept inactive in the cytoplasm by binding to the inhibitory protein I κ B- α . However, I κ B- α is phosphorylated and dissociated in response to stimulation, and thus the p65 subunit of NF- κ B is liberated and translocated into the nucleus, where it activates the transcription of NF- κ B-dependent genes. NF- κ B activation in IECs results in increased levels of IEC-derived inflammatory cytokines^[25-26]. Consistent with our previous *in vitro* study, LPS treatment led to the activation of NF- κ B, as confirmed by I κ B- α degradation and p65 nuclear translocation. CNP decreased the degradation of I κ B- α and translocation of NF- κ B p65 in the intestinal epithelium of LPS-inflamed piglets. Therefore, it is likely that CNP alleviates the LPS-induced inflammatory response by weakening the activation of the NF- κ B pathway.

Conclusions

In summary, CNP can suppress the activation of NF- κ B signaling pathway in piglets by inhibiting the degradation of I κ B. Impaired translocation of NF- κ B p65 leads to reduced production of markers of inflammation and intestinal damage. It is speculated that CNP alleviates the inflammatory response to LPS stimulation via inhibition of the NF- κ B signaling pathway in piglets. This underlying mechanism provides a theoretical basis for the application of CNP as a functional feed additive in piglets.

Abbreviations

CNP, Chitosan nanoparticles; LPS, lipopolysaccharide; GIT, gastrointestinal tract; TLR4, toll-like receptor 4; NF- κ B, nuclear factor κ B; IL, interleukin; IFN, interferon; ICAM-1, intercellular adhesion molecule-1; IEC,

Declarations

Author Contributions

Conceptualization: Y.X. P.G. and C.Y. methodology, Q.L. and Y.X. Data curation, Y.X. and H.M. Writing—original draft preparation, Y.X. and P.G. Writing—review and editing, C.W. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

Not applicable

Ethics approval and consent to participate

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Zhejiang A&F University and approved by the Animal Ethics Committee of Zhejiang A&F University (SYXKzhe2016-087).

Consent for publication

All the authors read and agree to the content of this paper and its publication.

Conflict of Interest

None declared.

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Figures

Figure 1

Atomic Force Micrograph of chitosan nanoparticles

Duodenum

Jejunum

Ileum

Figure 2

H&E staining of middle duodenum, jejunum and ileum tissue respectively (100×). A-C: control; D-F: LPS; G-I: CNP; J-L: CNP+ LPS. Scale bar = 10 μm.

Figure 3

Immunohistochemistry staining of neutrophils(CD177) in jejunum(100×). The microphotographs were analyzed by Image pro-plus 6.0 software, the values of integral optical density(IOD) per stained area (IOD/area) were calculated and analyzed in ordinary one-wayANOVA followed by Tukey's multiple comparisons test. * $p<0.05$, ** $p<0.01$ vs the control group. # $p<0.05$, ## $p<0.01$ vs the LPS group.

Figure 4

ELISA analysis of concentrations of ICAM1, IL-8 and IFNγ in jejunum. The respective ELISA kits were used, the determination of cytokine concentrations were carried out as per the manufacturer's instructions and the OD value was determined at corresponding wave length. The average values of six repetitions of each group were calculated and analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. * p < 0.05, ** p < 0.01 vs the control group. # p < 0.05, ## p < 0.01 vs the LPS group.

Figure 5

Western blot analysis of LPS activated IkB-α degradation in cytoplasm and p65 accumulation in nucleus. The density ratios were measured by densitometry. The average values of three male piglets of each group were calculated and analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. * p<0.05, ** p<0.01 vs the control group. # p<0.05, ## p<0.01 vs the LPS group.

Figure 6

Immunofluorescence analysis of the localization of the p65 subunit of the NF- κ B complex (Line a) p65 stained with FITC; (Line b) nuclei stained with DAPI; (Line c) merged image.