Carbohydrate Polymers 90 (2012) 197–200

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

α-Chitin nanofibrils improve inflammatory and fibrosis responses in inflammatory bowel disease mice model

Kazuo Azuma[a], Tomohiro Osaki[a,*], Shinsuke Ifuku[b], Hiroyuki Saimoto[b], Takeshi Tsuka[a], Tomohiro Imagawa[a], Yoshiharu Okamoto[a], Saburo Minami[a]

[a] Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan
[b] Graduated School of Engineering, Tottori University, Tottori 680-8550, Japan

ARTICLE INFO

Article history:
Received 27 March 2012
Received in revised form 1 May 2012
Accepted 5 May 2012
Available online 11 May 2012

Keywords:
α-Chitin nanofibrils
Inflammatory bowel disease
Nuclear factor κB
Anti-inflammatory
Anti-fibrosis

ABSTRACT

We evaluated the anti-inflammatory and anti-fibrosis effects of α-chitin nanofibrils in a mouse model of dextran sulfate sodium (DSS)-induced acute ulcerative colitis (UC). α-Chitin nanofibrils decreased positive areas of nuclear factor-κB staining in the colon tissue (7.2 ± 0.5%/fields in the α-chitin nanofibrils group *vs.* 10.7 ± 0.9%/fields in the control group; $p < 0.05$). α-Chitin nanofibrils also decreased serum monocyte chemotactic protein-1 concentration in DSS-induced acute UC (24.1 ± 7.8 pg/ml in the α-chitin nanofibrils group *vs.* 53.5 ± 3.1 pg/ml in the control group; $p < 0.05$). Moreover, α-chitin nanofibrils suppressed the increased positive areas of Masson's trichrome staining in colon tissue (6.8 ± 0.6%/fields in the α-chitin nanofibrils group *vs.* 10.1 ± 0.7%/fields in the control group; $p < 0.05$). On the other hand, α-chitin powder suspension did not show these effects in DSS-induced acute UC mice model. Our results indicated that α-chitin nanofibrils have the anti-inflammatory effect via suppressing NF-κB activation and the anti-fibrosis effects in DSS-induced acute UC mice model.

1. Introduction

Chitin (β-(1-4)-poly-*N*-acetyl-D-glucosamine) is widely distributed in nature and are the second abundant polysaccharide after cellulose (Muzzarelli, 2011a). Two chitin polymorphs are known: the α- and β-chitins (Khoushab & Yamabhai, 2010), of which the α-form, which is mainly obtained from crab and shrimp shells, is prevalent. The α-chitin chains are aligned in the anti parallel fashion, which gives rise to strong hydrogen bonding and consequently makes it more stable (Sikorski, Hori, & Wada, 2009).

The methods employed to prepare chitin nanofibrils (CNs) include acid hydrolysis (Gopalan & Dufresne, 2003; Revol & Marchessault, 1993), and ultrasonication of squid pen β-chitin under acidic conditions for the preparation of 3–4 nm wide chitin nanofibrils of relatively lower cristallinity (Fan, Saito, & Isogai, 2008). Recently, Ifuku et al. (2009) demonstrated that α-chitin nanofibrils with uniform widths of approximately 10–20 nm could be prepared from crab chitin flakes by a grinding method leading to fiber disassembly and high yield.

Chitin nanofibrils are gels, and dissolved in water (Ifuku et al., 2009). Chitin nanofibrils are considered to have great potential for applications in tissue engineering scaffolds, drug delivery, and wound dressing (Muzzarelli et al., 2007; Muzzarelli, 2011b, 2011c, 2012a, 2012b). It is also expected that chitin nanofibrils have a potential for a new functional food or drink in various diseases. Previously, we reported the beneficial and preventive effects of α-chitin nanofibrils in dextran sodium sulfate (DSS)-induced acute ulcerative colitis (UC) mice model. α-Chitin nanofibrils improved clinical symptoms and suppressed UC. Furthermore, α-chitin nanofibrils suppressed myeloperoxidase activation in the colon and decreased serum interleukin-6 concentrations (Azuma et al., 2012). However, the protective mechanisms of α-chitin nanofibrils are not unknown. In this study, we examined protective mechanism of α-chitin nanofibrils focusing on anti-inflammatory and anti-fibrosis effects in DSS-induced acute UC mice model.

2. Experimental

2.1. Reagents

DSS (molecular weight, 36–50 kDa; reagent grade) was purchased from MP Biomedicals LLC (Solon, OH, USA). Chitin powder from crab shells was purchased from Nacalai Tesque (Lot No.: M0A3811; Kyoto, Japan). The average particle diameter of chitin powder was approximately 200 μm. α-Chitin nanofibrils gels

* Corresponding author at: Faculty of Agriculture, Tottori University, 4-101 Koyama-minami, Tottori 680-8553, Japan. Tel.: +81 857 31 5434; fax: +81 857 31 5434.
E-mail address: tosaki@muses.tottori-u.ac.jp (T. Osaki).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.05.023

(1% α-chitn nanofibrils with 0.3% acetic acid; hereafter referred to as α-chitin nanofibrils) was prepared using a previously described method with slight modification (Ifuku et al., 2009). Briefly, the purified wet chitin from dry crab shells was dispersed in water at 1 wt%, and acetic acid was added to adjust the 0.3% (v/v) to facilitate fibrillation. A chitin powder suspension (1% α-chitin powder suspension with 0.3% acetic acid; hereafter referred to as chitin-PS) was prepared.

2.2. Animals

Thirty C57BL/6 mice (female, 5 weeks old) were purchased from CLEA Japan (Osaka, Japan). The animals were maintained under conventional conditions. The use of these animals and the procedures they underwent were approved by the Animal Research Committee of Tottori University.

2.3. Study design

Mice ($n = 30$) were randomized into 6 groups: the control (+) group was administered only DSS ($n = 5$); the control (−) group was administered tap water ($n = 5$); the α-chitin nanofibrils (+) group was administered α-chitin nanofibrils and DSS ($n = 5$); the α-chitin nanofibrils (−) group was administered only α-chitin nanofibrils ($n = 5$); the chitin-PS (+) group was administered chitin-PS and DSS ($n = 5$); and the chitin-PS (−) group was administered only chitin-PS ($n = 5$). To induce colitis, mice were administered 3% DSS *ad libitum* for 5 days from day 0 to day 5. For 7 days before starting the administration of DSS, α-chitin nanofibrils (+), α-chitin nanofibrils (−), chitin-PS (+), and chitin-PS (−) groups were administered 0.1% α-chitin nanofibrils, or chitin-PS dissolved in tap water *ad libitum*. Blood collection and colon sampling were done on days 5 in all groups. Colon tissues were fixed in 10% buffered formalin.

2.4. Masson's trichrome (MT) staining

In the DSS-induced UC, the fibrosis of mucosal and submucosal layers of the colon was observed at acute and chronic phase (Suzuki et al., 2011). To measure the fibrosis area of the mucosal and submucosal layers of the colon, we performed quantitative digital morphometri analysis of extracellular matrix (ECM) for colonic sections with MT staining according to a protocol adapted from that described in detail by Suzuki et al. (2011). In brief, 10 randomly chosen high-power fields (200× magnification) for each cross section were photographed with a digital camera attached to an Olympus microscope system (Olympus Corporation, Tokyo, Japan). The color wavelengths of the copied image were transformed into digital readings, by using Lumina Vision software (Mitani Corporation, Tokyo, Japan) allowing for quantification of the various color wavelengths with pixels as the unit of measure. By using the original image for comparison, the color spectra were analyzed and those corresponding to ECM were quantified. The percentage of the ECM tissues in mucosal and submucosal layers was calculated by dividing the total pixel area of the ECM by the total pixel area corresponding to the total colonic tissue in the field of view. The colons of three mice were analyzed in each group. The mean scores for 30 fields were considered the percentages of fibrosis areas for each group.

2.5. Immunohistochemical detection of nuclear factor-κB (NF-κB) in the colon

NF-κB has been reported to be activated in inflamed colonic mucosa of IBD (Reed et al., 2005; Visekruna et al., 2006). We evaluated the effects of α-chitin nanofibrils and β-CN on NF-κB activations in inflammatory colon. Colon tissue sections (3 μm) on glass slides were deparaffinized, washed by ethanol and water and soaked by PBS. The sections were treated by microwave with 0.01 M citrate buffer (pH 6.0) for 5 min. Then, the sections were washed with PBS and incubated with 1% hydrogen peroxide methanol for 30 min at room temperature. Washing with PBS, the sections were incubated with rabbit polyclonal anti-NF-κB p65 antibody (1:500, sc-372; Santa Cruz Biotechnology, Inc., California, USA) for 60 min at room temperature. The slides were washed with PBS, and envisioned for 30 min at room temperature (Code No. K3466, Dako, Glostrup, Denmark). Tissue sections were visualized by incubating with diaminobenzidine tetrahydrochloride, and counterstained with hematoxylin.

We calculated the positive areas of NF-κB in colon epithelium. The imaging analysis of NF-κB was performed as well as those of colon fibrosis. The colons of three mice were analyzed in each group. The mean scores for 30 fields were considered the percentages of fibrosis areas for each group.

2.6. Measurements of serum monocyte chemotactic protein 1 concentrations

Serum monocyte chemotactic protein 1 (MCP-1/CCL2) were quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) using commercial mouse MCP-1 ELISA kit (Quantikine®, R&D Systems Inc., Minneapolis, USA) according to the manufacturer's protocol.

2.7. Statistical analysis

The data are expressed as the mean ± S.E. Statistical analyses were performed using 1-way ANOVA followed by Tukey–Kramer's test or Steel–Dwass test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of α-chitin nanofibrils on fibrosis of the colon in DSS-induced acute UC mice

To investigate the effects of α-chitin nanofibrils on the fibrosis of mucosal and submucosal layers of the colon, MT staining was performed. The results of MT staining were shown in Fig. 1A. The area of collagen deposition was shown by arrows. In the control (+) and chitin-PS (+) group, much collagen deposition was observed (Fig. 1A(a) and (c)). In the α-chitin nanofibrils (+) group, the areas of collagen deposition were meekly decreased (Fig. 1A(b)).

For evaluating the area of collagen deposition in mucosal and submucosal layers, we performed digital image analysis. The percentages of collagen deposition areas in mucosal and submucosal layers are shown in Fig. 1B. In the α-chitin nanofibrils (+) group, the score was significantly lower than that in the control (+) group ($p < 0.05$). In the control (−), α-chitin nanofibrils (−), and chitin-PS (−) groups, the scores were 1.5–1.9%.

3.2. Effects of α-chitin nanofibrils on NF-κB of the colon epithelium in DSS-induced acute UC mice

To evaluate the effects of α-chitin nanofibrils on NF-κB of the colon epithelium, immunohistochemical detections of NF-κB were performed. The results of immunohistochemical detections of NF-κB were shown in Fig. 2A. In the control (+) and chitin-PS (+) group, much positive areas of NF-κB in epithelium cells were observed. In the α-chitin nanofibrils (+) group, positive areas of NF-κB in epithelium cells were markedly decreased.

To evaluate the effects of α-chitin nanofibrils on NF-κB activations in the epithelium cells, we performed digital image analysis.

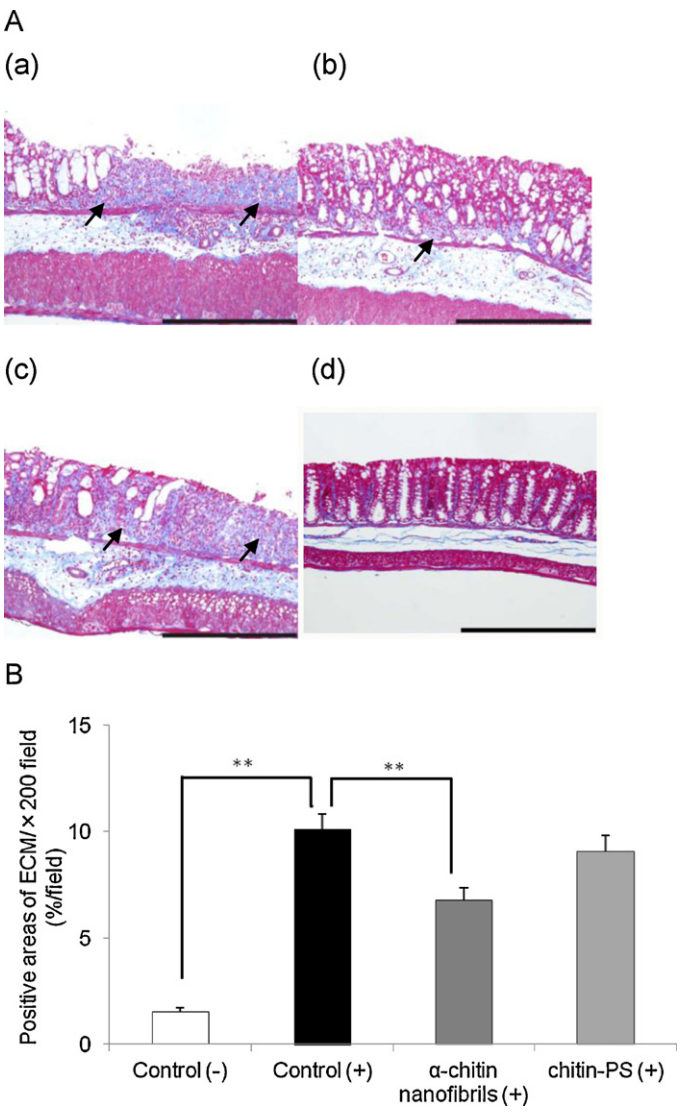

Fig. 1. Effects of α-chitin nanofibrils on colon fibrosis in a DSS-induced acute UC mouse model. A. Masson's trichrome staining results are shown. Data are presented for 1 mouse each from the control (+) (a), α-chitin nanofibrils (+) (b), chitin-PS (c), and control (−) groups. Areas of collagen deposition are indicated by arrows. Bar = 200 μm. B. Data represent the means ± S.E. of 30 fields/×100 field in each group. The statistical analyses were performed with a Steel–Dwass test. $^{*}p < 0.05$, $^{**}p < 0.01$.

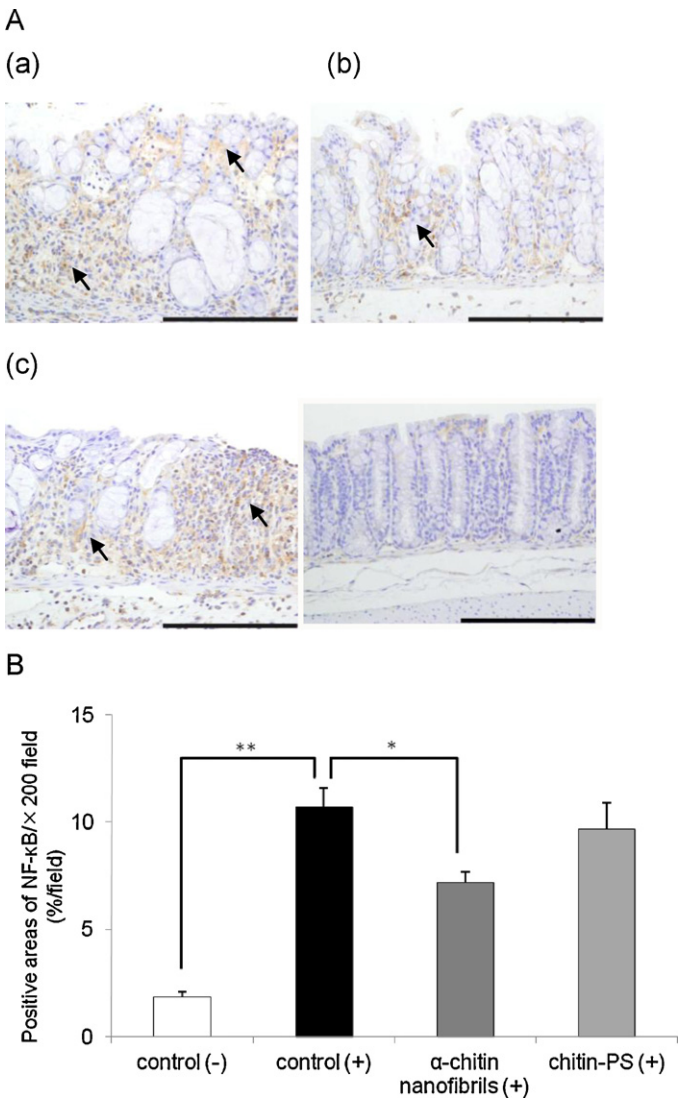

Fig. 2. Effects of α-chitin nanofibrils on colon NF-κB activation in a DSS-induced acute UC mouse model. A. NF-κB-positive areas are shown by arrows. Data are presented for 1 mouse each from the control (+) (a), α-chitin nanofibrils (+) (b), chitin-PS (c), and control (−) (d) groups. Bars = 100 μm. B. Data represent the means ± S.E. of 30 fields/×100 field in each group. The statistical analyses were performed with a Steel–Dwass test. $^{*}p < 0.05$, $^{**}p < 0.01$.

The percentages of positive areas of NF-κB in epithelium cells are shown in Fig. 2B. In the α-chitin nanofibrils (+) group, the score was significantly lower than that in the control (+) group ($p < 0.05$). In the chitin-PS (+) groups, the scores were slightly suppressed. In the control (−), α-chitin nanofibrils (−), and chitin-PS (−) groups, the scores were 1.8–3.0%.

3.3. *Effects of α-chitin nanofibrils on serum MCP-1 concentrations in DSS-induced acute UC mice*

The results were shown in Fig. 3. In the α-chitin nanofibrils (+) group, serum MCP-1 concentration was significantly lower than the control (+) groups ($p < 0.05$).

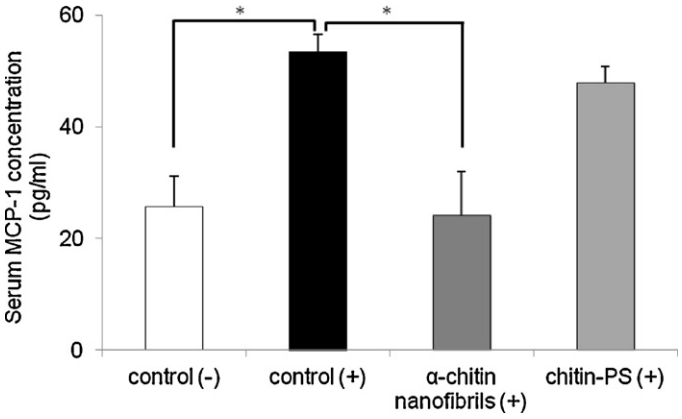

Fig. 3. Effects of α-chitin nanofibrils on serum MCP-1 concentrations in a DSS-induced acute UC mouse model. Data represent the means ± S.E. in each group ($n = 5$). The statistical analyses were performed with a Tukey–Kramer test. $^{*}p < 0.05$, $^{**}p < 0.01$.

4. Discussion

We previously reported that α-chitin nanofibrils improved clinical symptoms, colon inflammation and histological tissue

injury in the DSS-induced acute UC mouse model (Azuma et al., 2012). Likewise our previous report, α-chitin nanofibrils suppressed clinical symptoms and tissue injury of the colon in this study.

In the DSS-induced UC model mice, fibrosis in the colon was observed not only chronic phase but also acute phase (Suzuki et al., 2011). It is described that MCP-1 induces fibrogenic response of the gut in IBD model (Motomura et al., 2006). α-Chitin nanofibrils suppressed the fibrosis and decreased serum MCP-1 concentration in DSS-induced acute UC mouse model. These results indicated that α-chitin nanofibrils have the suppressive effects of fibrosis in DSS-induced acute UC mouse model. It was indicated one mechanism of suppressive effects on fibrosis by α-chitin nanofibrils came from suppressing the action of MCP-1.

NF-κB occupies a pivotal position in several innate immune signaling pathways. So far, it has been shown that NF-κB is the critical transcription factor needed to express genes associated with a proinflammatory response (Elson et al., 2005). NF-κB activity is increased in the colon during active episodes of IBD (Zarubin & Han, 2005). α-Chitin nanofibrils suppressed the activation of NF-κB in colon epithelium in DSS-induced acute colitis model. MCP-1 plays an important role in the pathogenesis of experimental colitis model to the recruitment of immune and enterochromaffin cells (Khan et al., 2006). The absence of MCP-1 is associated with a significant reduction in inflammation in experimental colitis model (Khan et al., 2006). Ju et al. demonstrated that pro-inflammatory cytokine induced the expression of MCP-1 via p38 mytogen-activated protein kinase (MAPK) and NF-κB signaling (Ju, Hua, Sakamoto, Ogawa & Nagaoka, 2008). α-Chitin nanofibrils decreased serum MCP-1 concentration compared with control (+) group. These results indicated that α-chitin nanofibrils suppressed the increase of MCP-1 in serum via suppressing NF-κB activation.

NF-κB stimulate cyclooxygenase-2, prostaglandin E2 and pro-inflammatory cytokines (interleukin-6, tumor necrosis factor-α and MCP-1) (Karrasch & Jobin, 2008). α-Chitin nanofibrils suppressed serum interleukin-6 concentrations in DSS-induced acute colitis mice (Azuma et al., 2012). However, relationships between α-chitin nanofibrils and other inflammatory mediators are still unclear. Further studies to evaluate the relationships α-chitin nanofibrils and inflammatory mediators are needed to understand the anti-inflammatory mechanism of α-chitin nanofibrils.

Currently, many medical treatments are used for IBD patients: 5-aminosalicylic acid drugs, sulfasalazine or balsalazide, immunomodulators such as thiopurines (azathioprine, 6-mercaptopurine), methotrexate, and biologic therapies that target tumor necrosis factor (TNF)-α or interleukin (IL)-6 (Morrison, Headon, & Gibson, 2009; Nakamura, Honda, Mizutani, Akiho, & Harada, 2006). However, these drugs have not only beneficial effects but also adverse effects in IBD patients. For example, 5-aminosalicylic acids drugs are expensive. Immunomodulators and biologic therapies increase the risk of serious infection (Morrison et al., 2009). By these backgrounds, the necessities of the functional food for IBD patients are increasing. Our previous report and these results indicate α-chitin nanofibrils have a potency as a new functional food for IBD patients. To use α-chitin nanofibrils in IBD patients, safety evaluation of α-chitin nanofibrils must be performed in long and short terms.

So far, it is not unclear how α-chitin nanofibrils absorb and metabolize *in vivo* and *in vitro*. To understand the anti-inflammatory mechanism of α-chitin nanofibrils, the study focusing on absorption and metabolism of α-chitin nanofibrils must be performed.

In conclusion, α-chitin nanofibrils have anti-inflammatory actions via suppressing NF-κB and MCP-1 activations in DSS-induced acute UC mice model. α-chitin nanofibrils also suppressed fibrosis in DSS-induced acute UC mouse model. These results indicate that α-chitin nanofibrils has a potency as a new functional food for IBD patients. The effect of α-chitin nanofibrils on human IBD should be carefully evaluated in the future.

References

Azuma, K., Osaki, T., Wakuda, T., Ifuku, T., Saimoto, H., Tsuka, T., et al. (2012). Beneficial and preventive effects of chitin nanofibrils in a dextran sulfate sodium-induced acute ulcerative colitis model. *Carbohydrate Polymers*, *87*(2), 1399–1403.

Elson, C. O., Cong, Y., McCracken, V. J., Dimmitt, R. A., Lorenz, R. G., & Weaver, C. T. (2005). Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunological Reviews*, *206*(1), 260–276.

Fan, Y., Saito, T., & Isogai, A. (2008). Preparation of chitin nanofibers from squid pen beta-chitin by simple mechanical treatment under acid conditions. *Biomacromolecules*, *9*(7), 1919–1923.

Gopalan, N. K., & Dufresne, A. (2003). Crab shell chitin whisker reinforced natural rubber nanocomposites. 1. Processing and swelling behavior. *Biomacromolecules*, *4*(3), 657–665.

Ifuku, S., Nogi, M., Abe, K., Yoshioka, M., Morimoto, M., Saimoto, H., et al. (2009). Preparation of chitin nanofibers with a uniform width as alpha-chitin from crab shells. *Biomacromolecules*, *10*(6), 1584–1588.

Ju, Y., Hua, J., Sakamoto, K., Ogawa, H., & Nagaoka, I. (2008). Modulation of TNF-alpha-induced endothelial cell activation by glucosamine, a naturally occurring amino monosaccharide. *International Journal of Molecular Medicine*, *22*(6), 809–815.

Karrasch, T., & Jobin, T. (2008). NF-κB and the intestine: friend or foe? *Inflammatory Bowel Disease*, *14*(1), 114–124.

Khan, W. I., Motomura, Y., Wang, H., El-Sharkawy, R. T., Verdu, E. F., Verma-Gandhu, M., et al. (2006). Critical role of MCP-1 in the pathogenesis of experimental colitis in the context of immune and enterochromaffin cells. *American Journal of Physiology: Gastrointestinal and Liver Physiology*, *291*(5), G803–G811.

Khoushab, F., & Yamabhai, M. (2010). Chitin research revisited. *Marine Drugs*, *8*(7), 1988–2012.

Morrison, G., Headon, B., & Gibson, P. (2009). Update in inflammatory bowel disease. *Australian Family Physician*, *38*(12), 956–961.

Motomura, Y., Khan, W. I., El-Sharkawy, R. T., Verma-Gandhu, M., Verdu, E. F., Gauldie, J., et al. (2006). Induction of a fibrogenic response in mouse colon by overexpression of monocyte chemoattractant protein 1. *Gut*, *55*(5), 662–670.

Muzzarelli, R. A. A., Morganti, P., Morganti, G., Palombo, P., Palombo, M., Biagini, G., et al. (2007). Chitin nanofibrils/chitosan glycolate composites as wound medicaments. *Carbohydrate Polymers*, *70*(3.), 274–284.

Muzzarelli, R. A. A. (2011a). Chitin nanostructures in living organisms. In N. Gupta (Ed.), *Chitin: formation and diagenesis* (pp. 1–34). Dordrecht: Springer.

Muzzarelli, R. A. A. (2011b). New techniques for optimization of surface area and porosity in nanochitins and nanochitosans. In R. Jayakumar, A. Prabaharan, & R. A. A. Muzzarelli (Eds.), *Advances in polymer science: chitosan for biomaterials* (pp. 167–186). Berlin: Springer-Verlag.

Muzzarelli, R. A. A. (2011c). Biomedical exploitation of chitin and chitosan via mechano-chemical disassembly, electrospinning, dissolution in imidazolium ionic liquids, and supercritical drying. *Marine Drugs*, *9*(9), 1510–1533.

Muzzarelli, R. A. A. (2012a). Chemical and technological advances in chitins and chitosans useful for the formulation of biopharmaceuticals. In B. Sarmento, & J. DasNeves (Eds.), *Chitosan-based systems for biopharmaceuticals* (pp. 3–22). New York: Wiley.

Muzzarelli, R. A. A. (2012b). Nanochitins and nanochitosans, paving the way to eco-friendly and energy-saving exploitation of marine resources. In J. McGrath, & R. Hoefer (Eds.), *Comprehensive polymer science: Vol. 10. Polymers for sustainable environment and energy*. Heidelberg: Springer.

Nakamura, K., Honda, K., Mizutani, T., Akiho, H., & Harada, N. (2006). Novel strategies for the treatment of inflammatory bowel disease: selective inhibition of cytokines and adhesion molecules. *World Journal of Gastroenterology*, *12*(29), 4628–4635.

Reed, K. L., Fruin, A. B., Gower, A. C., Gonzales, K. D., Stucchi, A. F., Andry, C. D., et al. (2005). NF-κB Activation precedes increases in mRNA encoding neurokinin-1 receptor, proinflammatory cytokines, and adhesion molecules in dextran sulfate sodium-induced colitis in rats. *Digestive Disease and Science*, *50*(12), 2366–2378.

Revol, J. F., & Marchessault, R. H. (1993). In vitro chiral nematic ordering of chitin crystallites. *International Journal of Biological Macromolecules*, *15*(6), 329–335.

Sikorski, P., Hori, R., & Wada, M. (2009). Revisit of alpha-chitin crystal structure using high resolution X-ray diffraction data. *Biomacromolecules*, *10*(5), 1100–1105.

Suzuki, K., Sun, X., Nagata, M., Kawase, T., Yamaguchi, H., Sukumaran, V., et al. (2011). Analysis of intestinal fibrosis in chronic colitis in mice induced by dextran sulfate sodium. *Pathology International*, *61*(4), 228–238.

Visekruna, A., Joseris, T., Seidel, D., Kroesen, A., Loddenkemper, C., Zeitz, M., et al. (2006). Proteasome-mediated degradation of IκBα and processing of p105 in Crohn disease and ulcerative colitis. *Journal of Clinical Investigation*, *116*(12), 3195–3203.

Zarubin, T., & Han, J. (2005). Activation and signaling of the p38 MAP kinase pathway. *Cell Research*, *15*(1), 11–18.