Doctor's Thesis

The United Graduate School of Veterinary Science

Yamaguchi University

Biological Effects of Orally Administered Chitin and its Derivatives

経口投与したキチンおよびその誘導体の生物学的効果

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

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Suppressive effects of N-acetyl-D-glucosamine on rheumatoid arthritis mouse models

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Effects of Oral Glucosamine Hydrochloride Administration on Plasma Free Amino Acid Concentrations in Dogs

Publications

The contents of this thesis were published in the following journals.

Chapter I:

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Chapter II:

Azuma, K., Osaki, T., Ifuku, S., Saimoto, H., Tsuka, T., Imagawa, T., Okamoto, Y., & Minami, S. (2012). α-Chitin nanofibrils improve inflammatory and fibrosis responses in mice with inflammatory bowel disease. *Carbohydrate Polymers* 90(1), 197-200.

Chapter III:

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Chapter IV:

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Abstract

Chitin and its monomer (Glucosamine hydrochloride (GlcN) and N-acetyl-d-glucosamine (GlcNAc)) are widely used as dietary supplements. Several reports revealed bioactivities of chitin and its derivatives. However, the action mechanisms of chitin and its derivatives *in vivo* are not established.

In the chapter I, we evaluated the beneficial and preventive effects of chitin nanofibrils on inflammatory bowel disease (IBD) mouse model. Chitin nanofibrils improved clinical symptoms, inhibited colonic inflammation, and prevented tissue injury in IBD mouse model. Furthermore, chitin nanofibrils inhibited mucosal inflammation by suppressing the myeloperoxidase (MPO)-positive cells such as leukocytes and decreasing serum IL-6 concentrations. Conversely, chitin powder was not effective in our DSS-induced acute UC model.

In the chapter II, we revealed the anti-inflammatory effects of chitin nanofibrils by suppressing nuclear factor- κ B (NF-κB) activation in IBD model mouse. Chitin nanofibrils have anti-inflammatory actions via suppressing NF-κB and MCP-1 activations in IBD mice model. Chitin nanofibrils also suppressed fibrosis in IBD mouse model. These results indicate that chitin nanofibrils has a potency as a new functional food for IBD patients.

In the chapter III, we evaluated the suppressive effects of GlcNAc in rheumatoid arthritis (RA) model mouse. GlcNAc suppressed not only finger, wrist, and ankle swelling but also the histological changes in experimental RA mouse

models. Compared to GlcN, GlcNAc had different mechanisms of action on RA, especially in increasing serum IL-10, TGF-β1, and IL-2 concentrations. Our results indicate that GlcNAc is also a potential supplement for RA patients.

In the chapter IV, our results suggested the existence of species-specific differences in PFAA dynamics after oral administration of GlcN. Our results also suggested the existence of differences in PFAA dynamics after oral administration of GlcN and GlcNAc in dogs. . Our results also might indicate that anti-inflammatory effects of GlcN in horse come from inducing autophagy.

Our results revealed anti-inflammatory effects of chitin derivatives (chitin nanofibrils, GlcNAc and GlcN). Especially, we revealed anti-inflammatory effect of chitin nanofibrils via suppressing NF-κB activation. Compared to GlcN, GlcNAc had different mechanisms of action in experimental inflammation. We also revealed that anti-inflammatory effects of GlcN in horse come from inducing autophagy. Our results indicated chitin derivatives have potency as functional foods for inflammatory disease.

General Introduction

Chitin (β-(1-4)-poly-*N*-acetyl-D-glucosamine) is widely distributed in nature and is the second abundant polysaccharide after cellulose (Gupta, 2011). The nonspecific antiviral and antitumor activities of chitin or chitin derivatives were described (Shibata *et al.*, 2000; Shibata *et al.*, 2001). Glucosamine hydrochloride (GlcN) and N-acetyl-D-glucosamine (GlcNAc), monomer of chitin and chitosan, are widely used as supplements for treating osteoarthritis (Anderson *et al.*, 2005; Chen *et al.*, 2010). Some studies revealed the bioactivities of chitin or its derivatives. For example, it was suggested that the size of chitin influences its effects on immune cells (Da Silva *et al.*, 2008; Lee *et al.*, 2008).

Chitins in crustacean shells are highly crystalline: in the α-chitin, the microfibers consist of nanofibrils approximately 2-5 nm in diameter and 300 nm in length embedded in a protein matrix (Raabe *et al.*, 2006; Chen *et al.*, 2008). Isolated chitin nanofibrils are considered to have great potential for applications in tissue engineering scaffolds, drug delivery, and wound dressing (Muzzarelli, *et al.*, 2007).

The methods employed to prepare chitin nanofibrils include acid hydrolysis (Revol & Marchessault, 1993; Gopalan & Dufresne, 2003), 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 *et al.*, 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. However, no study has

described the in vivo effects of chitin nanofibrils after oral administration.

Some reports indicate different bioactivities between GlcN and GlcNAc *in vitro*. Differences in GlcN and GlcNAc uptake and their subsequent effects on glucose transport, glucose transporter (GLUT) expression, and synthesis of sulfated glycosaminoglycans (sGAGs) and hyaluronan have been reported (Shikhman *et al.*, 2009). GlcN suppressed interleukin (IL)-8, prostaglandin E_2, and nitric oxide synthesis on IL-1β mediated synoviocytes; however, GlcNAc did not show such effects (Hua *et al.*, 2007). The beneficial effects of GlcN on experimental rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) were reported (Hua *et al.*, 2005; Yomogida *et al.*, 2008).

In an experimental rabbit model of cartilage injury, oral administration of GlcN or GlcNAc led to regeneration of both glycosaminoglycan and proteoglycan (Tamai *et al.*, 2002; Tamai *et al.*, 2003). GlcN has a potential to exert a chondroprotective action on an experimental induced osteoarthritis (OA) by inhibiting type II collagen degradation and enhancing type II collagen synthesis in the articular cartilage (Naito *et al.*, 2010). These results suggested that synthesis of type II collagen and proteoglycan core protein as well as glycosaminoglycan occurs upon GlcN and GlcNAc supplementation. However, no reports to date had been investigated the relationship between oral administration of amino monosaccharide and amino acid synthesis.

In the chapter I, we evaluated the beneficial and preventive effects of chitin nanofibrils on IBD model mouse model. In the chapter II, we revealed the anti-inflammatory effects of chitin nanofibrils by suppressing nuclear factor- κ B

activation in IBD model mouse. In the chapter III, we evaluated the suppressive effects of GlcNAc in RA model mouse. The results of serum cytokine concentrations suggested that compared to GlcN, GlcNAc has a different suppressive mechanism in experimental RA models. In the chapter IV, we revealed that species-specific differences in PFAA dynamics might be existed after oral administration of GlcN. Our results also suggested the existence of differences in PFAA dynamics after oral administration of GlcN and GlcNAc in dogs

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Chapter I.

Beneficial and preventive effect of chitin nanofibrils in a dextran sulfate sodium-induced acute ulcerative colitis model

Abstract

Chitin nanofibrils, which are prepared from dried crab shells by a grinding method, are newly developed natural materials with uniform widths of approximately 10–20 nm. The bioactivities of chitin nanofibrils have not been investigated. In this study, we examined the preventive effects of chitin nanofibrils in a mouse model of dextran sulfate sodium (DSS)-induced acute ulcerative colitis. The results indicated that chitin nanofibrils improved clinical symptoms and suppressed ulcerative colitis. Furthermore, chitin nanofibrils suppressed myeloperoxidase activation in the colon and decreased serum interleukin-6 concentrations. Conversely, chitin powder did not suppress DSS-induced acute ulcerative colitis. Our results suggested that chitin nanofibrils have potential as a functional substance for inflammatory bowel disease patients.

1. Introduction

Chitin (β-(1-4)-poly-*N*-acetyl-d-glucosamine) is widely distributed in nature and is the second abundant polysaccharide after cellulose (Gupta, 2011). The nonspecific antiviral and antitumor activities of chitin or chitin derivatives were

described (Shibata *et al.*, 2000; Shibata *et al*, 2001). Recently, it was suggested that the size of chitin influences its effects on immune cells (Da Silva *et al.*, 2008; Lee *et al.*, 2008). Chitins in crustacean shells are highly crystalline: in the α-chitin, the microfibers consist of nanofibrils approximately 2-5 nm in diameter and 300 nm in length embedded in a protein matrix (Raabe *et al.*, 2006; Chen *et al.*, 2008). Isolated chitin nanofibrils are considered to have great potential for applications in tissue engineering scaffolds, drug delivery, and wound dressing (Muzzarelli *et al.*, 2007).

The methods employed to prepare chitin nanofibrils include acid hydrolysis (Revol & Marchessault, 1993; Gopalan & Dufresne, 2003), 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 *et al.*, 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. However, no study has described the in vivo effects of chitin nanofibrils after oral administration.

Inflammatory bowel disease (IBD) is common and refers to a group of conditions characterized by inflammation in the intestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) account for the majority of the cases of these conditions (Morrison *et al.*, 2009). Currently, some experimental animal models are used in IBD research. A model of dextran sulfate sodium (DSS)-induced colitis is one common model of IBD, in which animals develop acute and chronic colitis resembling UC (Melgar *et al.*, 2005).

Glucosamine hydrochloride is likely to suppress the cytokine-induced activation

of intestinal epithelial cells *in vivo*, thereby possibly exerting anti-inflammatory effects in a DSS-induced rat UC model (Yomogida *et a.l*, 2008). However, no study has investigated the effects of chitin or chitin deliveries in a DSS-induced UC model. The aim of this study was to evaluate the preventive effects of chitin nanofibrils compared with those of chitin in a mouse model of DSS-induced acute UC.

2. Materials and Methods

2.1 Reagents

DSS (molecular weight, 36–50 kDa; reagent grade) was purchased from MP Biomedicals LLC (Solon, OH, USA). Chitin powder was purchased from Nacalai Tesque (Lot No.: M0A3811; Kyoto, Japan). The average diameter of chitin powder was approximately 200 μm. Chitin nanofibrils gel (1%, pH 3; hereafter referred to as chitin nanofibrils) was prepared using a previously described method (Ifuku *et al.*, 2009). A chitin powder suspension (1%, pH 3; hereafter referred to as chitin-PS) was prepared; the percentage of deacetylated chitin-PS in the suspension was 3.9%.

2.2 Animals

Sixty-eight C57BL/6 mice (female, 6 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 = 68) were randomized into 6 groups: the control (+) group was administered only DSS (n = 17); the control (−) group was administered tap water (n = 5); the chitin nanofibrils (+) group was administered chitin nanofibrils and DSS (n = 17); the chitin nanofibrils (−) group was administered only chitin nanofibrils (n = 7); the chitin-PS (+) group was administered chitin-PS and DSS (n = 16); and the chitin-PS (−) group was administered only chitin-PS (n = 6). To induce colitis, mice were administered 3% DSS *ad libitum* for 6 days from day 0 to day 6. For 7 days before starting the administration of DSS, chitin nanofibrils (+), chitin nanofibrils (−), chitin-PS (+), and chitin-PS (−) groups were administered 0.1% chitin nanofibrils or chitin-PS dissolved in tap water *ad libitum*. Blood collection and colon sampling were done on days 3 and 5 in control (+), chitin nanofibrils (+), and chitin-PS groups (each n = 5) and on day 6 in all groups (each group: n = 5–7).

2.4 Clinical analysis

UC was evaluated using the disease activity index (DAI) as described by Melger *et al* (Melgar *et al.*, 2005) with a slight modification by using the parameters of body weight loss, stool consistency, and bleeding (Table I-1). The length and weight of the colon were measured, and tissue obtained from each colon was processed for further analysis.

2.5 Histological evaluation of colitis

Colon tissues were fixed in 10% buffered formalin. Thin sections (3 μm) were

made from each sample for histological observation after hematoxylin-eosin staining. Each section was examined microscopically, and histological scoring was performed as described by Ohkawara *et al* (2005). In brief, tissue damage was classified using 6 grades: 0: normal mucosa; 1: infiltration of inflammatory cells; 2: shortening of the crypt by less than half of the height; 3: shortening of the crypt by more than half of the height; 4: crypt loss; 5: destruction of epithelial cells. Histological scoring was performed in 10 fields at ×100 magnification using 3 mice in each group. The mean scores for 30 fields were considered the histological score for each group.

2.6 Myeloperoxidase (MPO) staining

MPO staining, which is a marker of leukocyte invasion into tissue (Schindhelm *et al.*, 2009), was performed in a routine manner as described previously (Ohtsuka *et al.*, 2001). Counts of MPO-positive cells in the submucosal layer were performed in 20 fields at ×400 magnification using 3 mice in each group. The mean scores for 60 fields were considered the number of MPO-positive cells for each group.

2.7 Measurements of serum IL-6 concentrations

Serum IL-6 was quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) using a Mouse IL-6 ELISA kit (Thermo SCIENTIFIC, Rockford, IL, USA) according to the manufacturer's protocol.

2.8 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. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1 Effects of chitin nanofibrils on DAI in DSS-induced acute UC mice

Weight loss, loose stools, and bleeding were observed on day 3 in the control (+) and chitin-PS (+) groups and on day 4 in the chitin nanofibrils (+) group (Table I-2). The chitin nanofibrils (+) group exhibited a significantly reduced DAI on days 4–6 compared with that in the control (+) group ($p < 0.05$) and on days 5 ($p < 0.01$) and 6 ($p < 0.05$) compared with that in the chitin-PS (+) group (Table I -2). No change of DAI was observed in the control (−), chitin nanofibrils (−), and chitin-PS (−) groups (date not shown).

3.2 Effects of chitin nanofibrils on colon length and the colon weight/length ratio in DSS-induced acute UC mice

The administration of 3% DSS shortened colon length and increased the colon weight/length ratio (mg/cm) in C57BL/6 mice (Melgar *et al.*, 2005). In the chitin nanofibrils (+) group, colon lengths were significantly longer than those in the control (+) group on days 3, 5, and 6 ($p < 0.05$ for days 3 and 5, $p < 0.01$ for day 6). Moreover, colon length in the chitin nanofibrils (+) group was significantly longer than that in the chitin-PS (+) group on days 3, 5, and 6 ($p < 0.05$ for days 3 and 5, $p < 0.01$ for day 6, Table I-3a). No change of colon length was observed

in the control (−), chitin nanofibrils (−), and chitin-PS (−) groups (data not shown).

The colon weight/length ratio was decreased in the chitin nanofibrils (+) group on days 5 and 6 compared with that in the control (+) group. On day 5, the colon weight/length ratios of the chitin nanofibrils (+) and chitin-PS (+) groups were significantly decreased compared with that of the control (+) group ($p < 0.05$). On day 6, the colon weight/length ratio of the chitin nanofibrils (+) group was significantly decreased compared with those of the control (+) and chitin-PS (+) groups ($p < 0.01$, Table I-3b). No change of the weight/length ratio was observed in the control (−), chitin nanofibrils (−), and chitin-PS (−) groups (date not shown).

3.3 Effects of chitin nanofibrils on histological changes in DSS-induced acute UC mice

Damage in the intestinal mucosa was microscopically evaluated by histological scoring. No histological change was observed in the control (−), chitin nanofibrils (−), and chitin-PS (−) groups. On day 3 in the control (+), chitin nanofibrils (+), and chitin-PS (+) groups, inflammatory cell infiltration was observed. On day 5, erosions, shortening or destruction of the crypt, and edema were observed in the control (+) and chitin-PS (+) groups. Some erosions were observed on day 5 in the chitin nanofibrils (+) group; however, shortening or destruction of the crypt was markedly suppressed, and edema was slightly suppressed. On day 6 in the control (+) and chitin-PS (+) groups, severe erosions, crypt destruction, and edema were observed; moreover, some ulcers were observed. In the chitin

nanofibrils (+) group, erosions, crypt destruction, and edema were markedly suppressed compared with those in the control (+) and chitin-PS (+) groups (Figure I-1).

In addition, the severity of tissue damage was evaluated by histologically scoring hematoxylin-eosin–stained sections. The histological scores of the chitin nanofibrils (+) group were significantly decreased on day 5 compared with those of the control (+) group ($p < 0.01$) and on day 6 compared with those of the control (+) and chitin-PS (+) groups ($p < 0.01$, FigureI- 2).

3.4 Effects of chitin nanofibrils on the number of MPO-positive colon cells in DSS-induced acute UC mice

The results of MPO staining on day 6 are shown in Figure I-3, and the numbers of MPO-positive cells in each group are shown in Figure I-4. In the control (−), chitin nanofibrils (−), and chitin-PS (−) groups, 0–1 MPO positive cells were observed per ×400 field (data not shown). In the control (+) and chitin nanofibrils (+) groups, the numbers of MPO-positive cells gradually increased from day 3 to day 6. In the chitin nanofibrils (+) group, however, the numbers of MPO-positive cells were significantly lower than those in the control (+) group on days 3, 5, and 6 ($p < 0.01$). Moreover, significantly fewer MPO-positive cells were counted in the chitin nanofibrils (+) group than in the chitin-PS (+) group on days 3, 5, and 6 ($p < 0.01$ for days 3 and 5, $p < 0.05$ for day 6).

3.5 Effects of chitin nanofibrils on serum IL-6 concentrations in DSS-induced

acute UC mice

On day 5, the serum IL-6 concentration was significantly lower in the chitin nanofibrils (+) group (85.8 ± 1.2 pg/ml) than in the control (+) group (237.1 ± 41.9 pg/ml) ($p < 0.01$).

4. Discussion

In this study, we evaluated the preventive effects of chitin nanofibrils in a mouse model of DSS-induced experimental acute UC. Chitin nanofibrils improved clinical symptoms, colon inflammation, and histological tissue injury in the DSS-induced acute UC mouse model. As MPO is a marker of oxidative stress, high MPO activities were observed in a DSS-induced UC model (Naito *et al.*, 2007; Schindhelm *et al.*, 2009). IL-6 is a central cytokine in IBD that contributes to enhanced T-cell survival and apoptosis resistance in the lamina propria at sites of inflammation (Mudeter & Neurath, 2007). Thereby, chitin nanofibril suppressed inflammation caused by acute UC by suppressing the MPO-mediated activation of inflammatory cells such as leukocytes and decreasing serum IL-6 concentrations.

Chitin-PS is a solid, whereas chitin nanofibrils is a gel. Solid chitin induces chemotactic neutrophil migration in a concentration-dependent manner and activates the complement system in an alternative pathway depending on its degree of deacetylation (Usami *et al*, 1994; Minami *et al*, 1998). However, 50% deacetylated chitin in a homogeneous system becomes water-soluble (Kurita *et al.*, 1977) and loses its effects on the complement system (Suzuki *et al*, 2000). Whether chitin nanofibrils can activate the complement system is unclear;

however, the water solubility (dispersion) of chitin nanofibrils is resembles that of 50% deacetylated chitin. We speculate that the differences in the findings between chitin-PS and chitin nanofibrils groups are mostly due to the difference in their hydrophilicities.

It is suggested that the size of chitin determines its effects on immune cells (Da Silva *et al.*, 2008). This can readily be observed via comparisons of large chitin polymers that are biologically inert and intermediately sized (40–70 μm) fragments that trigger inflammation and inflammatory cytokine production (Lee *et al.*, 2008). The chitin powder used in this study has a diameter of approximately 200 μm. In our results, chitin-PS had no suppressive effects on UC model mice. It is suggested that the preparation of chitin nanofibrils confers new bioactivity to chitin. In a clinical study of CD patients, the oral administration of a chitosan and ascorbic acid mixture did not affect disease activity (Tsujikawa *et al.*, 2003). However, to the best of our knowledge, no previous study has reported preventive or therapeutic effects of chitin or chitin derivatives on IBD.

Currently, many medical treatments are used for IBD patients: 5-aminosalicylic acid drugs such as sulfasalazine or balsalazide, immunomodulators such as thiopurines (azathioprine, 6-mercaptopurine), methotrexate, and biologic therapies that target TNF-α or IL-6 (Nakamura *et al.*, 2006; Morrison *et al.*, 2009). 5-Aminosalicylic acids drugs are expensive drugs that are well tolerated by most people with a low rate of adverse events (Morrison *et al.*, 2009). However, 5-aminosalicylic acid has low efficacy against moderate UC (Carter *et al.*, 2004). Immunomodulators are the mainstay of treatment in maintenance therapy for patients with more than mild CD and for chronically active or frequently relapsing

UC where 5-aminosalicylic acid drugs have failed (Lichenstein *et al.*, 2006). Biologic therapies are used for induction (to get the disease under control) and the long-term maintenance of moderately to severely active disease that has not responded to conventional treatment. However, immunomodulators and biologic therapies increase the risk of serious infection (Morrison *et al.*, 2009). Optimal therapy for IBD is not still established. It was described that some nutritional supplements are beneficial for IBD including amino acids (Coëffier *et al.*, 2010), omega-3 fatty acids (Rajendran & Kumar, 2010), d-glucosamine hydrochloride (Yomogida *et al.*, 2008), dietary fibers (Rodríguez-Cabezas *et al.*, 2002), and probiotics (Vanderpool *et al.*, 2008). Our results suggested that chitin nanofibrils has potential as a nutritional supplement for IBD patients. In our study, chitin nanofibrils did not cause body weight losses and show side effects in gross pathology (data not shown). However, more careful evaluation of the side effects of chitin nanofibrils is necessary before its use in IBD patients. In IBD patients, mucosal lymphocytes and intracellular markers (mitogen-activated protein kinase and nuclear factor-κB) are related with mucosal inflammation (Scaldaferri *et al.*, 2010). Studies that focus on the cells and molecules in the mucosa are expected to elucidate the anti-inflammatory mechanism of chitin nanofibrils.

In conclusion, our results indicated that chitin nanofibrils improved clinical symptoms, inhibited colonic inflammation, and prevented tissue injury in DSS-induced acute UC mice. Furthermore, chitin nanofibrils inhibited mucosal inflammation by suppressing the MPO-positive cells such as leukocytes and decreasing serum IL-6 concentrations. Conversely, chitin powder was not effective in our DSS-induced acute UC model. More developmental research is

necessary before the use of chitin nanofibrils as a functional food for IBD patients.

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Figures and tables

Table I-1. Scoring of inflammation based on clinical parameters during treatment

Score	Weight loss	Diarrhea score	Visible fecal blood
0	<5%	Normal	Normal
1	5–10%	Slightly loose feces	Slightly bloody
2	10–20%	Loose feces	Bloody
3	>20%	Water diarrhea	Blood in entire colon

Table I-2. Effect of chitin nanofibrils administration on the DAI in DSS-induced acute UC mice

	Day0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	1.1 ± 0.4	3.6 ± 0.3	6.9 ± 0.5
Chitin nanofibrils	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1*	2.3 ± 0.3*††	5.1 ± 0.4*†
Chitin-PS	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	1.0 ± 0.4	4.0 ± 0.5	7.0 ± 0.8

*$p < 0.05$ compared with chitin nanofibrils (+) and control (+) groups, ††$p < 0.01$ compared with chitin nanofibrils (+) and chitin-PS (+) groups, and †$p < 0.05$ compared with chitin nanofibrils (+) and chitin-PS (+) groups.

Table I-3. Effect of chitin nanofibrils administration on colon length and the colon weight/length ratio (mg/cm) in DSS-induced acute UC mice

(a)

	(−)	day 3	day 5	day 6
Control	7.4 ± 0.1	6.7 ± 0.2	5.1 ± 0.2	4.9 ± 0.1
Chitin nanofibrils	7.3 ± 0.1	7.5 ± 0.2*†	7.0 ± 0.1**††	6.3 ± 0.1**††
Chitin-PS	7.4 ± 0.2	6.6 ± 0.2	5.7 ± 0.1	5.3 ± 0.2

(b)

	(−)	day 3	day 5	day 6
Control	26.8 ± 0.8	31.9 ± 1.5	50.2 ± 3.9	40.8 ± 2.7
Chitin nanofibrils	29.8 ± 0.3	29.8 ± 0.5	38.4 ± 1.2*	33.3 ± 1.2*†
Chitin-PS	28.8 ± 1.7	27.2 ± 0.5	37.4 ± 1.4*	41.7 ± 2.5

*$p < 0.05$, **$p < 0.01$ compared with the control (+) on the respective day; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ compared with the control (+) on the respective day.

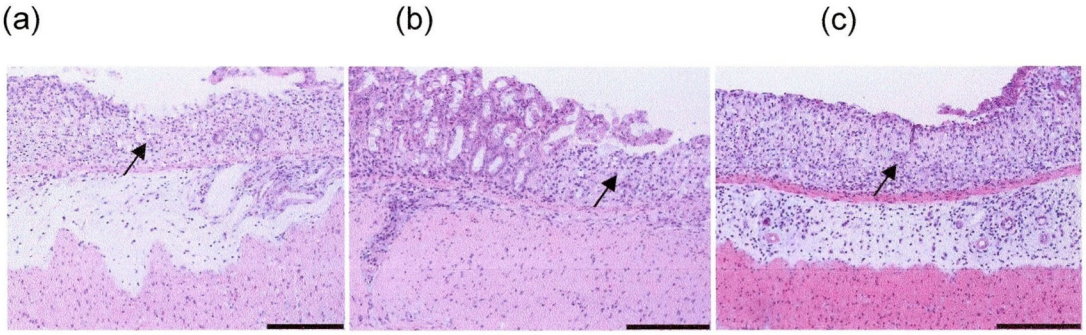

Figure I-1. Effect of chitin nanofibrils administration on histopathological changes in DSS-induced acute UC mice.

The colon was fixed, and tissue sections were stained with hematoxylin and eosin. Data are presented for 1 mouse each from the control (+) (a), chitin nanofibrils (+) (b), and chitin-PS (c) groups on day 6. Erosion indicated by allow. Bar = 100 μm.

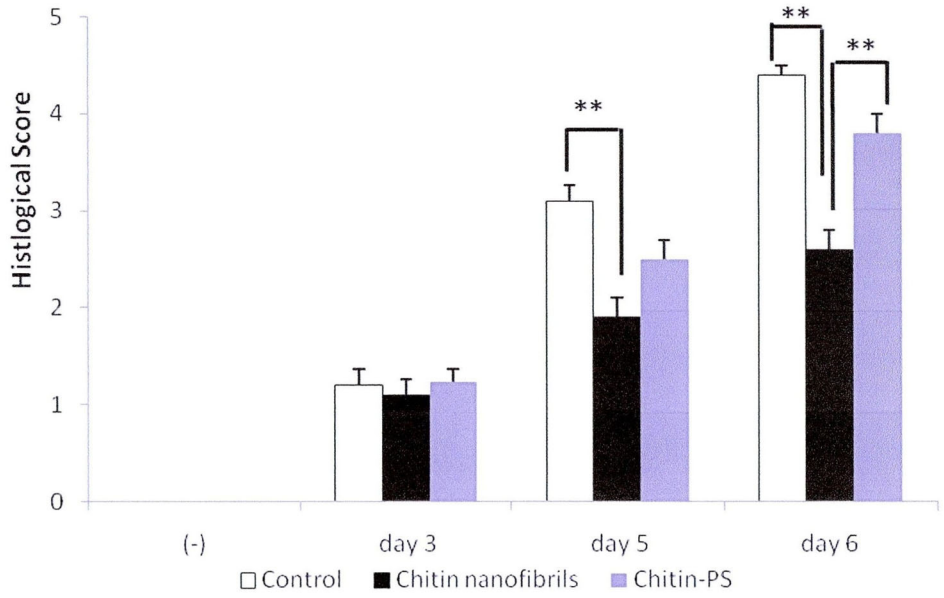

Figure I-2. Effect of chitin nanofibrils administration on the histological damage score of the intestinal mucosa in DSS-induced acute UC mice.

Data represent the means ± S.E. of 30 fields/×100 Field in each group. Values are compared among control (+), chitin nanofibrils (+) and chitin-PS (+) groups. **p<0.01

(a) (b) (c)

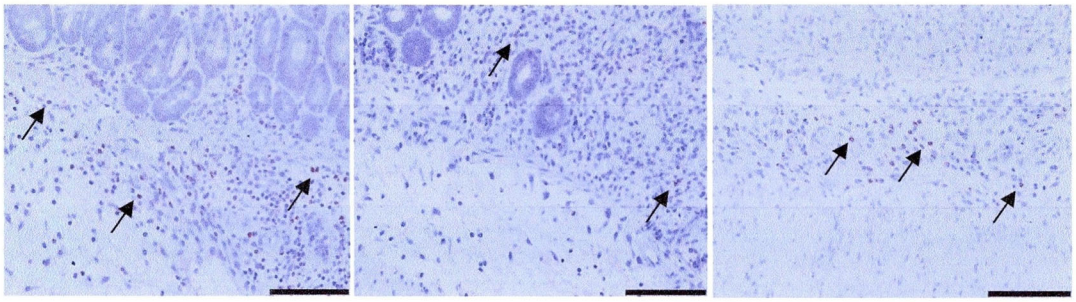

Figure I-3. Effect of chitin nanofibrils administration on the number of MPO-positive cells in the colons of DSS-induced acute UC mice.

MPO positive cells indicated by arrows. Data are from one of 3 mice in the control. (+) (a), chitin nanofibrils (+) (b) and chitin-PS (+) (c) on day 6. Bar: 100μm

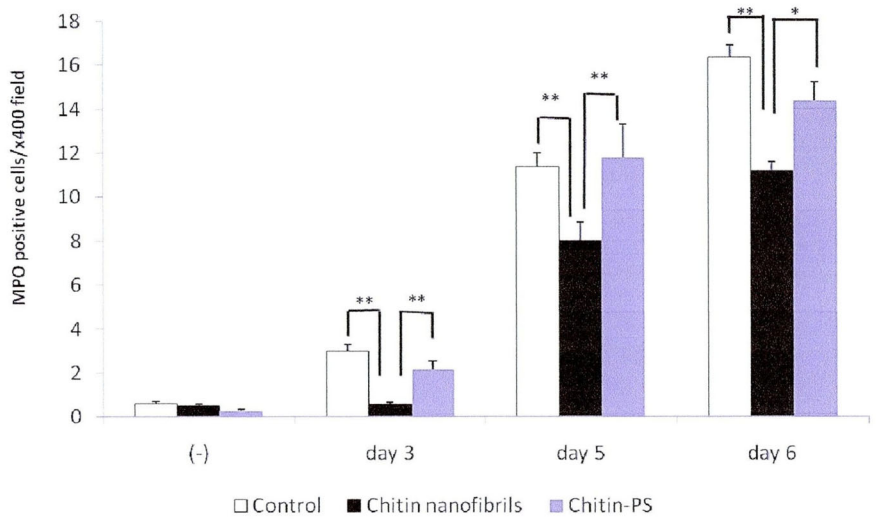

Figure I-4. Effect of chitin nanofibrils administration on the MPO-positive cells counts/ ×400 field in the colons of DSS-induced acute UC mice.

Data represent the means ± S.E. of 60 fields/×400 Field in each group. Values are compared among control (+), chitin nanofibrils (+) and chitin-PS (+) groups. **p<0.01

Chapter II

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

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-κ B 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) are widely distributed in nature and are the second abundant polysaccharide after cellulose (Muzzarelli , 2011a). Two chitin polymorphs are known: the α- and β-chitins (Khouhsab & 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 *et al.*, 2009).

The methods employed to prepare chitin nanofibrils (CNs) include acid hydrolysis (Revol & Marchessault, 1993; Gopalan & Dufresne, 2003), 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 *et al.*, 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; Muzzarelli, 2011c; Muzzarelli, 2012a; Muzzarelli, 2012b). It is also expected that chitin nanofibrils have a potential for a new functional food or drink in various disease. 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. Materials and Methods

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 (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 wavelength 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-κ B (*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 minutes. Then, the sections were washed with PBS and incubated with 1% hydrogen peroxide methanol for 30 minutes 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 minutes 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 were 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 were performed. The results of MT staining were shown in FigureII-1 A. The area of collagen deposition was shown by arrows. In the control (+) and chitin-PS (+) group, much collagen deposition

were observed (FigureII-1 A (a) and (c)). In the α-chitin nanofibrils (+) group, the areas of collagen deposition were meekly decreased (FigureII-1 A (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 FigureII-1 B. 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 FigureII-2 A. 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. The percentages of positive areas of NF-κB in epithelium cells are shown in FigureII-2 B. 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 Figure II-3. In the α-chitin nanofibrils (+) group, serum MCP-1 concentration was significantly lower than the control (+) groups ($p<0.05$).

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-kB occupies a pivotal position in several innate immune signaling

pathways. So far, it has been shown that NF-kB 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 *et al.*, 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 (Nakamura *et al.*, 2006; Morrison *et al.*, 2009). 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.

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Figures and tables

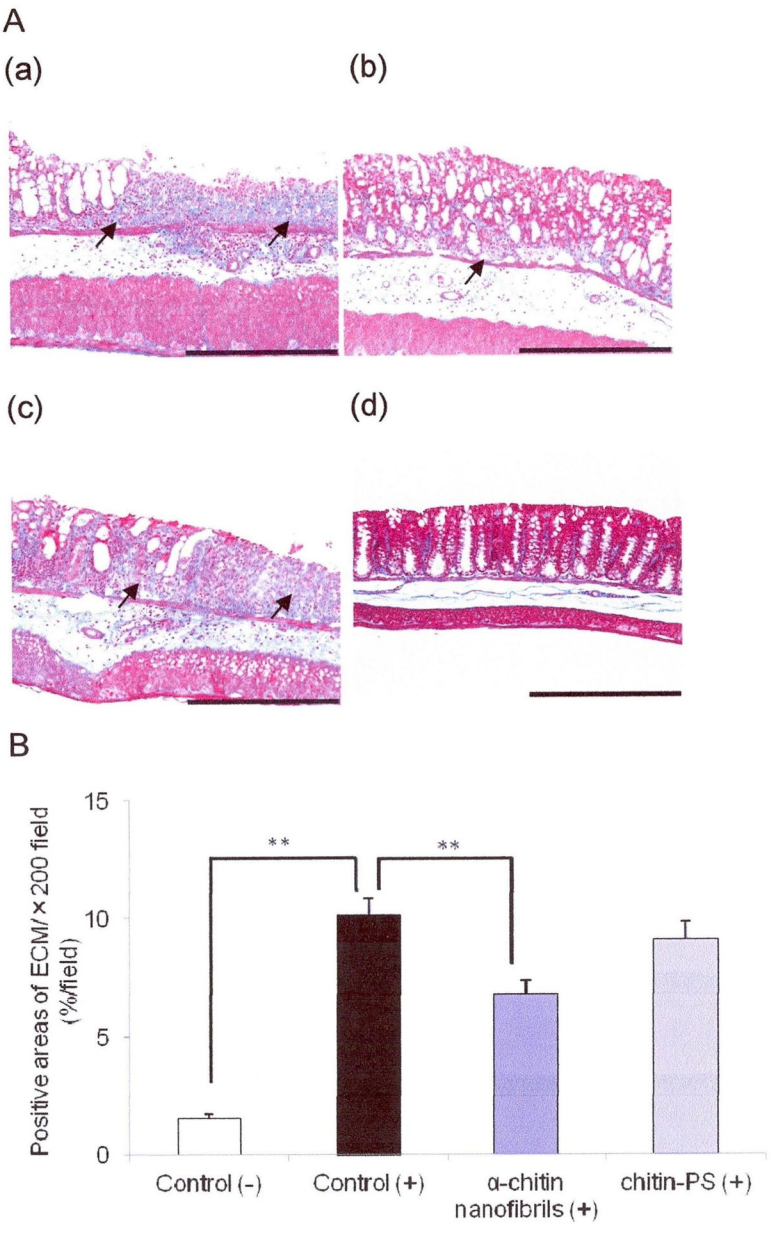

Figure II-1. Effects of α-chitin nanofibrils on colon fibrosis in a DSS-induced acute UC mouse model.

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. *: $p < 0.05$, **: $p < 0.01$

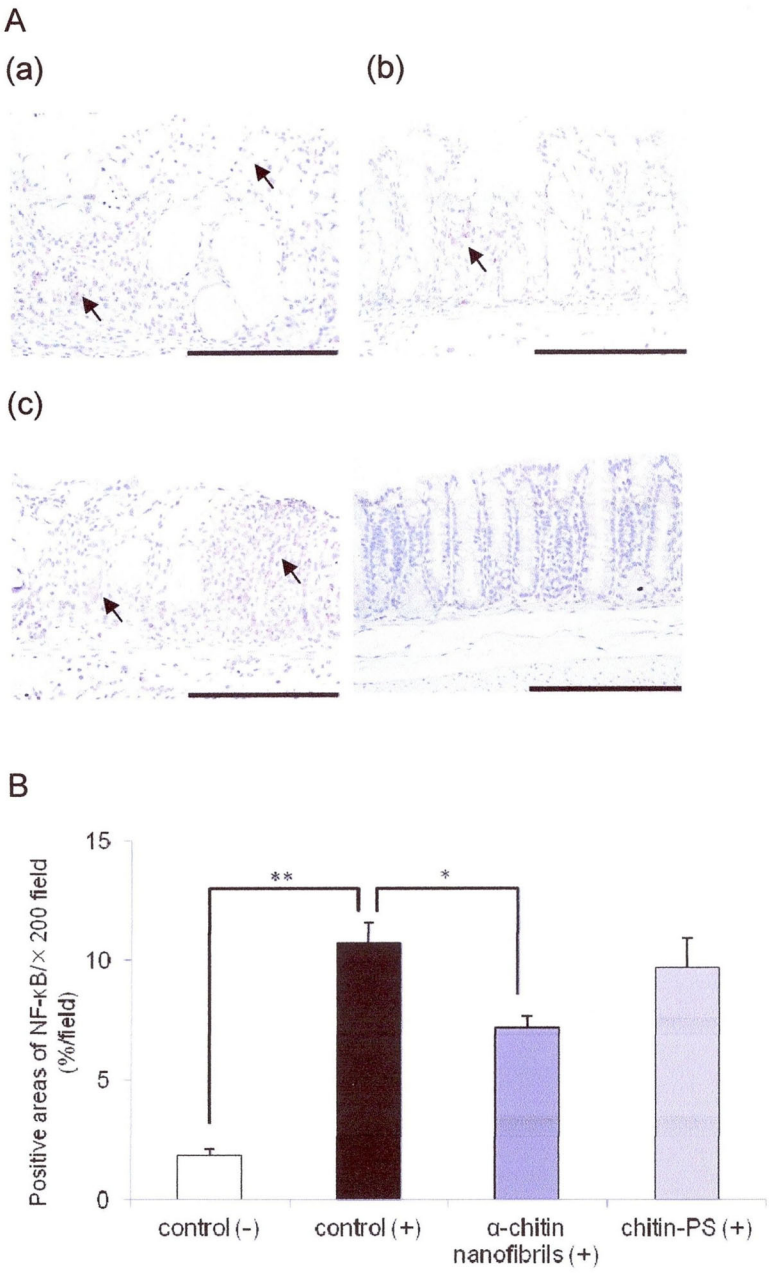

Figure II-2. Effects of α-chitin nanofibrils on colon NF-κB activation in a DSS-induced acute UC mouse model.

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. *: $p < 0.05$, **: $p < 0.01$

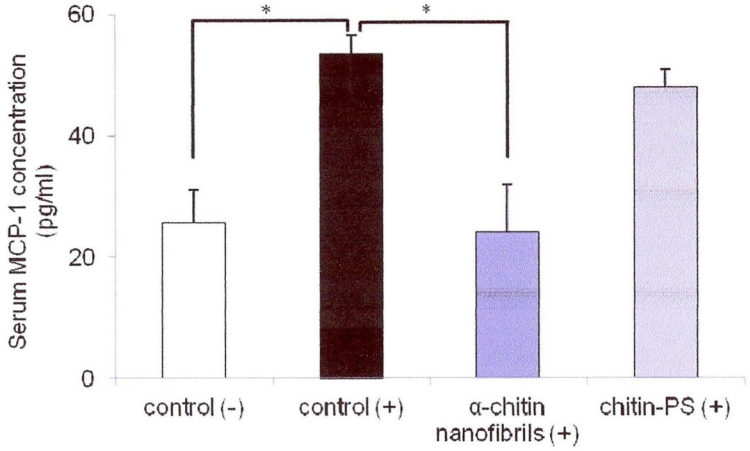

Figure II-3. Effects of α-chitin nanofibrils on serum MCP-1 concentrations in a DSS-induced acute UC mouse model.

Data represent the means ± S.E. in each group (n = 5). The statistical analyses were performed with a Tukey-Kramer test. *: $p < 0.05$, **: $p < 0.01$

Chapter III.

Suppressive effects of N-acetyl-D-glucosamine on rheumatoid arthritis mouse models

Abstract

We examined effects of N-acetyl-D-glucosamine (GlcNAc) on rheumatoid arthritis (RA) mouse models and effects of GlcNAc and glucosamine hydrochloride (GlcN) on several serum cytokine productions in RA mouse models. SKG/jcl mice were divided into control, GlcNAc, and GlcN groups. For 56 days, the control group received normal food, the GlcNAc group received 0.5% GlcNAc-containing food, and the GlcN group received 0.5% GlcN-containing food. GlcNAc and GlcN equally suppressed arthritis scores and histopathological scores compared to the control group. In the GlcN group, serum tumor necrosis factor-α and interleukin (IL)-6 concentrations were significantly decreased compared to the control group. In the GlcNAc group, serum IL-10, transforming growth factor-β-1, and IL-2 concentrations were significantly increased compared to the control group. Our results indicated that GlcNAc also has suppressive effects on experimental RA in mouse models. The results of serum cytokine concentrations suggested that compared to GlcN, GlcNAc has a different suppressive mechanism in experimental RA models.

1. Introduction

Glucosamine hydrochloride (GlcN) and N-acetyl-D-glucosamine (GlcNAc) are widely used as supplements for treating osteoarthritis (Anderson, Nicolosi, & Borzelleca, 2005; Chen, Shen, & Liu, 2010). Some reports indicate different bioactivities between GlcN and GlcNAc *in vitro*. Differences in GlcN and GlcNAc uptake and their subsequent effects on glucose transport, glucose transporter (GLUT) expression, and synthesis of sulfated glycosaminoglycans (sGAGs) and hyaluronan have been reported (Shikhman *et al.*, 2009). GlcN suppressed interleukin (IL)-8, prostaglandin E_2, and nitric oxide synthesis on IL-1β mediated synoviocytes; however, GlcNAc did not show such effects (Hua *et al.*, 2007). Corticosteroids, nonsteroidal anti-inflammatory drugs, and disease-modifying anti-rheumatic drugs (methotrexate and biologics) are used for rheumatoid arthritis (RA) patients. However, these drugs have various side effects (Ngian, 2010). GlcN and GlcNAc are safe supplements (Anderson *et al.*, 2005; Chen *et al.*, 2010). GlcN has preventive effects on experimental rat models of RA (Hua *et al.*, 2005). However, there have been no reports of the effect of GlcNAc on experimental mouse models of RA *in vivo*.

The SKG/Jcl mouse is thought to be an appropriate animal model for human RA (Sakaguchi *et al.*, 2003). In SKG/jcl mice, cytokines such as tumor necrosis factor-α (TNF-α), IL-6, and IL-10 play an important role in the development of arthritis (Hata *et al.*, 2004).

The aim of this study was to identify the effects of GlcNAc on experimental models of RA using SKG/jcl mice. Furthermore, we investigated differences in the mechanism of action between GlcNAc and GlcN on an experimental RA

mouse model using serum cytokine measurements.

2. Materials and Methods

2.1. Animals

Seventeen SKG/Jcl mice (female; 6 weeks old; weight, 18–22 g) were purchased from CREA Japan (Osaka, Japan). The animals were kept under conventional conditions, and all experiments were performed with the approval of the Animal Research Committee of Tottori University. To induce severe chronic arthritis in SKG mice, a single intraperitoneal injection of 30 mg laminarin (Sigma Chemical Co.; St. Louis, USA) was administered to the mice after 7 days of acclimation (Yoshitomi *et al.*, 2005).

2.2. Materials

N-acetyl-D-glucosamine (GlcNAc) was supplied by Yaizu SuisannKagaku Industry Co., Ltd., (Shizuoka, Japan). D-Glucosamine hydrochloride (GlcN) was supplied by Koyo Chemical Co., Ltd., (Tokyo, Japan). Mice were divided into the following 3 groups: control group (control; n = 6), GlcNAc-fed group (GlcNAc; n = 6), and GlcN-fed group (GlcN; n = 5). Control mice were fed a normal powdered diet (CE-2; CREA Japan, Osaka, Japan). GlcNAc and GlcN mice were fed a powdered diet with addition of 0.5% GlcNAc or GlcN for 56 days. GlcNAc and GlcN mice were fed 500–600 mg/kg/day of GlcNAc or GlcN.

2.3. Experimental Protocol

Arthritis scores were measured 7 days after the laminarin injection (day 0) until

day 56, every 7 days. Blood was collected via cardiac puncture from each mouse under 4% isoflurane inhalation anesthesia. After 1 hour at room temperature, serum was recovered by centrifugation of the blood at 1,000 ×g for 10 minutes at 4°C. The serum samples were stored at -80°C until cytokine analysis. After blood collection, animals were immediately sacrificed by cervical dislocation, and the left hand and foot were harvested.

2.4. Arthritis scores

Joint swelling was monitored through inspection by an unbiased observer, who compared reference images and allotted scores as follows: 0, no joint swelling; 0.1, swelling of 1 finger joint; 0.5, mild swelling of wrist or ankle; 1.0, severe swelling of wrist or ankle. Scores for all fingers of forepaws and hind paws and wrists and ankles were totaled for each mouse (Sakaguchi *et al.*, 2003).

2.5. Histopathological evaluation

Wrist and ankle joints were fixed in 10% buffered formalin. Thin sections (3 μm) were prepared from each sample for histological observation with hematoxylin-eosin staining. Histopathological index in the ankle and foot were scored (Lee *et al.*, 2005) as follows: 0, normal; 1, weak leukocyte infiltration without erosion; 2, modest infiltration and weak erosion; 3, severe infiltration and invasion into bones; and 4, loss of bone integrity. Scores for left hands and feet were totaled for each mouse.

2.6. Evaluation of serum cytokine

Serum cytokine concentrations were measured with a Mouse Th1/Th2 10plex Kit (eBioscience, San Diego, USA) for tumor necrosis factor-α, IL-2, IL-6, and IL-10. A sandwich enzyme-linked immune sorbent assay kit was used for measurement of transforming growing factor-β1 (TGF-β1, R&D Systems, Minneapolis, USA). Cytokines were measured according to the manufacturer's protocols.

2.7. Statistical Analysis

Statistical analyses were performed on all results using one-way ANOVA and Tukey-Kramer's test. All data were reported as mean ± SD. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Arthritis score

Swelling of the finger joints was observed at day 28 in all groups. Swelling of wrist and ankle joints appeared at day 35 in the control group and day 42 in the GlcNAc and GlcN groups. At days 35, 42, 49, and 56, arthritis sores were significantly depressed in the GlcNAc and the GlcN groups compared to the control group (Figure III-1).

3.2. Histopathological evaluation

Histopathological findings of the ankle joints (hematoxylin-eosin stained specimens) are shown in Figure III-1. Severe synovial hyperplasia, bone

erosion, pannus formation, and leukocyte invasion of the wrist and ankle joints were observed in the control mice (Figure III-2 a). The GlcNAc and the GlcN group mice had less of these changes compared to the control mice (Figure III-2 b and c).

The histopathological indices at day 56 were similar to the arthritis scores of each group. The histopathological indices of the GlcNAc and GlcN groups were significantly lower than those of the control group ($p < 0.05$, Table III-1).

3.3. Evaluation of serum cytokine concentration

Serum concentrations of TNF-α and IL-6 were significantly decreased in the GlcN group compared to the control group (Table III -1). Serum IL-10 concentration in the GlcNAc group was significantly increased compared to the control and GlcN groups ($p < 0.01$, Table III-1). Serum transforming growth factor-β1 and IL-2 concentrations of the GlcNAc group were significantly increased compared to the control group ($p < 0.05$, Table III-1).

4. Discussion

GlcN suppressed the progression of adjuvant arthritis in rats (Hua *et al.*, 2005). Consistent with previous reports, our results indicated that GlcN suppressed the progression of arthritis in SKG/jcl mice, which are human RA mouse models. However, no studies have reported the effects of GlcNAc on experimental RA mice in vivo. Our results indicate that GlcNAc has suppressive effects on experimental RA mouse models.

In RA patients, corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), and disease-modifying anti-rheumatic drugs (methotrexate and biologics) are used to control clinical symptoms (Hawkey *et al.*, 1998). In fact, meloxicam, which is an NSAID, suppressed strongly clinical symptoms in SKG/jcl mice, compared to GlcNAc and GlcN mice (date are not shown). However, meloxicam has been found to induce side effects such as gastrointestinal ulcers in 13% patients (Hawkey *et al.*, 1998). No side effects have been reported with administration of GlcNAc or GlcN (Anderson *et al.*, 2005; Chen *et al.*, 2010).

Both GlcNAc and GlcN significantly suppressed arthritis scores and histopathological indices. In the GlcN group, serum TNF-α and IL-6 concentrations significantly decreased compared to the control group. TNF-α and IL-6 are important cytokines that contribute to joint inflammation in SKG/jcl mice (Hata *et al.*, 2004). GlcN suppresses IL-1β-induced phosphorylation of p38 mitogen-activated protein kinase in synoviocytes (Hua *et al.*, 2007). GlcN also reduces the production of nitric oxide and prostaglandin E2 in plasma in vivo (Hua *et al.*, 2005). GlcN moderates IL-1 induced activation of chondrocytes by increasing the expression of mRNA encoding type II IL-1β receptor and decreasing the activation of nuclear factor κB (Bianchi *et al.*, 2002). These reports indicate that GlcN has anti-inflammatory effects in RA. In the present study, GlcN may have produced anti-inflammatory effects on RA via the previously described mechanisms (Bianchi *et al.*, 2002; Hua *et al.*, 2005; Hua *et al.*, 2007).

In the GlcNAc group, serum IL-10, TGF-β1, and IL-2 concentrations were increased compared to the control group. Recently, CD4+ CD25+ Foxp3+

regulatory T-cells (Treg) have been reported as a therapeutic target of autoimmune diseases. Forkhead box P3 (Foxp3), a transcription factor, is a key regulatory gene for the development of Tregs (Sakaguchi *et al.*, 2006). IL-2 is important for proliferation and maintenance of Treg. IL-10 and TGF-β1 are released from Tregs (Nistala & Wedderbum, 2009). Our results suggest that GlcNAc stimulates Treg function, and predominant Treg conditions may suppress arthritis. However, no studies have reported the effects of GlcNAc or GlcN on Treg or Foxp3 in vitro or in vivo. Further investigation of GlcNAc or GlcN on Treg regulation, including Foxp3 in peripheral blood and joint tissue, should be performed to understand the mechanism underlying RA regulation by GlcNAc. Our results indicate that GlcNAc and GlcN have different mechanisms of action. A synergistic effect may be expected with the combination of GlcNAc and GlcN.

In conclusion, GlcNAc suppressed not only finger, wrist, and ankle swelling but also the histological changes in experimental RA mouse models. Compared to GlcN, GlcNAc had different mechanisms of action on RA, especially in increasing serum IL-10, TGF-β1, and IL-2 concentrations. Our results indicate that GlcNAc is also a potential supplement for RA patients.

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Figures and Tables

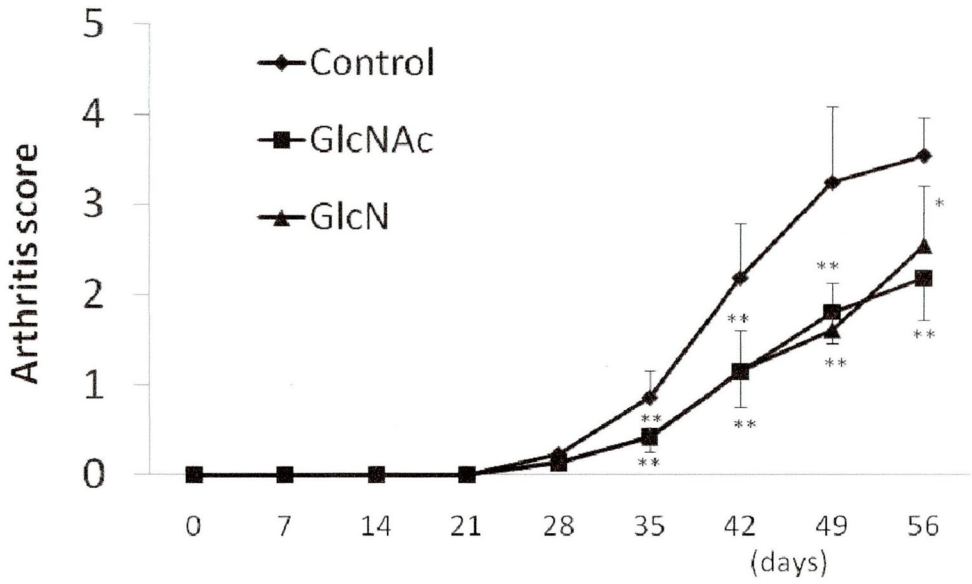

Figure III-1. Effects of administration of GlcNAc on arthritis score in SKG/jcl mice.

Each data represents the mean ± S.D. of 5–6 mice in each group. **: $p < 0.01$ compared to the control group. *: $p < 0.05$ compared to the control group.

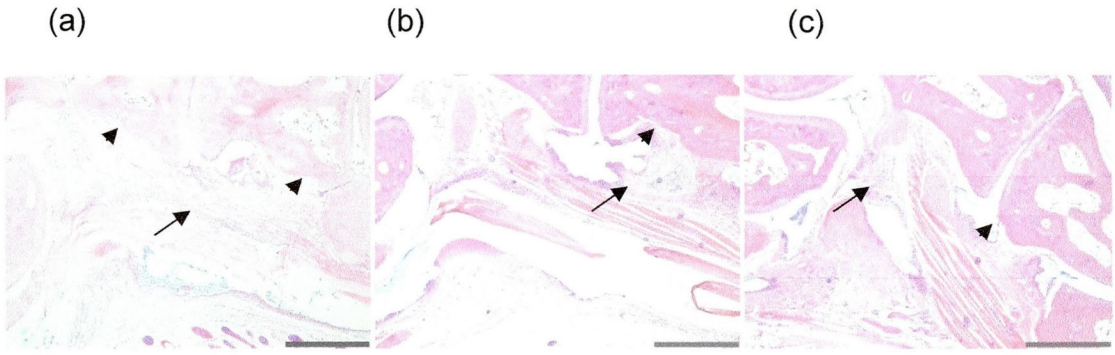

Figure III-2. Effects of GlcNAc and GlcN on histopathological changes of ankle joints in SKG/jcl mice.

All images are from 1 of 5–6 mice in the control (a), GlcNAc (b), and GlcN (c) groups. Synovial hyperplasia and bone erosion are indicated by the arrow and arrowhead. Bar: 500 μm

Table III-1. Effects of GlcNAc on histopathological score and serum cytokine concentration in SKG/jcl mice.

	Control	GlcNAc	GlcN
Histopathological index	6.5 ± 1.3	4.5 ± 1.0*	4.3 ± 0.6*
Serum TNF-α (pg/ml)	13.5 ± 1.7	4.5 ± 4.4	3.8 ± 0.9**
IL-6 (pg/ml)	73.3 ± 18.6	44.9 ± 30.8	25.2 ± 3.2*
IL-2 (pg/ml)	13.9 ± 9.4	46.0 ± 17.8*	19.6 ± 2.5
IL-10 (pg/ml)	18.7 ± 12.4	104.5 ± 39.3**†	25.2 ± 4.2
TGF-β1 (pg/ml)	230.4 ± 33.6	334.6 ± 52.1*	282.9 ± 159.7

Each value represents the mean ± S.D. of 3–4 mice in each group. **: $p < 0.01$ compared to control group. *: $p < 0.05$ compared to control group. †: $p < 0.05$ compared to GlcN group.

Chapter IV.

Effects of Oral Glucosamine Hydrochloride Administration on Plasma Free Amino Acid Concentrations in Dogs

Abstract

We examined the effects of oral glucosamine hydrochloride (GlcN) and N-acetyl-D-glucosamine (GlcNAc) administration on plasma free amino acid (PFAA) concentrations in a horse and dogs. In the horse, PFAA concentrations increased after oral GlcN administration. Plasma ammonia concentration increased after administration of GlcN. In contrast, in dogs, PFAA concentration decreased after GlcN administration compared to controls. No change in PFAA concentration was observed after administration of GlcNAc. Our results suggested the existence of species-specific differences in PFAA dynamics after oral administration of GlcN. Our results also suggested the existence of differences in PFAA dynamics after oral administration of GlcN and GlcNAc in dogs

1. Introduction

Glucosamine hydrochloride (GlcN) and N-acetyl-D-glucosamine (GlcNAc) are components of glycosaminoglycan and now widely used as dietary supplements (Gregorym, Speny, & Wilson, 2008). Moreover, GlcN is useful for the treatment

of joint diseases both in humans and in veterinary medicine, including dogs and horses (Goodrich, 2006; Minami et al., 2011). The bioavailability of GlcN has been reported as 26% in humans (Barclay, Tsourounis, & McCart, 1998), 19% in rats (Aghazadeh-Habashi, Sattari, Pasutto, & Jamali, 2002), 12% in dogs (Adebowale, Du, Liang, Leslie, & Eddington, 2002), and 2–6.1% in horses (Du, White, & Eddington, 2004; Laverty et al., 2005; Meulyzer et al., 2008). These results suggest the presence of a species-specific difference in GlcN absorption and metabolism. Different biological activities between GlcN and GlcNAc have also been demonstrated *in vitro*. For example, differences in GlcN and GlcNAc uptake and their subsequent effects on glucose transport, glucose transporter (GLUT) expression, and sulfated glycosaminoglycans (sGAG) and hyaluronan synthesis have been reported (Shikhman, Brinson, Valbracht, & Lotz, 2009).

In an experimental rabbit model of cartilage injury, oral administration of GlcN or GlcNAc led to regeneration of both glycosaminoglycan and proteoglycan (Tamai et al., 2002; Tamai et al., 2003). GlcN has a potential to exert a chondroprotective action on an experimental induced osteoarthritis (OA) by inhibiting type II collagen degradation and enhancing type II collagen synthesis in the articular cartilage (Naito et al., 2010). These results suggested that synthesis of type II collagen and proteoglycan core protein as well as glycosaminoglycan occurs upon GlcN and GlcNAc supplementation. However, no reports to date have investigated the relationship between oral administration of amino monosaccharide and amino acid synthesis.

The aim of this study was to examine the effects of oral GlcN and GlcNAc administration on plasma free amino acid (PFAA) concentrations, as well as

species-specific differences in these effects. Using dogs and a horse, we investigated (PFAA) dynamics after oral administration of GlcN, GlcNAc, or glucose (Glc), which is source of glycosaminoglycan and proteoglycan in a body.

2. Materials and Methods

2.1. Materials

Glucosamine hydrochloride (GlcN) was supplied by Koyo Chemical Co., Ltd., (Tokyo, Japan). N-acetyl-D-glucosamine (GlcNAc) was supplied by Yaizu SuisannKagaku Industry Co., Ltd., (Shizuoka, Japan). D-Glucose (Glc), molecular weight 180.16, was purchased from Wako Pure Chemical (Osaka, Japan).

2.2. Animals

Six healthy beagle dogs, mean age of 4 years (range 2–6 years) and mean body weight 9 kg (range 7–12 kg) and a healthy male pony, 7 years of age and with a body weight of approximately 200 kg were used. The use of these animals and the procedures they underwent were approved by the Animal Research Committee of Tottori University.

2.3. Administration and blood sampling

Dogs were separated into the following groups (n=3 for each group): usual dog food (Hill's-Colgate (Japan) Ltd, Science Diet, Tokyo, Japan) (Control), usual dog food plus GlcN, usual dog food plus GlcNAc, and usual dog food plus Glc. Each saccharide dissolved in water was orally administered at approximately

500 mg/kg to dog. Dog's blood was collected (0 h) in the morning before being feed, then 35 kcal/kg body weight dog food with each saccharide dissolved in 10 ml water (500 mg/kg body weight, single dose) was fed (10 ml water for control group) (first feeding). After blood collection at 1, 2, 4, and 6 hour, all the dog was fed with 35 kcal/kg dog food without saccharide (second feeding), and the blood was collected at 18 hour after second feeding.

In the horse, material was dissolved in tap water and administered at 500 mg/kg body weight using a nasal catheter. This administration was repeated 3 times with at less 7 days interval. The horse was pasture-fed freely before and after administration.

Blood was collected from the jugular vein using heparin as an anti-coagulant. The blood was centrifuged at 3,000 rpm for 10 min, and the plasma was then separated promptly and frozen at -80°C until measurement of PFAA concentrations.

2.4. Measurement of PFAA concentrations

Plasma samples were mixed with equal volumes of 3% (w/w) sulfosalicylic acid, and left to stand at 4°C for 1 h. Samples were then centrifuged (4°C, 15 min, 1,500 rpm), and precipitated protein was removed. The amino acid concentrations were measured by an automatic amino acid analyzer (JLC-500/V2, AminoTac; JEOL, Tokyo, Japan). The amino acids measured are listed in Table 1.

2.5. Measurement of plasma GlcN and GlcNAc concentrations

GlcN or GlcNAc dissolved in water was orally administered at approximately 300 mg/kg to dogs (n = 3 in each group). Blood samples were collected before administration and 0.5, 1, 2, 4, and 24 h after administration. Blood was collected from the jugular vein using heparin as an anti-coagulant. The blood was centrifuged at 3,000 rpm for 10 minutes, and the plasma was then separated promptly. Plasma samples were mixed with four equal volumes of ethanol and centrifuged, and precipitated protein was removed. These samples were treated using a *p*-ethyl 4-aminobenzoate carbohydrate chain labeling kit (Seikagaku Kogyo, Tokyo, Japan). Samples were analyzed quantitatively using high performance liquid chromatography fitted with a reversed-phase column (Honenpak C18, 75 mm × 4.6 mm I.D.) and fluorometer (Ex. 305 nm, Em. 360 nm).

2.6. Statistical analysis

Each amino acid concentration, total amino acid concentration, essential amino acid concentrations and nonessential amino acid concentrations were used for the evaluation. In dogs, total amino acid concentrations were expressed as the percentage of pre-administration values. Student's t-tests were used to assess differences at each time point. A probability of 5% or less was considered statistically significant.

3. Results

Amino acids measured in dogs were shown in Table IV-1. The PFAA concentrations increased in the control dogs and the GlcNAc treated dogs

whereas that from the GlcN- or the Glc-treated dogs significant decrease after one hour (Figure IV-1). The levels of Glu, Gly, and Ala concentrations were significant lower than GlcNAc-treated dogs (Table IV-2). After administration of GlcNAc, no remarkable change was observed in either PFAA concentration or each amino acid level compare to the control.

No change in total or individual amino acid concentrations was observed after administration of GlcNAc compared to the control group. The maximum concentration of GlcNAc in dogs reached about 20 μM after 300 mg/kg GlcNAc administration (Figure IV-2).

Total PFAA concentration increased from 1 h after administration of GlcN. At 6 h after administration, total PFAA concentration (1510.9 ± 210.0 nmol/ml) significantly increased compared to pre-administration levels (930.2 ± 145.5 nmol/ml) (Figure IV-3). Plasma free NH_3 concentration increased significantly at 4 h after administration (116.3 ± 26.1 nmol/ml) compared to pre-administration (19.4 ± 3.6 nmol/ml), 1 h post-administration (33.2 ± 10.8 nmol/ml), and 24 h post-administration (19.3 ± 1.0 nmol/ml) (Figure IV-3).

4. Discussion

In healthy human's report, postprandial PFAA concentrations were raised compared to those before meal (Tokubo, & Ando, 2010). Because of feeding, PFAA increased in the Control dogs and the GlcNAc-treated dogs. However, PFAA decreased compared to the control group after administration of GlcN or

Glc. In an *in vitro* study using mesenchymal stem cells, treatment with 100 μM or 1,000 μM GlcN increased expression of aggrecan and type II collagen. Moreover, 100 μM GlcN treatment led to increased sGAG content (Derfoul, Miyoshi, Freeman, & Tuan, 2007). In plasma GlcN concentration reached 150–300 μM after oral administration of 20 mg/kg GlcN (Reginster, 2004). In dogs, plasma GlcN concentration was reported to reach 50 μM after oral administration of 125 mg/kg GlcN (Adebowale, Du, Liang, Leslie, & Eddington, 2002), and reached 100 μM after oral administration of 300 mg/kg GlcN (Figure 2). Although the maximum plasma GlcN concentration achieved after oral administration of 500 mg/kg GlcN has not been reported, a previous study indicated that it exceeds 100 μM. These findings suggested that high levels of GlcN were provided to tissue by the circulatory system in dogs. Naito K *et al.* (2010) described GlcN has a potential to exert a chondroprotective action on an experimental induced OA by inhibiting type II collagen degradation and enhancing type II collagen synthesis in the articular cartilage. Therefore, proteoglycan and type II collagen were likely to be synthesized actively in cartilage. Gly, Ala and Glu are main component of type II collagen (Deshmukh, & Nimmi, 1973), and the levels of these amino acids became lower than those of the control and GlcNAc after administration of GlcN or Glc. These results suggest GlcN or Glc stimulated proteoglycan and type II collagen synthesis in the dogs.

We did not directly confirm type II collagen and proteogrycan synthesis in dogs after administration of GlcN. However, oral administration of GlcN induced obvious functional recovery in various kinds of canine orthopedic diseases (Minami *et al.*, 2011). To confirm these phenomena occur in dogs is the task to

understand mechanism of GlcN in dog joint diseases.

Equine intestinal absorption of carbohydrates such as GlcN and Glc occurs through glucose transporters located primarily in the duodenum (Dyer et al., 2002), which also expresses high levels of the glucose/glucosamine transporter GLUT-2, a low-affinity transporter of glucose (Km = ± 3.617 mM) and high-affinity transporter of glucosamine (Km = 0.8 mM) (Uldry *et al.*, 2002). In catabolic and metabolic GlcN pathways, glucosamine-6-phosphate deaminase (GNPDA) catalyzes the conversion of glucosamine-6-phosphate to fructose-6-phosphate, resulting in NH3 synthesis (Wolosker *et al.*, 1998). In this study, plasma free NH3 concentration increased significantly at 4 h after oral GlcN administration. This finding indicated that part of the administered GlcN was converted to fructose-6-phosphate and NH3 by deamination of GNPDA.

Recently, it was suggested that glucosamine induces autophagy via mammalian target of rapamycin (Shintani *et al.* 2010). Autophagy has important functions in quality control and anti-aging of the cells. In processing autophagy, glutamine-dependent ammonia production supports basal autophagy and protects against TNF-α-induced cell death (Eng *et al.* 2010). In the intestinal epithelium, autophagy reduces the inflammatory response by inhibiting nuclear factor-κB activation (Fujishima *et al.* 2011). Nagaoka *et al.* (2011) indicated that glucosamine suppressed nuclear factor-κB activation in various inflammatory conditions in vitro and in vivo. Our results indicate the anti-inflammatory effects of GlcN in horse might come from inducing autophagy. Further study must be performed whether GlcN induce autophagy in horse by *in vitro* and *in vivo* experiments.

In this study, we investigated differences in plasma amino acid dynamics after oral administration of GlcN to a horse and dogs. Our results suggested the presence of species-specific differences in the absorption and metabolism of orally administered GlcN. Moreover, our findings indicated differences in absorption and metabolism of GlcN and GlcNAc in dogs. Our results also might indicate that anti-inflammatory effects of GlcN in horse come from inducing autophagy.

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Figures and Tables

Table IV-1. Amino acids measured in this study.

Essential amino acids (EAA)	Nonessential amino acids (NEAA)
Threonine (Thr)	Asparate (Asp)
Valine (Val)	Serine (Ser)
Methionine (Met)	Asparagine (Asn)
Isoleucine (Ile)	Glutamate (Glu)
Leucine (Leu)	Glutamine (Gln)
Phenylalanine (Phe)	Glycine (Gly)
Lysine (Lys)	Alanine (Ala)
Histidine (His)	Tyrosine (Tyr)
Tryptophan (Trp)	Proline (Pro)
Arginine (Arg)	Citrulline (Cit)
	Ornithine (Orn)
	Hydroxyproline (Hypro)

Table IV-2. Significant changing of serum amino acid concentration at 1 h after administration of each saccharide (GlcN, GlcNAc, and Glc) to the dog.

	Control	GlcN	GlcNAc	Glc
Glu	128.3 ± 6.4	98.6 ± 9.0*	184.0 ± 20.7	103.6 ± 10.1*
Gly	126.8 ± 12.3	95.3 ± 5.8*	145.0 ± 6.1	100.9 ± 2.9*
Ala	135.3 ± 21.9	82.8 ± 4.1*	148.3 ± 4.1	85.4 ± 5.6*

Each plasma free amino acid concentration in the pre-administration was considered as 100%.*: $p<0.05$, compared to the levels of the GlcNAc or the control at 1 hour after administration. Data represent the mean ± SE of three dogs in each group. Control dogs were fed only dog food, and the other dogs were fed dog food with each saccharide.

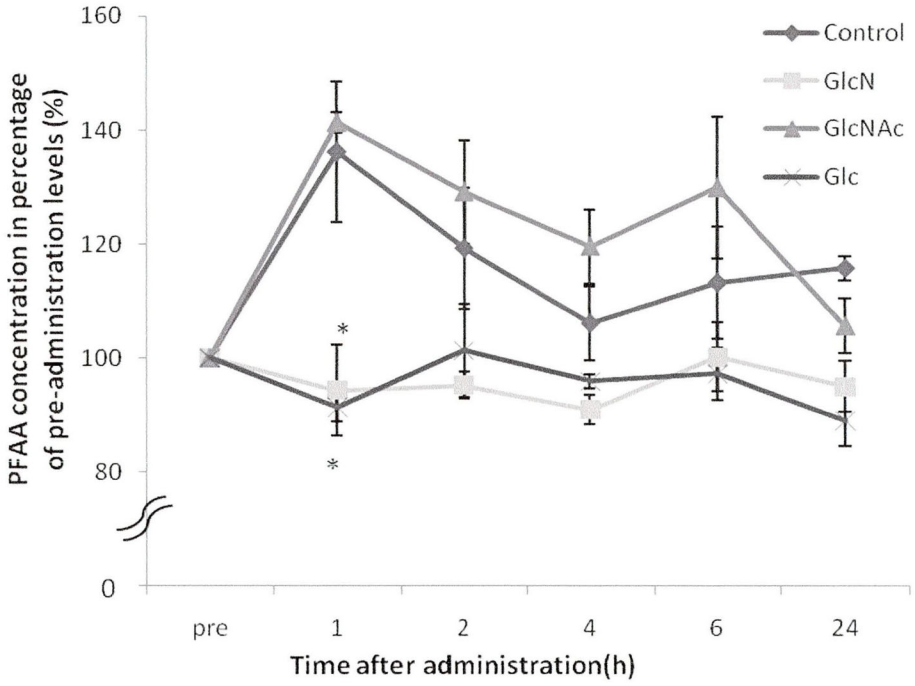


Figure IV-1. Changes in plasma total free amino acid (PFAA) concentration in dogs after each saccharide (GlcN, GlcNAc, and Glc) administration.

Plasma total free amino acid concentration in the pre-administration was considered as 100%.*: $p<0.05$, compared to the level of the control at each hour. Data represent the mean ± SE of three dogs in each group.

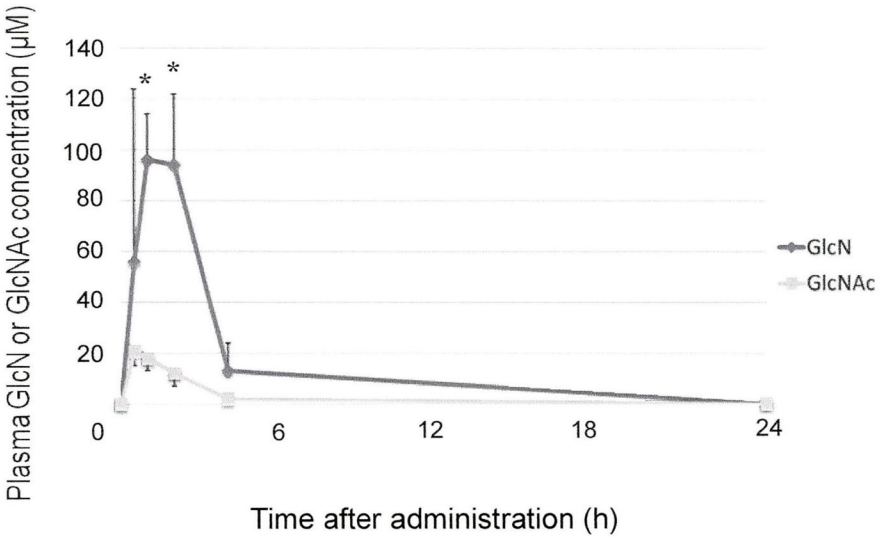
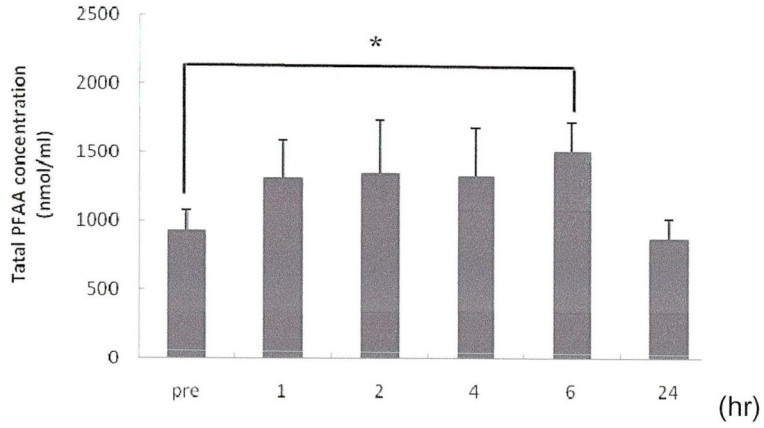


Figure IV-2. Changes in plasma GlcN or GlcNAc concentration after oral GlcN or GlcNAc administration. All data indicate mean ± S.D. *: $P < 0.05$ compared to GlcN group.

(a)



(b)

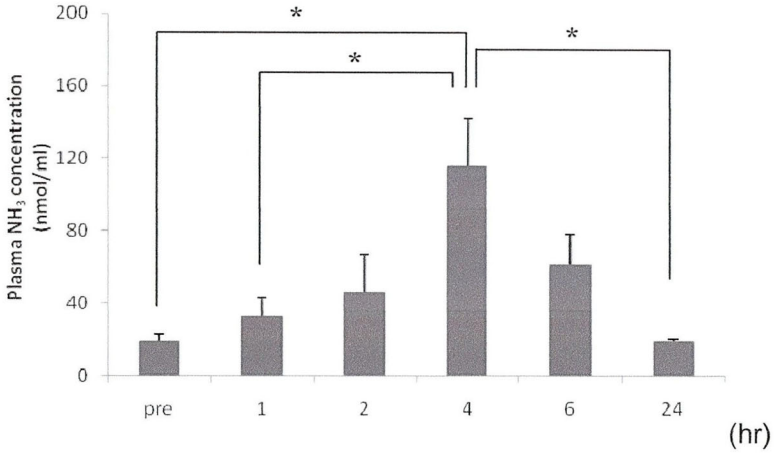


Figure IV-3. Changes in plasma total amino acid and ammonia concentrations after oral GlcN administration in horse. All data indicate mean ± S.D. *: $P < 0.05$.

Conclusions

- Chitin nanofibrils improved clinical symptoms, inhibited colonic inflammation, and prevented tissue injury in IBD mouse model.
- Chitin nanofibrils inhibited mucosal inflammation via suppressing NF-κB activation.
- GlcNAc suppressed experimental inflammation by different mechanisms of action compared to GlcN.
- In the horse, anti-inflammatory effects of GlcN might be come from inducing autophagy.

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