

Chitin nanofibrils suppress skin inflammation in atopic dermatitis-like skin lesions in NC/Nga mice

Ryotaro Izumi^{a,1}, Kazuo Azuma^{b,*}, Hironori Izawa^a, Minoru Morimoto^c, Masaaki Nagashima^d, Tomohiro Osaki^b, Takeshi Tsuka^b, Tomohiro Imagawa^b, Norihiko Ito^b, Yoshiharu Okamoto^b, Hiroyuki Saimoto^a, Shinsuke Ifuku^{a,*}

^a Graduate School of Engineering, Tottori University, Tottori 680-8533, Japan

^b Department of Veterinary Clinical Medicine, Tottori University, Tottori 680-8533, Japan

^c Division of Instrumental Analysis, Research Center for Bioscience and Technology, Tottori University, Tottori 680-8550, Japan

^d Regional Industrial Academic Cooperation, Tottori University, Tottori 680-0833, Japan

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ABSTRACT

We evaluated the effect of chitin nanofibril (CNF) application via skin swabs on an experimental atopic dermatitis (AD) model.

AD scores were lower, and hypertrophy and hyperkeratosis of the epidermis were suppressed after CNF treatment. Furthermore, inflammatory cell infiltration in both the epidermis and dermis was inhibited. CNFs also attenuated histological scores. The suppressive effects of CNFs were equal to those of corticosteroid application; however, chitin did not show these effects. CNF application might have anti-inflammatory effects via suppression of the activation of nuclear factor-kappa B, cyclooxygenase-2, and inducible nitric oxide synthase.

In an early-stage model of experimental AD, CNFs suppressed AD progression to the same extent as corticosteroids. They also suppressed skin inflammation and IgE serum levels. Our findings indicate that CNF application could aid in the prevention or treatment of AD skin lesions.

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1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is increasingly prevalent and has reached epidemic proportions in children worldwide (Odhambo et al., 2009; Williams et al., 2008). Topical corticosteroids are the first-line therapy for the treatment of AD (Eichenfield et al., 2014). Systemic administration of glucocorticoids is effective against AD (Sidbury & Hanifin, 2000), but there are many side effects, including cushingoid features, cataracts, hyperglycemia, moon facies, body weight

loss, hypokalemia, and osteopenia (Harada et al., 2009; Sidbury & Hanifin, 2000).

Previously, the immunologic effects of chitin have been explored (Bae, Shin, Kim, Kim, & Shon, 2013; Da Silva et al., 2009; Nishimura et al., 1984; Shibata, Foster, Bradfield, & Myrvik, 2000). For example, exogenous chitin activates adaptive type-2 allergic inflammation (Shibata et al., 2000). Da Silva et al. (2009) described the size-dependent regulation of innate immunity by chitin. It is described only small chitin (SC; <40 μm, largely 2–10 μm) induced IL-10 elaboration. The effects of intermediate-sized chitin (40–70 μm) were mediated by pathways that involve toll like receptor (TLR) 2, dectin-1, and nuclear factor κB (NF-κB). In contrast, the effects of SC were mediated by TLR2-dependent and -independent, as well as dectin-1-dependent pathways that involved the mannose receptor and spleen tyrosine kinase.

Recently, several methods of preparing chitin nanofibrils have been reported (Azuma, Isuku, Osaki, Okamoto, & Minami, 2014; Ifuku, 2014; Ifuku & Saimoto, 2012). Ifuku et al. (2009) prepared chitin nanofibrils (CNFs) via a simple grinding treatment under an acidic condition. The beneficial effects of CNFs to the skin have previously been reported (Morganti, Muzzarelli, & Muzzarelli, 2006;

* Corresponding authors at: 4-101 Koyama-Minami, Tottori 680-8533, Japan.

E-mail addresses: jastam0ment@gmail.com (R. Izumi),

kazu-azuma@muses.tottori-u.ac.jp (K. Azuma), h-izawa@chem.tottori-u.ac.jp

(H. Izawa), morimoro@chem.tottori-u.ac.jp (M. Morimoto),

nagashima@cjr.tottori-u.ac.jp (M. Nagashima), tosaki@muses.tottori-u.ac.jp

(T. Osaki), tsuka@muses.tottori-u.ac.jp (T. Tsuka), imagawat@muses.tottori-u.ac.jp

(T. Imagawa), taro@muses.tottori-u.ac.jp (N. Ito), yokamoto@muses.tottori-u.ac.jp

(Y. Okamoto), saimoto@chem.tottori-u.ac.jp (H. Saimoto),

sifuku@chem.tottori-u.ac.jp (S. Ifuku).

¹ These authors contributed equally to this work.

Morganti & Morganti, 2008). Their enormous surface allows CNFs to interact with enzymes, platelets, and other cell compounds present in living tissues. Thus, the peculiarity and ability for granulation tissue is accompanied by angiogenesis and regular deposition of collagen fibers with the consequent enhanced and correct repair of dermoepidermal lesions (Morganti & Morganti, 2008; Morganti et al., 2006). Recently, Ito et al. (2014) reported beneficial effects of CNFs to the skin in an experimental skin model. The application of CNFs to skin improved the epithelial granular layer and increased granular density. Furthermore, CNF application to the skin resulted in a lower production of TGF- β compared to that in the control group. Izumi et al. (2015) reported that surface-modified CNFs promote wound healing. In this study, it is showed that CNFs, before surface modification, induce only a slight inflammation in the skin. This evidence indicates that CNFs have a high biocompatibility to the skin.

To the best of our knowledge, the effects of CNFs on skin inflammations like AD has never been evaluated. Herein, we applied CNFs in an experimental AD-like skin lesion model. We evaluated the effects of CNFs on a developmental or early-stage model of AD. Furthermore, its effects were compared to that of corticosteroid treatment.

2. Materials and methods

2.1. Animals

NC/Nga mice (5-week-old, females) were purchased from SLC, Inc. (Shizuoka, Japan). Mice were kept under conventional conditions. The animals were reared in a room with the temperature controlled at $22 \pm 2^\circ\text{C}$, humidity at $50 \pm 5\%$, and lighting set at 12:12-h light/dark cycles (light cycle, 7:00–19:00 h). All mice had a normal diet, with free access to food (CE-2, Japan CLEA, Osaka, Japan) and tap water during the experimental periods. The animal procedures in this study were approved by the Animal Research Committee of Tottori University.

2.2. Reagents and drugs

α -Chitin powder from red snow crab was purchased from Koyo Chemical. Degree of substitution of amino groups calculated from the C and N content in the elemental analysis data was 3.9%. The degree of the relative crystalline index of original chitin determined from the X-ray diffraction profiles was 88%. Sodium hydroxide, 2,4,6-trinitrochlorobenzene (TNCB), and acetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). The commercial drug prednisolone valerate acetate (LID-MEX, Kowa Co., Ltd., Osaka, Japan) was used as a reference.

2.3. Preparation of CNFs

CNFs were prepared by following a previously reported procedure (Ifuku et al., 2009). Chitin (2.0 g) was dispersed in 200 mL of distilled water. The dispersion was roughly crushed with a grinder (MKCA6-3; Masuko Sanyo Co., Ltd.) at 1500 rpm. The crushed dispersion was passed through a Star Burst system (Star Burst Mini, HJP-25001S, Sugino Machine Co., Ltd.) equipped with a ball-collision chamber. The slurry was ejected from a small nozzle with a diameter of 100 μm under a high pressure of 200 MPa and collided with a ceramic ball with a diameter of 12.7 mm. The suspension was subjected to 30 mechanical treatments. The average width of the nanofiber calculated from SPM images was 6.0 nm (Supplementary Fig. S1, Dutta, Izawa, Morimoto, Saimoto, & Ifuku, 2013). After mechanical treatments, degree of acetyl groups and relative crystalline indices did not change.

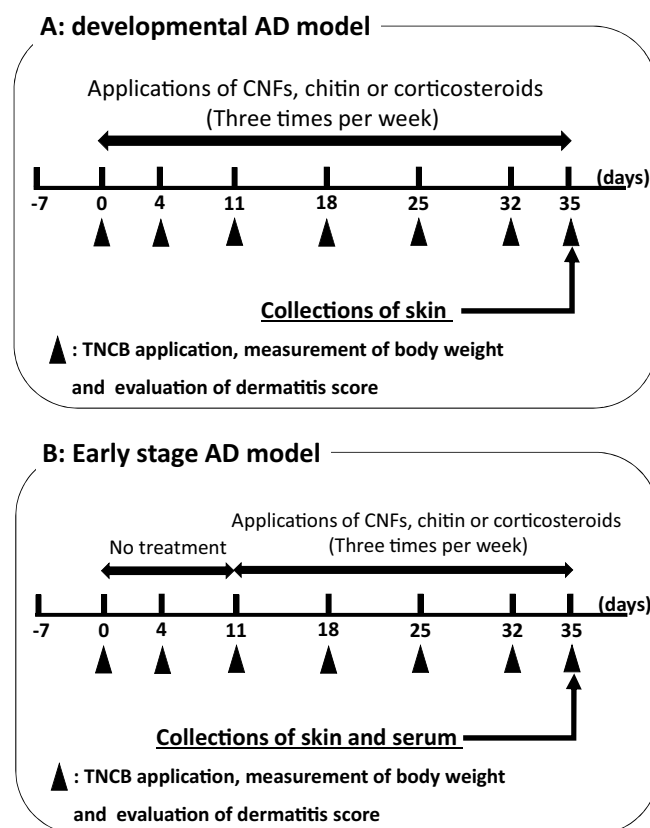


Fig. 1. Diagram of the study design.

The experimental procedures for the developmental (A) and early-stage (B) models of AD are shown.

2.4. Study design of a developing AD model

Mice were divided into 5 groups: (1) the non-treated (NT) group ($n=6$), (2) the control group ($n=6$), (3) the CNFs group which was swabbed with 1% (w/v) CNFs ($n=6$), (4) the chitin group which was swabbed with a 1% (w/v) chitin suspension ($n=5$), and (5) the steroid (S) group which was swabbed with steroid ($n=6$). AD-like skin lesions were prepared according to a previously published method with slight modifications (Sugiyama et al., 2010). The experimental timeline is summarized in Fig. 1. Hair of the thoracic, abdominal, and dorsal regions of the mice was shaved with a hair clipper after inhalation of 2–3% isoflurane. The mice in the control, CNFs, Chitin, and S groups were sensitized on day 0 by the application of 150 μL (thoracic area, 50 μL ; abdominal area, 50 μL ; hind paws, 50 μL) 2% TNCB dissolved in an ethanol and acetone mixture (4:1). These mice were challenged on day 4 with 190 μL (dorsal area, 150 μL ; both sides of the right and left ears, 10 μL) of a 1% TNCB solution to induce AD-like skin lesions. After the first challenge (day 0), 1% TNCB solution was repeatedly applied to the same skin area on days 4, 11, 18, 25, and 32. From days 0–35, each mouse was swabbed each sample (dorsal area, 150 μL ; at each side of both the right and left ear, 10 μL) three times per week. In the control group, no application was performed to the skin except for stimulation with TNCB. In the NT group, no treatments were performed except for shaving.

2.5. Study design of an early stage model of AD

Mice were divided into 3 groups: (1) the control group ($n=5$), (2) the CNF group which was swabbed with 1% (w/v) CNFs ($n=3$), and (3) the steroid (S) group which was swabbed with steroid ($n=4$).

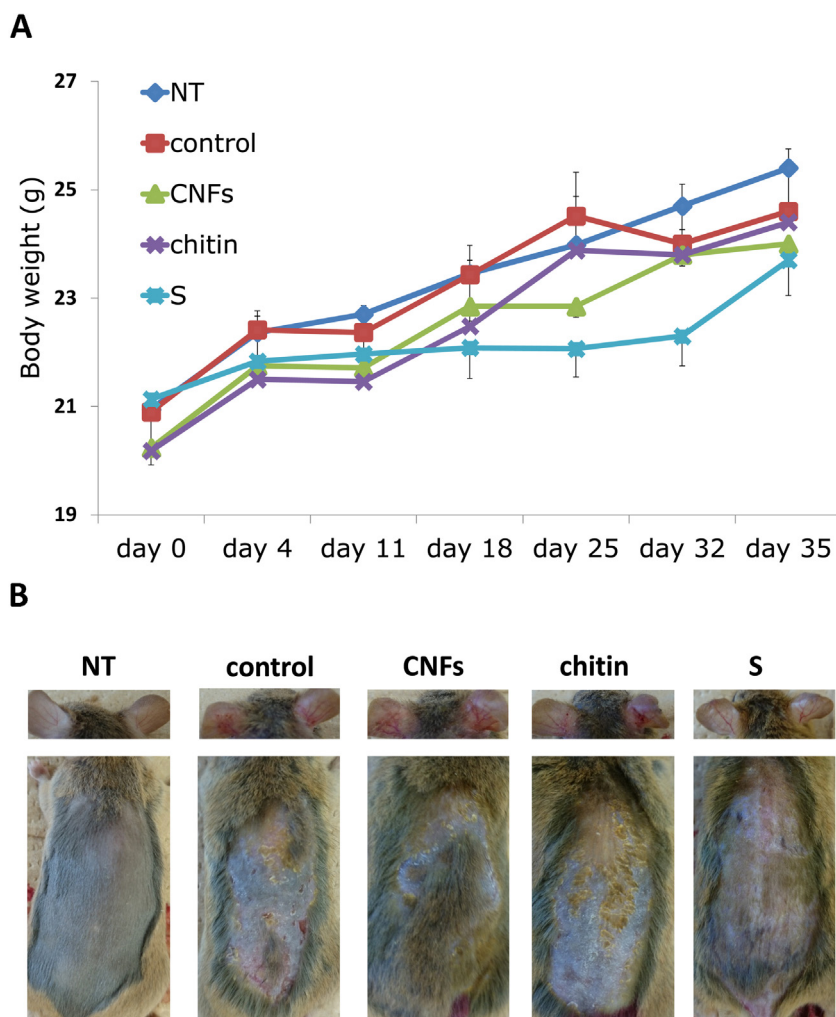


Fig. 2. Effects of topical application of CNFs on body weight and gross symptoms in a developmental AD model. Changes in body weight (A) and gross appearance on day 35 (B) are shown. Data present mean $\pm$ S.E. ($n = 5-6$ in each group).

AD-like skin lesions were prepared according to the same ways as for the developing AD model. The experimental timeline is summarized in Fig. 2. The thoracic, abdominal, and dorsal regions of the mice were shaved with a hair clipper after anesthetization via inhalation of 2–3% isoflurane. In the control, CNFs, and S groups, mice were sensitized on day 0 by the application of 50 μ L of 2% TNCB dissolved in an ethanol and acetone mixture (4:1) at each of the following regions: the thoracic area, the abdominal area, and the hind paws. These mice were challenged on day 4 with 190 μ L of 1% TNCB solution (150 μ L at the dorsal area, and 10 μ L at each side of both the right and left ear) to induce AD-like skin lesions. After the first challenge (day 0), 1% TNCB solution was repeatedly applied to the same skin areas on days 11, 18, 25, and 32. From days 11–35, each mouse was swabbed each sample (dorsal area, 150 μ L; each sides of both the right and left ears, 10 μ L) three times per week. In the control group, no application was performed to the skin except for the stimulation with TNCB.

2.6. Clinical evaluation

The severity of dermatitis was assessed macroscopically according to a previously described method (Matsuda et al., 1997). The skin lesions on the back and on the ears were scored on days 0, 4, 11, 18, 25, 32, and 35. The dermatitis score (minimum 0; maximum 12) was defined as the sum of the individual scores for each lesion,

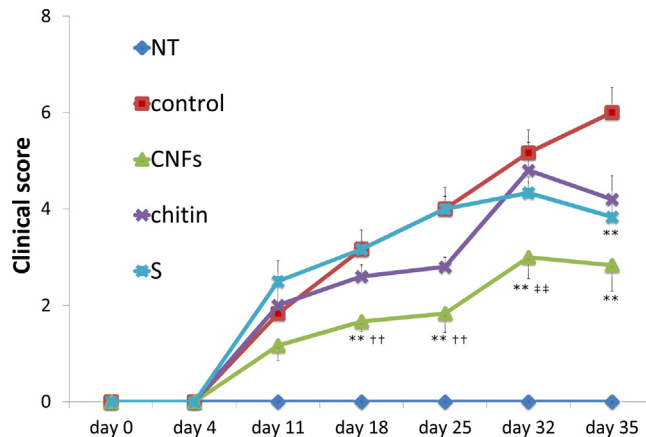


Fig. 3. Effects of topical application of CNFs on clinical scores. The skin lesions on the back and on the ears were scored on days 0, 4, 11, 18, 25, 32, and 35. The dermatitis score (minimum 0; maximum 12) was defined as the sum of the individual scores for each lesion, graded as 0 (absent), 1 (mild), 2 (moderate), and 3 (severe), for each of the following symptoms: (1) erythema/hemorrhage, (2) edema, (3) excoriation/erosion, and (4) scaling/dryness. Data present mean $\pm$ S.E. ($n = 5-6$ in each group), ** $p < 0.01$ compared to the control group, †† $p < 0.01$ compared to the S group, ‡‡ $p < 0.01$ compared to the chitin group. Statistical differences were assessed using the Steel-Dwass test.

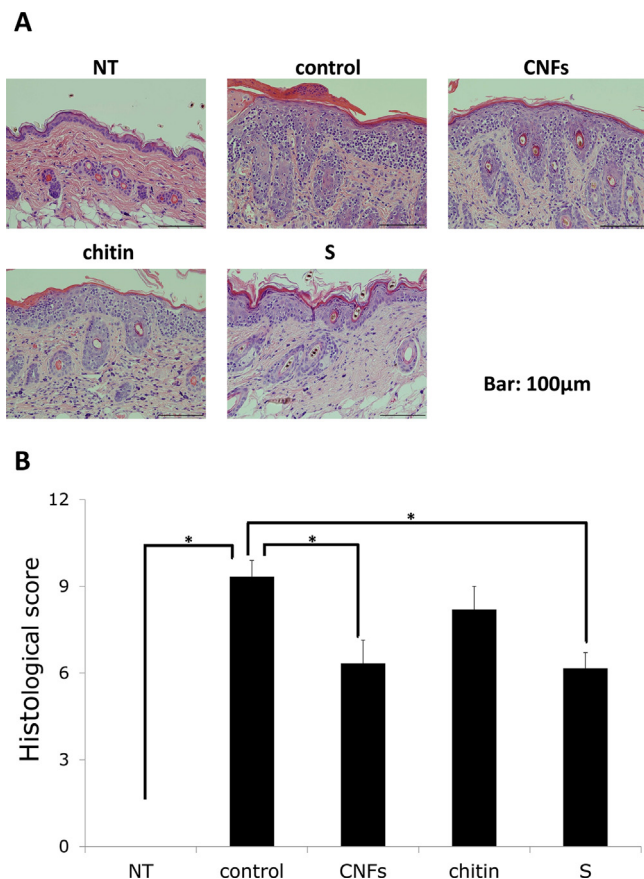


Fig. 4. Histological assessment of the effects of topical CNF application on a developmental AD model.

Skins were harvested on day 35, and provided for histological evaluation. Histological changes (A) and histological scores (B) are shown. A: In the control group, hypertrophy and hyperkeratosis of the epidermis were observed. Inflammatory cells had infiltrated in both the epidermis and dermis. Notably, in the CNFs and S groups, hypertrophy and hyperkeratosis of the epidermis, as well as infiltration of inflammatory cells, were markedly suppressed compared to that in the control group. Each scale bar indicates 100 μm. (B) The severity of dermatitis was assessed on the epidermis (hypertrophy, hyperkeratosis, and infiltration by inflammatory cells) as well as on the dermis (infiltration by inflammatory cells). Each score was expressed as the sum of the individual scores, graded from 0 (no symptoms) to 12 (severe). Data are presented as the mean ± S.E. (n = 5–6 in each group), **p < 0.01 compared to the control group, *p < 0.05 compared to the control group. Statistical differences were assessed using the Steel-Dwass test.

graded as 0 (absent), 1 (mild), 2 (moderate), and 3 (severe), for each of the following symptoms: (1) erythema/hemorrhage, (2) edema, (3) excoriation/erosion, and (4) scaling/dryness. The body weight of each mouse was also measured on days 0, 4, 11, 18, 25, 32, and 35.

2.7. Histopathological observations

The auricular and dorsal skin was harvested on day 35. Skins were fixed in 10% buffered formaldehyde for histopathological examination. Thin sections (4 μm) were obtained for each sample for histological observation after hematoxylin-eosin (HE) staining. Each section was examined microscopically, and histological scoring was performed according to a previously described method (Taniguchi et al., 2003; Watanabe et al., 2009). In brief, the severity of dermatitis was assessed on the epidermis (hypertrophy, hyperkeratosis, and infiltration by inflammatory cells) as well as on the dermis (infiltration by inflammatory cells). Each score was expressed as the sum of the individual scores, graded from 0 (no symptoms), to 1 (mild), 2 (moderate), and 3 (severe).

Table 1

Positive areas of NF-κB, COX-2 and iNOS in developmental skin lesion.

	NT	C	CNFs	chitin	S
NF-κB	2.2 ± 0.3	12.7 ± 1.0	4.0 ± 0.3 ^{**} , †	12.0 ± 0.8	7.0 ± 1.0 ^{**}
COX-2	0.9 ± 0.1	5.1 ± 0.4	2.7 ± 0.2 ^{**}	3.4 ± 0.3	2.3 ± 0.1 ^{**}
iNOS	2.7 ± 0.2	4.6 ± 0.3	3.2 ± 0.3 ^{**}	4.0 ± 0.4	3.7 ± 0.4

Positive areas of NF-κB, COX-2 and iNOS in the skin were calculated. Data indicate mean ± S.E.

^{**} p < 0.01 compared to the control group.

† p < 0.05 compared to the chitin group.

2.8. Immunohistochemistry (IHC) of nuclear factor-κB (NF-κB), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS)

Effects of CNF on NF-κB, COX-2, and iNOS activation in the skin were evaluated using our previously developed methods (Azuma et al., 2014, 2015). Sections of skin tissue prepared on glass slides were deparaffinized, washed with ethanol and water, and soaked in phosphate-buffered saline (PBS). Then, sections were microwaved with 0.01 M citrate buffer (pH 6.0) for 5 min. Thereafter, they were washed with PBS and incubated with 1% hydrogen peroxide-methanol for 30 min at room temperature.

For IHC of NF-κB, after washing PBS, sections were incubated with rabbit polyclonal anti-NF-κB p65 antibody (sc-372; 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 min at room temperature, followed by another wash with PBS. Tissue sections were then visualized by staining with diaminobenzidine tetrahydrochloride (Code No. K3466; Dako, Glostrup, Denmark) and counterstaining with hematoxylin for 30 min at room temperature.

For the IHC analyses of COX-2, after washing with PBS, blocking was carried out in PBS with 1% bovine serum albumin (BSA) for 30 min at room temperature. Sections were incubated with rabbit monoclonal anti-COX-2 antibody (1:400 dilution; Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C, followed by a wash with PBS. Subsequently, the envision method was employed for 30 min at room temperature (Dako). Then, tissue sections were stained with 3,3'-diaminobenzidine (DAB; Dako) and counterstained with hematoxylin.

For the IHC analyses of iNOS, after washing with PBS, blocking was undertaken in PBS with 1% BSA for 30 min at room temperature. Sections were incubated with rabbit monoclonal anti-iNOS antibody (1:200 dilution; Abcam, Cambridge, UK) overnight at room temperature, followed by a wash with PBS. Subsequently, the envision method was employed for 30 min at room temperature (Dako). Tissue sections were stained with DAB (Dako) and counterstained with hematoxylin.

We calculated the positive areas of NF-κB, COX-2 and iNOS in skin. The skin mice were analyzed in each group. The mean scores for 5 fields were considered the percentages of positive areas for each mouse.

2.9. Measurement of serum total immunoglobulin E (IgE) levels

Blood samples were collected on day 35, and serum was obtained by centrifugation at 1200g, for 15 min at 4 °C. Total IgE levels in the serum were measured by enzyme-linked immunosorbent assay (ELISA) using the mouse IgE ELISA kit.

2.10. Statistical analysis

Data are represented by the mean ± standard error (SE). Statistical analyses were carried out using the Steel-Dwass test or

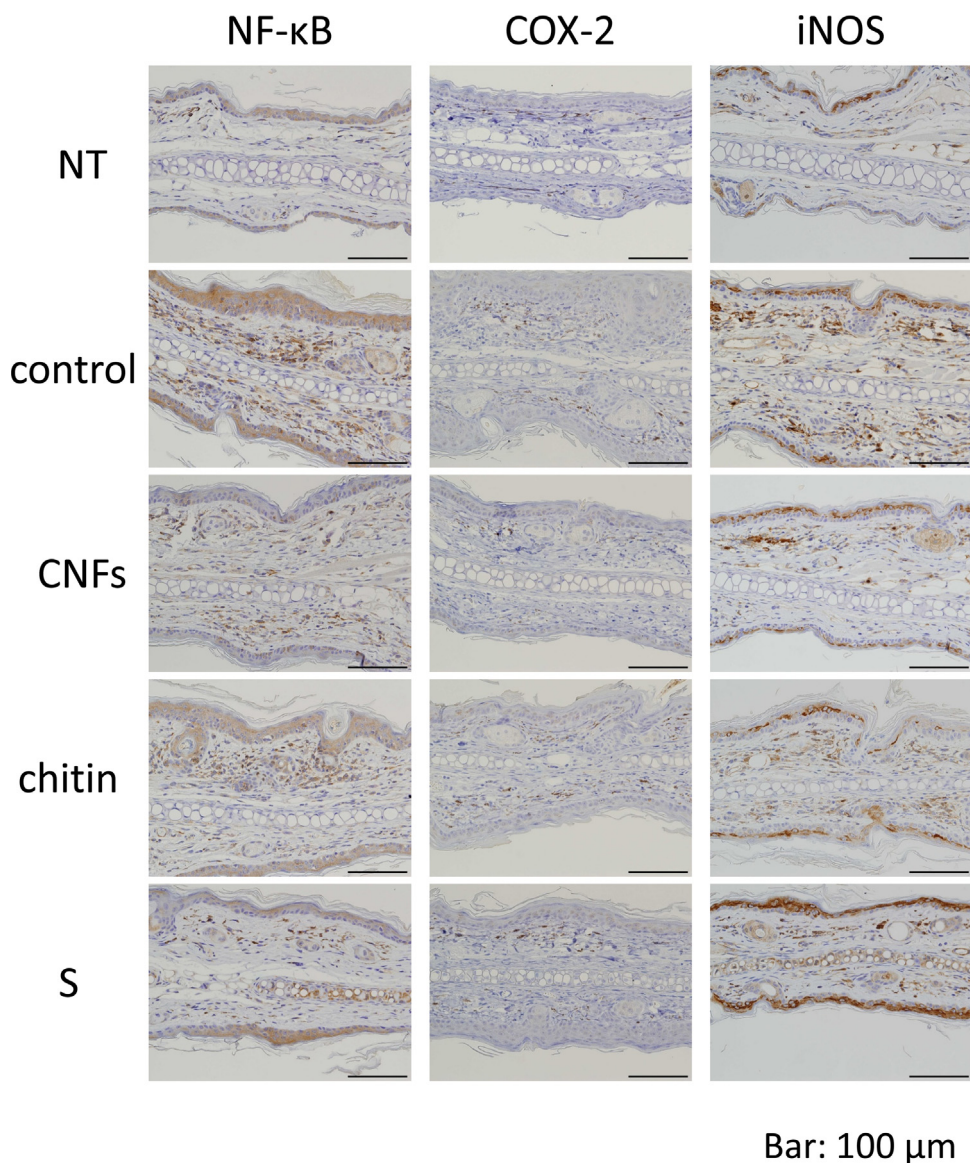


Fig. 5. Effects of topical application of CNFs on NF- κ B, COX-2, and iNOS in a developmental model of AD.

Representative images of IHC staining of NF- κ B, COX-2, and iNOS in the ear skins are shown. In the control group, there was widespread expression of NF- κ B, COX-2, and iNOS in the epithelial cells. In the CNFs and S groups, the positive area of each of these molecules is markedly decreased. Each scale bar represents 100 μ m.

one-way ANOVA followed by the Tukey-Kramer test, and $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Clinical and histological changes upon application of CNFs in development of AD-like skin lesion

The body weight changes during the experimental period are shown in Fig. 2A. No change in body weight was observed during the experimental period. Gross changes in the appearance of the skin are shown in Fig. 2B. The clinical scoring of dermatitis severity is presented in Fig. 3. In the NT group, the dermatitis score increased over time during the experimental period. In the CNFs group, the dermatitis score also increased, however, the scores were lower than those of the control and chitin group between day 18 and 32 ($p < 0.01$). In the CNFs and S group, the dermatitis scores were significantly lower than in the chitin group on day 35 ($p < 0.01$). In the CNFs and S groups, the dermatitis scores were also lower than that

of the control group on day 35 ($p < 0.01$). There was no difference in dermatitis score between the chitin group and the control group. In the CNFs and S groups, serum IgE levels were slightly decreased compared with that of the control group. However, there was no significant difference (data are not shown).

The results of the HE stains are shown in Fig. 4. In the control group, hypertrophy and hyperkeratosis of the epidermis were observed. Inflammatory cells such as mast cells, eosinophils, neutrophils, and lymphocytes had infiltrated in both the epidermis and dermis. Notably, in the CNFs and S groups, hypertrophy and hyperkeratosis of the epidermis, as well as infiltration of inflammatory cells, were markedly suppressed compared to that in the control group. The histological scores for the samples obtained from the CNFs and S groups were significantly lower than that of the control group ($p < 0.05$; Fig. 4). The histological score for the chitin group was not significantly different from that of the control group.

The results of the present study strongly indicate that CNFs exert a suppressive effect on developmental AD symptoms, as application of CNFs suppressed the developmental of AD-like skin lesions.

AD-like lesions were induced in NC/Nga mice by recurrent application of TNCB. In contrast to spontaneous AD-like lesion formation, this method allows for the reproducible induction of skin lesions in NC/Nga mice (Shiohara, Hayakawa, & Mizukawa, 2004; Tomimori, Tanaka, Goto, & Fukuda, 2005). In our developmental AD model, no differences in body weight were observed between the NT, control, CNFs, and chitin groups. Although there was no significant difference, the body weight was slightly lower in the S group than in the other groups. The clinical and histological scores of the CNFs and S group exhibited a similar decrease compared to those of the control group. In the chitin group, on the other hand, there was no suppressive effect in our developmental AD model.

3.2. CNF application reduced the levels of NF- κ B, COX-2 and iNOS in the skin

The IHC results are shown in Fig. 5. And the percentage of each positive area is shown in Table 1. In the control group, there was widespread expression of NF- κ B, COX-2, and iNOS in the epithelial cells. In the CNFs and S groups, the positive area of each of these molecules is markedly decreased. In contrast, the staining of NF- κ B, COX-2, and iNOS for the chitin group was not different from what was observed for the control group. In the CNFs group, positive areas of NF- κ B, COX-2 and iNOS were significantly decreased compared with those of the control group ($p < 0.01$). In the S group, positive areas of NF- κ B and COX-2 were significantly decreased compared with those of the control group ($p < 0.01$).

In the present study, application of CNFs led to a decrease in NF- κ B, COX-2, and iNOS levels in the skin as compared to that in the control group. NF- κ B occupies a pivotal position in several signaling pathways involved in innate immunity. It stimulates the expression of COX-2, prostaglandin E2, and pro-inflammatory cytokines (Karrasch & Jobin, 2008). NF- κ B is a critical transcription factor for the activation of pro-inflammatory responses (Elson et al., 2005; emphasizing the importance of NF- κ B in allergic disorders such as asthma, rheumatoid arthritis, and AD (Barnes & Karin, 1997; Yamamoto & Gaynor, 2001; Nakamura et al., 2002). In fact, NF- κ B is one of the clinical target molecules for AD (Tanaka et al., 2007). COX-2 expression is markedly increased in inflammatory cells in response to cytokine stimulation in inflammatory or other immune reactions (Luca & Perretti, 2003). Nitric oxide (NO) plays a role in vasodilation, neurotransmission, blood coagulation, and immune regulation. iNOS is involved in the massive

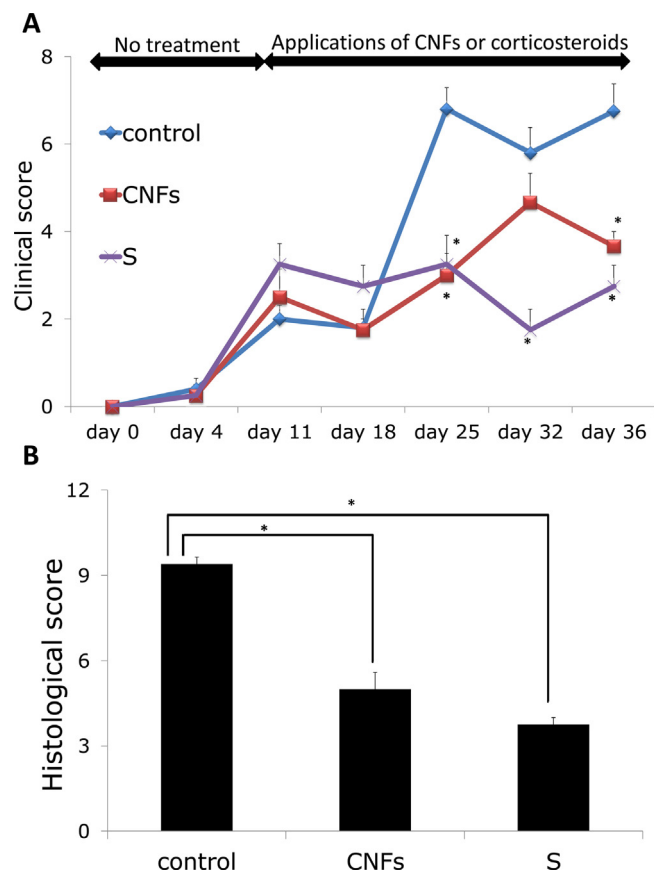


Fig. 6. Effects of topical application of CNFs on clinical and histological scores in an early-stage AD model.

The dermatitis score (minimum 0; maximum 12) was defined as the sum of the individual scores for each lesion, graded as 0 (absent), 1 (mild), 2 (moderate), and 3 (severe), for each of the following symptoms: (1) erythema/hemorrhage, (2) edema, (3) excoriation/erosion, and (4) scaling/dryness. The severity of dermatitis was assessed on the epidermis (hypertrophy, hyperkeratosis, and infiltration by inflammatory cells) as well as on the dermis (infiltration by inflammatory cells). Each score was expressed as the sum of the individual scores, graded from 0 (no symptoms) to 12 (severe). Data are represented by the mean $\pm$ S.E. ($n = 3$ –5 in each group), ** $p < 0.01$ compared to the control group, * $p < 0.05$ compared to the control group. Statistical differences were assessed using the Steel-Dwass test.

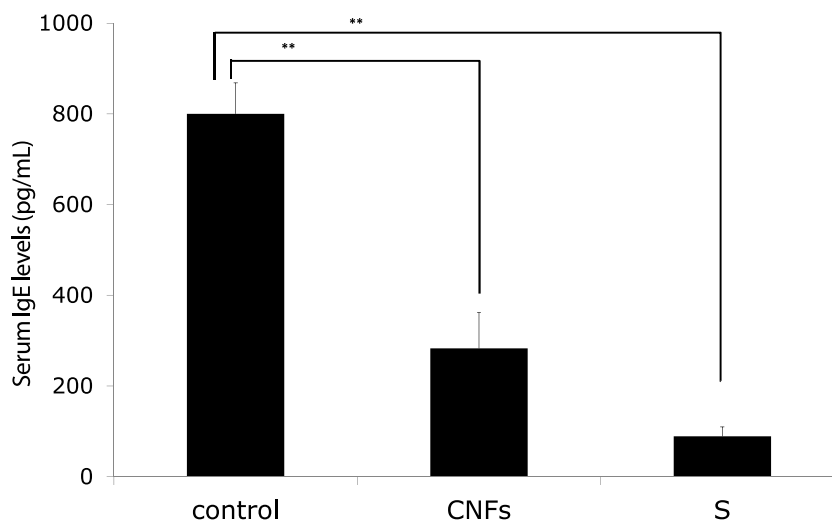


Fig. 7. Effects of topical application of CNFs on serum IgE levels.

Data are presented as the mean $\pm$ S.E. ($n = 5$ –6 in each group), ** $p < 0.01$ compared to the control group, Statistical differences were assessed using the Tukey-Kramer's test.

production of NO and it is activated by various cytokines and by bacterial lipopolysaccharide (LPS). Excessive NO levels aggravate the inflammatory response (Kharitonov et al., 1994). During inflammation, NF- κ B controls the expression of many inflammatory proteins including COX-2 and iNOS (Schottelius, Mayo, Sartor & Baldwin, 1999; Ding et al., 2003). The results of the present study indicate that the suppressive effects of CNF application during the development of AD might be via suppression of NF- κ B, COX-2, and iNOS in the skin. Da Silva et al. (2009) described a kind of chitin suppressed the NF- κ B activation via involving TLR-2 and dectin-1. We also reported CNFs but not chitin suppressed NF- κ B activation in colon inflammation (Azuma et al., 2012). As it is unknown how CNFs suppress these activities, further study must be performed for this point.

3.3. Application of CNFs for early-stage AD

No change in body weight was observed during the experimental period (data are not shown). Clinical and histological scores for the early stage AD mouse model are shown in Fig. 6. In the CNFs group, the clinical scores were significantly decreased compared with those of the control group on days 25 and 35 ($p < 0.05$). In the S group, the clinical scores were significantly lower than in the control group on days 25, 32, and 35 ($p < 0.05$). The histological scores of the CNFs and S groups were significantly lower than that of the control group as well ($p < 0.05$). The total IgE serum levels are shown in Fig. 7. In the CNFs and S groups, the serum levels of IgE were significantly lower than in the control group ($p < 0.05$). The activations of NF- κ B, COX-2 and iNOS were also suppressed on day 35 in the CNFs and S groups (data are not shown).

In the early-stage AD model, swabs of CNFs suppressed clinical and histological scores to a similar extent as corticosteroid application in the S group. Topical application of corticosteroids is an efficient therapy for AD patients that show signs of skin inflammation (Callen et al., 2008). However, corticosteroids have not only beneficial but also adverse effects.

Topical corticosteroid application may lead to local side effects, including steroid acne, flushing, skin atrophy, hypertrichosis, striae, telangiectasia, and occasional allergic contact dermatitis in the treated area (Hengge, Ruzicka, Schwartz, & Cork, 2006). In some cases, topical use of corticosteroids also induces systemic side effects (Mooney et al., 2015). In both models used in this study, a change in body weight was observed in the S group. Systemic corticosteroid treatment causes rapid muscle atrophy in animals and humans, leading to serious clinical side effects such as wasting and debilitation (Seene, Kaasik, Pehme, Alev, & Riso, 2003). Thus, it is possible that the decrease in body weight observed for the S group might be attributed to corticosteroids. However, the effect of topical corticosteroid application on body weight has not been established.

In the CNFs group, serum IgE levels were significantly decreased. Some reports have indicated that oral administration of chitin suppresses allergic responses. Shibata et al. (2000) described how oral administration of chitin down-regulates serum IgE levels and lung eosinophilia in an allergy model. Bae et al. (2013) reported that oral administration of chitin prevents peanut-induced anaphylaxis in an experimental model. Although the symptom score was decreased, administrations of chitin did not lead to a decrease in serum IgE levels. To the best of our knowledge, there are no reports on the immunological effects of topical administration of chitin or chitin derivatives. Our results indicate that topical use of CNFs might affect systemic immunological reactions. Further studies are needed to explore this interaction in more detail.

Of all the skin layers, the certified layer acts as a protective barrier and, hence, is an important part of the epithelium. It protects the internal structures from desiccation and against

environmental changes (Rawlings & Harding, 2004). The essential role that natural moisturizing factors (NMFs) play in the moisturizing and barrier functions of the skin underscores the critical function of the granular layer and the associated granules because they are responsible for the secretion of both lipids and NMFs (Kubo, Nagao, Yokouchi, Sasaki, & Amagai, 2009). Ito et al. (2014) reported that CNFs increased granular density in a skin model. One possible mechanism that could explain the beneficial effects of CNFs in our experimental AD model might be the activation of the epithelial granular layer. To confirm and understand this mechanism, further research is needed.

More recently, the potency of modified CNF complexes for therapeutic approaches has been highlighted. Anraku et al. (2015) examined the use of surface-deacetylated CNFs (SD-CNFs) and hyaluronic acid (HA) interpolymer complexes (IPC) as a potential anti-oxidative compound in extended-release matrix tablets. An SD-CNFs/HA IPC was prepared and evaluated as an extended-release tablet matrix using famotidine (FMT) as a model drug. The release of FMT from the IPC tablet (SD-CNFs:HA = 1:1) was slower than that from a SD-CNF only. With these materials, it might become possible to develop controlled-release drugs for administration at topical sites, including the skin. To reduce the use of corticosteroids in AD patients, the therapeutic application of CNF derivatives could prove valuable. Efficiency of chitosan nanofibers for biomedical application was also indicated (Azuma et al., 2014). The effects of chitosan nanofibers for skin inflammation should be evaluated.

In conclusion, clinical and histological assessment demonstrated that the application of CNFs suppressed the development of experimental AD. CNFs suppressed the expression of inflammatory molecules including NF- κ B, COX-2, and iNOS. Moreover, topical CNF application suppressed the progression of AD in an early stage AD model. Our evidence indicates that CNFs have potential as new functional biomaterials for the suppression of AD.

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Appendix A. Supplementary data

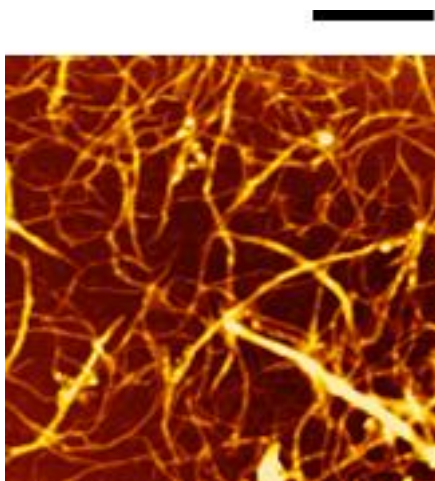
Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.carbpol.2016.03.068>.

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SMP image of CNFs



SMP image of chitin nanofiber after 30 passes through the star burst system.
Scale bar: 1000 nm