

Inhibitory Effect of Chitosan-Containing Lotion on Scratching Response of Hairless Mice with Atopic Dermatitis-Like Dry Skin

Masanori FUJII,^a Tatsuo SHIMIZU,^b Takeshi NAKAMURA,^a Fumiko ENDO,^a Shigekatsu KOHNO,^a and Takeshi NABE*,^a

^aDepartment of Pharmacology, Division of Pathological Sciences, Kyoto Pharmaceutical University; 5 Nakauchi, Misasagi, Yamashina, Kyoto 607-8414, Japan; and ^bResearch and Development Institute, High Performance Functional Plastics Company, Sekisui Chemical Co., Ltd.; 2-1 Hyakuyama, Shimamoto, Mishima, Osaka 618-0021, Japan.

Received August 2, 2011; accepted September 26, 2011; published online September 29, 2011

In this study, using a special diet-induced mouse model of atopic dermatitis, we tested the effect of chitosan-containing lotion (CL) on itch-related scratching associated with barrier-disrupted dry skin. HR-1 hairless mice fed a special diet exhibited apparent dry skin symptoms characterized by decreased skin hydration and increased transepidermal water loss. In the special diet-fed mice, scratching behavior was markedly enhanced for 60 min after oral administration of ethanol. When CL was applied once immediately after ethanol administration, the enhanced scratching response was significantly suppressed, but this effect was abolished within 30–40 min; when applied twice immediately and at 30 min, CL almost completely blocked scratching throughout 60 min. Comparison of CL and the chitosan-free vehicle showed that CL inhibited scratching more strongly and persistently than the vehicle, which transiently suppressed scratching only for 10 min after application. Although the decreased skin hydration was improved even by the vehicle, the increased transepidermal water loss was resolved only by CL. Skin surface temperature was much more reduced in CL-treated mice than in vehicle-treated mice. Collectively, CL has an antipruritic effect, which could be partly explained by recovery of skin barrier function and cooling of the skin.

Key words chitosan; itch; skin barrier; atopic dermatitis; hairless mouse

Itch is the predominant complaint of atopic dermatitis.¹⁾ In patients with atopic dermatitis, a vicious itch-scratch cycle is easily established, leading to exacerbation of disease.²⁾ Controlling itch is therefore an important issue in its treatment, but at present there are few effective remedies for itch. Dry skin, a typical dermatological feature of atopic dermatitis, is linked to impaired function of the skin barrier.^{3–5)} Since a clinical association between itch and dry skin has been well discussed,^{6,7)} dry skin could be one of the triggering factors of itch in atopic dermatitis. Although the underlying mechanism is not fully elucidated, one possibility may be that the impaired barrier function facilitates the easy entrance of irritants and itchy agents.⁸⁾ Thus, correcting skin barrier function as well as skin dryness is thought to be an important therapeutic strategy for itch in atopic dermatitis.

Animal disease models are useful for evaluating therapeutic drugs. We have reported the characteristics of a diet-induced mouse model of atopic dermatitis. HR-1 hairless mice fed a special diet, HR-AD, exhibited prolonged duration of spontaneous scratching bouts accompanied by atopic dermatitis-like symptoms, which were characterized by severe dry skin with barrier dysfunction, epidermal hyperplasia, dermal cellular infiltration, and elevation of the serum immunoglobulin E level.⁹⁾ The prolonged duration of scratching bouts was inhibited by an opioid antagonist,¹⁰⁾ suggesting that it is an itch-related response. Additionally, prolonged scratching coincided with dry skin symptoms and was suppressed depending on recovering barrier function by the application of petrolatum ointment,¹⁰⁾ implicating a causative role of skin barrier dysfunction in basal scratching. Furthermore, we recently found that orally administered ethanol markedly enhanced basal scratching possibly *via* its central actions, even though enhanced scratching was also suppressed by petrolatum application.¹¹⁾ Therefore, skin barrier

dysfunction seems to essentially underlie the scratching responses in this model.

Chitosan, a polysaccharide of β -(1,4) linked D-glucosamine and N-acetyl-D-glucosamine, is obtained from partial N-deacetylation of chitin, which is widely distributed in nature in the exoskeletons of crustaceans. Chitosan has attracted attention as a functional biopolymer for diverse applications, especially in pharmaceuticals and medicine,¹²⁾ because of a variety of biological activities, such as hemostatic, antimicrobial, and wound-healing properties; however, little has been reported on the effectiveness of chitosan against itch. Furthermore, we recently examined, using guinea pigs, the effect of application of chitosan-containing lotion (CL) on acute skin barrier disruption, and found that CL application improved skin barrier function (Shimizu *et al.*, unpublished observation). Therefore, in this study, we examined the effect of applying CL on the scratching response associated with skin barrier dysfunction in HR-AD-induced dermatitis model.

MATERIALS AND METHODS

Animals and Diets Female, 4-week-old, HR-1 hairless mice were purchased from Hoshino Laboratory Animal Inc. (Ibaraki, Japan). To develop atopic dermatitis-like symptoms, they were fed a special diet (HR-AD diet; Norsan Co., Yokohama, Japan) for 8–10 weeks. As a negative control, a normal diet (F-2; Funabashi Farm, Chiba, Japan) was provided. The ingredients of both diets have been described previously.⁹⁾ The animal study was approved by The Experimental Animal Research Committee at Kyoto Pharmaceutical University.

Agents Chitosan-containing lotion (CL) was supplied by Sekisui Chemical Co. (Osaka, Japan). This lotion contained

* To whom correspondence should be addressed. e-mail: nabe@mb.kyoto-phu.ac.jp

two types of chitosan (1% Flonac[®] C-60M-SEK and 0.5% Flonac[®]-NSW-SEK; both from Kyowa Technos Co., Chiba, Japan) and general cosmetic components, such as lactic acid, sodium hyaluronate, glycerol, urea and chlorhexidine gluconate. A vehicle with the chitosan component removed from the lotion was also provided by Sekisui Chemical Co. These solutions were stored at 4 °C, but returned to room temperature before use.

Measurements of Skin Hydration and Transepidermal Water Loss (TEWL) As a parameter of skin hydration, capacitance of dorsal skin was measured by Corneometer[®] CM825 (Courage and Khazawa, Cologne, Germany). To evaluate skin barrier function, TEWL on dorsal skin was measured by Tewameter[®] TM210 (Courage & Khazawa). Both measurements were conducted at a temperature of 22 ± 1 °C and 50 ± 10% humidity.

Analysis of Scratching Behavior Before measurement of scratching behavior, mice were acclimatized for at least 30 min in a polyvinyl chloride cage. Spontaneous scratching behavior was recorded for 1 h immediately after acclimatization. Alternatively, ethanol solution (30%, 10 ml/kg) was orally administered to enhance the scratching response as reported previously,¹¹⁾ and then scratching behavior was recorded for 1 h. Mice generally show a series of scratching (scratching bout), which consists of several repetitions of scratching movement of the hind paws. Regardless of the sites of scratching, the cumulative time and frequency of scratching bouts were measured by playing back the videotape with own counter, as described previously.⁹⁾ In addition, the duration of one bout was calculated by dividing the cumulative time by the frequency.

Evaluation of the Effects of CL Application on Ethanol-Induced Scratching, Dry Skin Symptoms and Skin Temperature Eight to 10 weeks after HR-AD feeding, skin hydration, TEWL and ethanol-induced scratching were measured. Based on these data, the mice were divided into different groups. The following day, the effect of CL application on scratching was evaluated in two protocols. In one protocol, CL (400—500 μ l/animal/time) was applied on the whole skin of mice once immediately after ethanol administration (designated “once”). In the other protocol, CL (400—500 μ l/animal/time) was applied on the whole skin of mice twice immediately and 30 min after ethanol administration (designated “twice”). To evaluate the influence of the vehicle, either CL or the chitosan-free vehicle was applied in the “twice” protocol. Scratching behavior was recorded for 1 h from the first drug application and analyzed as described above. Skin hydration and TEWL were measured at the end of scratching measurement.

To determine skin surface temperature, either CL or the vehicle was applied in the “twice” protocol after ethanol dosing. The temperature of the cervical dorsal skin was measured by a handheld thermometer (HA200K) connected to a thermocouple probe (N-211K-00-1-TC1-ASP; Anritsu Meter Co., Tokyo, Japan) at 10 min intervals during 1 h from the first drug application.

Statistical Analysis Data are presented as the mean ± S.E. Statistical differences were determined using Student's unpaired *t*-test or two-way analysis of variance (ANOVA) with the Bonferroni multiple test. Differences were considered significant when $p < 0.05$.

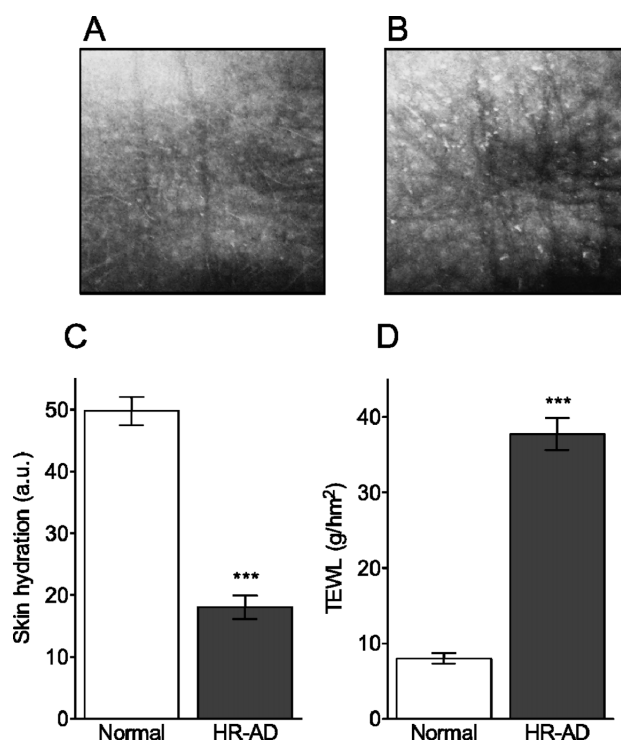


Fig. 1. Dry Skin Symptoms in HR-AD-Fed Mice

(A, B) Skin appearance of mouse fed a normal diet (normal, A) or a special diet (HR-AD, B) for 8 weeks from 4 weeks old. (C, D) Skin hydration (C) and transepidermal water loss (TEWL, D) of normal diet- and HR-AD-fed mice. These parameters were measured 8 weeks after feeding. Each column represents the mean ± S.E. of 5 animals. *** $p < 0.001$, Student's unpaired *t*-test.

RESULTS

Dry Skin Symptoms and Scratching Behavior in HR-AD-Fed Mice As reported previously,⁹⁾ the skin of mice fed HR-AD for 8 weeks displayed redness, wrinkling and desquamation (Fig. 1B), which affected the entire skin surface, while that of normal diet-fed mice showed no gross abnormality (Fig. 1A). Skin hydration (Fig. 1C) and TEWL (Fig. 1D) of HR-AD-fed mice decreased and increased, respectively, compared with those of mice fed the normal diet, indicating the development of barrier-disrupted dry skin by HR-AD feeding.

Similar to previous results,^{9–11)} neither the cumulative time nor frequency of spontaneous scratching bouts differed between groups (Figs. 2A, B), whereas the duration of one bout of HR-AD-fed mice was prolonged (** $p < 0.001$) (Fig. 2C). When ethanol (30%, 10 ml/kg) was orally administered (Figs. 2D–F) to normal diet-fed mice, neither the cumulative time nor frequency was significantly affected, while the duration of one bout was slightly prolonged ($\dagger p < 0.05$). In contrast, in HR-AD-fed mice, all scratching parameters were markedly increased ($\dagger\dagger\dagger p < 0.001$) (Figs. 2D–F). In the following experiments, the ethanol-induced increase in cumulative time was used as a parameter of itch-related scratching, because the magnitude of response was greater than that of spontaneous scratching with high reproducibility.

Effect of CL Application on the Enhanced Scratching Response Figures 3A and B respectively shows the total time and the time course of scratching when CL was applied once immediately after ethanol administration. In the sham

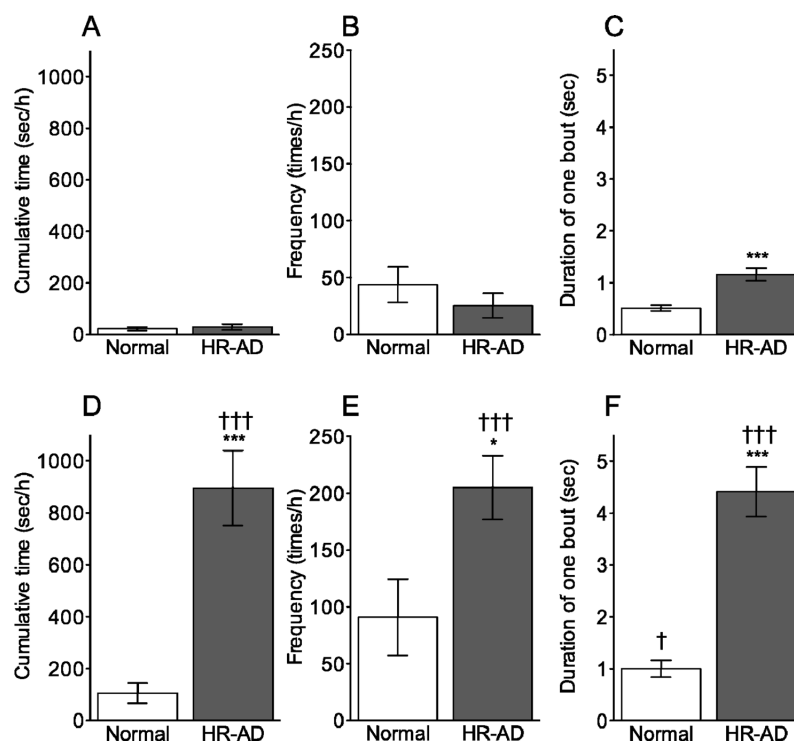


Fig. 2. Spontaneous and Ethanol-Induced Scratching Behaviors in HR-AD-Fed Mice

(A—C) Spontaneous scratching. Eight weeks after feeding, spontaneous scratching behaviors of normal diet- and HR-AD-fed mice were measured. (D—F) Ethanol-induced scratching. One day after the measurement of spontaneous scratching, ethanol (30%, 10 ml/kg) was orally administered, and scratching behaviors were measured. Cumulative time (A, D) and frequency (B, E) of scratching bouts were measured, and the duration of one bout (C, F) was calculated by dividing the cumulative time by the frequency. Each column represents the mean $\pm$ S.E. of 5 animals. * $p < 0.05$ and *** $p < 0.001$, normal vs. HR-AD, Student's unpaired *t*-test. † $p < 0.05$ and ††† $p < 0.001$, spontaneous scratching vs. ethanol-induced scratching, Student's unpaired *t*-test.

group, a marked scratching response with a peak at 0—10 min after ethanol treatment was observed, followed by a gradual decline until 60 min (Fig. 3B). Surprisingly, in the CL (400—500 μ l/animal/time)-treated group, the scratching response was almost completely inhibited during 0—30 min, but the degree of scratching during 40—60 min was comparable to that in the sham group (Fig. 3B).

Because the inhibitory effect on scratching disappeared around 40 min after application, CL was applied twice immediately and 30 min after ethanol dosing. As shown in Figs. 3C and D, when CL was applied twice, the scratching response was almost completely blocked for 60 min.

We next examined the influence of the vehicle of CL. When CL was applied twice, complete inhibition of scratching was observed (Figs. 3E, F). On the other hand, although the vehicle with chitosan components removed suppressed the scratching response during 0—10 min after the first application, this effect disappeared within 10—20 min; after the second application, a similar tendency was observed (Fig. 3F). Comparison of scratching response during 0—60 min between sham controls and the vehicle showed no statistical significance (Fig. 3E).

Effects of CL Application on Barrier-Disrupted Dry Skin and Skin Surface Temperature To consider how CL blocked the scratching response, the effects on dry skin symptoms were evaluated. From our preliminary experiment, the measurement of skin hydration and TEWL was greatly influenced by evaporation from the lotion itself within 30 min after application (data not shown). Thus, to avoid the influence as much as possible, parameters were measured just

after the end of scratching measurement. When CL or the vehicle was applied in the “twice” protocol, both CL and the vehicle similarly increased skin hydration (Fig. 4A). On the other hand, CL reduced TEWL, while the vehicle exerted no effect (Fig. 4B).

Because we felt that the application of lotion cooled the skin, we next investigated the time-course change in skin surface temperature when CL or the vehicle was applied twice after ethanol dosing. Consistent with previous result,¹¹⁾ skin surface temperature of sham controls slightly declined after ethanol dosing (Fig. 4C). Application of the vehicle resulted in a transient but marked decrease in skin surface temperature, which disappeared by 30 min after either the first or second application (Fig. 4C). On the other hand, application of CL produced a further reduction in skin surface temperature compared with the vehicle, especially after the second application (Fig. 4C).

DISCUSSION

As described in previous articles^{9–11)} and in the present study, in HR-AD-fed mice, neither the cumulative time nor frequency of spontaneous scratching bouts was changed consistently, whereas the duration of one bout, which was calculated by the cumulative time divided by the frequency, was reproducibly prolonged. Thus, we preliminarily examined the effect of CL on basal scratching using the prolonged duration of one bout as a parameter of itch. However, as a consequence, we could not evaluate this effect accurately because application of CL markedly blocked the frequency, in some

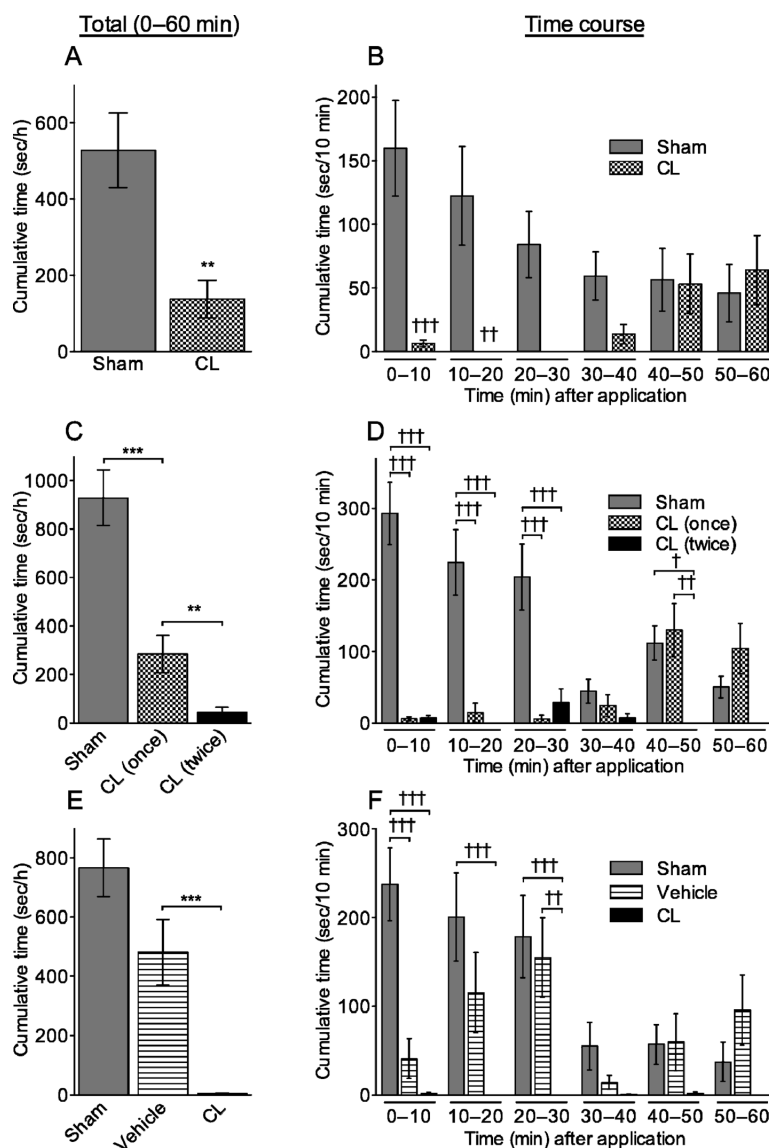


Fig. 3. Effect of Application of Chitosan-Containing Lotion (CL) on Ethanol-Induced Scratching in HR-AD-Fed Mice

(A,B) Effect of CL on scratching when CL was applied once immediately after ethanol dosing. Each column represents the mean $\pm$ S.E. of 17 animals. ** p < 0.01, Student's unpaired t -test. ++ p < 0.01 and +++ p < 0.001, two-way ANOVA with the Bonferroni multiple test. (C,D) Effect of CL on scratching when CL was applied twice immediately and at 30 min after ethanol dosing. Each column represents the mean $\pm$ S.E. of 11 or 12 animals. ** p < 0.01 and *** p < 0.001, Student's unpaired t -test. + p < 0.05, ++ p < 0.01 and +++ p < 0.001, two-way ANOVA with the Bonferroni multiple test. (E,F) Comparison of the effect on scratching between the vehicle and CL. The vehicle or CL was applied in the "twice" protocol. Each column represents the mean $\pm$ S.E. of 11 or 12 animals. *** p < 0.001, Student's unpaired t -test. ++ p < 0.01 and +++ p < 0.001, two-way ANOVA with the Bonferroni multiple test.

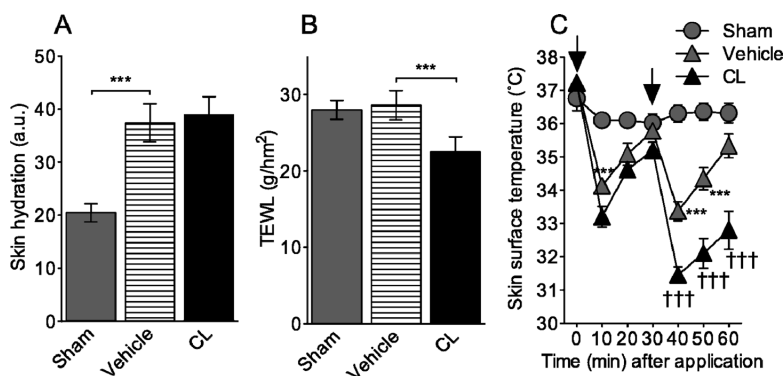


Fig. 4. Effects of Application of Chitosan-Containing Lotion (CL) on Dry Skin Symptoms and Skin Surface Temperature

(A,B) Effects of application of CL and the vehicle on dry skin symptoms, decreased skin hydration (A) and increased TEWL (B). Each column represents the mean $\pm$ S.E. of 11 or 12 animals. *** p < 0.001, Student's unpaired t -test. (C) Effect of application of CL and the vehicle on skin surface temperature. Application of CL or the vehicle is indicated by an arrow. Each point represents the mean $\pm$ S.E. of 5 or 6 animals. *** p < 0.001, sham vs. vehicle, two-way ANOVA with Bonferroni multiple test. +++ p < 0.001, vehicle vs. CL, two-way ANOVA with Bonferroni multiple test.

cases, resulting in no data on the duration of one bout (Fujii *et al.*, unpublished observation). Recently, we have found that oral administration of ethanol markedly enhanced scratching, and that the increased cumulative time was highly reproducible.¹¹ Thus, we reexamined the effect of CL on scratching using an ethanol-induced increase in cumulative time. When CL was applied once immediately following ethanol administration, the scratching response was almost completely inhibited, but this effect disappeared within 30–40 min (Fig. 3B); therefore, when applied twice immediately and at 30 min, CL blocked scratching for 60 min (Fig. 3D). Comparison of scratching response between CL and the chitosan-free vehicle showed that CL inhibited scratching more strongly than the vehicle, whereas the vehicle also suppressed scratching only for 10 min after application (Fig. 3F). Together, these results indicated that CL strongly inhibited the scratching of HR-AD-fed mice, and that the effect was largely dependent on the two types of chitosan contained.

Our previous studies^{10,11} have suggested that skin barrier dysfunction is involved not only in basal but also in ethanol-induced scratching of HR-AD-fed mice; therefore, in order to clarify the mechanism of inhibition by CL, the effect on dry skin symptoms was assessed. Although the decreased skin hydration was improved even by the vehicle (Fig. 4A), the increased TEWL was resolved only by CL (Fig. 4B), suggesting that the mechanism underlying the inhibition of scratching includes the recovery of skin barrier function but not the improvement of skin hydration. In the cosmetic field, chitosan is generally used as a humectant, but only a few reports are available on the effect of chitosan on skin barrier function. Denda *et al.*¹³ have examined, using mice, the effects of topical application of ionic polymers on acute skin barrier disruption made by tape stripping, and described that topical application of a cationic polymer such as chitosan delayed recovery of the skin permeability barrier. In contrast, in the present study, we found that CL improved skin barrier function (Fig. 4B), and such improvement was confirmed in our preliminary experiment using an acute skin disruption model of guinea pigs (Shimizu *et al.*, unpublished observation). Thus, the chitosan used in this study is thought to have the ability to recover the barrier function of skin. Although the reason for the inconsistent effects of chitosan on skin barrier function remains uncertain, it is likely due to differences in the models used for evaluation and/or the properties of chitosan (*e.g.*, molecular weight, degree of *N*-deacetylation, *etc.*). Furthermore, the mechanism underlying barrier recovery by CL remains to be determined. Since chitosan is known to have film-forming¹⁴ or bioadhesive¹⁵ properties, it is possible that such properties contributed to barrier recovery; however, the mechanism can only be speculated, and further investigations are needed.

In general, when certain lotions are applied topically, the liquid evaporates with a concomitant cooling sensation. Also, in the present study, we felt that the skin of mice was cooled after application of CL; therefore, to examine the relationship between inhibition of scratching and skin temperature, changes in skin temperature of vehicle- and CL-treated mice were measured. Although skin temperature was transiently but significantly reduced even by the vehicle, further reduc-

tion was detected by CL (Fig. 4C). The reduction of skin temperature by these lotions may be explained as follows. Firstly, liquid (nearly water) evaporation could contribute to temperature reduction. Because both CL and the vehicle were applied after recovery to room temperature (see Materials and Methods), cooling by lotion itself may also contribute to temperature reduction. Furthermore, chitosan is known to be hygroscopic; hence, cooling by lotion itself may be potentiated by chitosan contained. Since it is empirically well known that cold stimuli strongly suppress the itch sensation,¹⁶ the reduction of skin temperature could also be involved in the inhibition of scratching.

In conclusion, application of CL transiently but strongly inhibited itch-related scratching of special diet-fed hairless mice accompanied by barrier-disrupted dry skin. The inhibitory mechanism could be partly explained by recovery of skin barrier function and cooling of the skin. In the treatment of atopic dermatitis, along with anti-inflammatory agents such as corticosteroids and calcineurin inhibitors, regular applications of moisturizer have been recommended.^{17,18} Considering that chitosan is a non-toxic, biodegradable and biocompatible polymer, our findings suggest that the addition of chitosan to commonly used topical treatments may be a therapeutic option for treating itch and dry skin conditions in dermatological disorders, including atopic dermatitis.

Acknowledgement This study was supported in part by a Grant-in-Aid for Young Scientists (B) (21790096) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

REFERENCES

- 1) Koblenzer C. S., *J. Allergy Clin. Immunol.*, **104**, S109–S113 (1999).
- 2) Wahlgren C. F., *J. Dermatol.*, **26**, 770–779 (1999).
- 3) Werner Y., Lindberg M., *Acta Derm. Venereol.*, **65**, 102–105 (1985).
- 4) Lodén M., Olsson H., Axéll T., Linde Y. W., *Br. J. Dermatol.*, **126**, 137–141 (1992).
- 5) Seidenari S., Giusti G., *Acta Derm. Venereol.*, **75**, 429–433 (1995).
- 6) Ständer S., Steinhoff M., *Exp. Dermatol.*, **11**, 12–24 (2002).
- 7) Yosipovitch G., *Int. J. Cosmet. Sci.*, **26**, 1–7 (2004).
- 8) Yoshiike T., Aikawa Y., Sindhvananda J., Suto H., Nishimura K., Kawamoto T., Ogawa H., *J. Dermatol. Sci.*, **5**, 92–96 (1993).
- 9) Fujii M., Tomozawa J., Mizutani N., Nabe T., Danno K., Kohno S., *Exp. Dermatol.*, **14**, 460–468 (2005).
- 10) Fujii M., Nabe T., Tomozawa J., Kohno S., *Eur. J. Pharmacol.*, **530**, 152–156 (2006).
- 11) Fujii M., Nakamura T., Fukuno S., Mizutani N., Nabe T., Kohno S., *Eur. J. Pharmacol.*, **611**, 92–99 (2009).
- 12) Zhang J., Xia W., Liu P., Cheng Q., Tahirou T., Gu W., Li B., *Mar. Drugs*, **8**, 1962–1987 (2010).
- 13) Denda M., Nakanishi K., Kumazawa N., *Skin Pharmacol. Physiol.*, **18**, 36–41 (2005).
- 14) Pavinatto F. J., Caseli L., Oliveira O. N., *Biomacromolecules*, **11**, 1897–1908 (2010).
- 15) Peh K., Khan T., Ch'ng H., *J. Pharm. Pharm. Sci.*, **3**, 303–311 (2000).
- 16) Bromm B., Scharein E., Darsow U., Ring J., *Neurosci. Lett.*, **187**, 157–160 (1995).
- 17) Hanifin J. M., Cooper K. D., Ho V. C., Kang S., Krafchik B. R., Margolis D. J., Schachner L. A., Sidbury R., Whitmore S. E., Sieck C. K., Van Voorhees A. S., *J. Am. Acad. Dermatol.*, **50**, 391–404 (2004).
- 18) Saeki H., Furue M., Furukawa F., Hide M., Ohtsuki M., Katayama I., Sasaki R., Suto H., Takehara K., *J. Dermatol.*, **36**, 563–577 (2009).