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Effects of nobiletin on skin inflammation and pruritus in the ICR mouse model of anti-DNP monoclonal IgE antibody sensitization

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Abstract

We purified and chemically identified nobiletin (Nb) from the peel of *Citrus depressa* Rutaceae grown mainly in Okinawa, which contains abundant Nb. For the purpose of investigating the effects of Nb, which is present in citrus peel, on inflammation and pruritus in atopic dermatitis (AD), we evaluated the anti-pruritic effects of Nb using a topical application test involving a pruritus-induced guinea pig model developed by Taguchi et al., and also evaluated the anti-inflammatory and anti-pruritic effects of Nb by an internal use test using the ICR mouse model of pruritus associated with IgE-mediated auricular skin inflammatory reactions (IgE-mIP-ICR mouse) developed by Kano et al. First, the anti-pruritic effects were examined using guinea pigs with pruritus induced by histamine (HS) or kallikrein (KK). After a pruritus-inducing substance was intracutaneously injected, 10% glycerine aqueous solution containing 1% (w/v) Nb (100 μL) was immediately applied to the injection site. The control group received 10% glycerine aqueous solution alone. Nb significantly inhibited both the pruritus symptoms induced by HS and KK. Then, the cumulative duration of pruritic behavior in 2 hours was measured using the IgE-mIP-ICR mouse model to evaluate the anti-pruritic effects of the test substance. Also, Nb exhibited anti-pruritic effects, significantly reducing the cumulative duration of pruritic behavior within 2 hours after the topical application of the eliciting substance, dinitrofluorobenzene (DNFB). Using this latter model, the effects of the test substance were examined based on the inflammatory changes over 48 hours, and the changes in pruritus over 48 hours. The control vehicle consisted of 10% glycerine aqueous solution. After the topical application of DNFB, 0.3% CMC suspension containing 100 mg/kg Nb in control vehicle was immediately orally given orally. The control group received a suspension containing control vehicle. Skin inflammation and pruritus were observed at 1, 4, 24, and 48 hours after the topical application of DNFB. Nb significantly inhibited inflammatory reactions in IgE-mIP-ICR mice, with 2 peaks at 1 and 24 hours inceases of auricular thickness and also, significantly inhibited pruritus in IgE-mIP-ICR mice, showing 2 peaks at 1 and 24 hours, reducing the cumulative duration of pruritic behavior in 1 hour.

Therefore, from these results, lipophilic Nb significantly inhibited all skin inflammatory and pruritic symptoms in AD animal models. Nb appears, potentially, to be an effective nutrition supplement for the treatment of inflammation and pruritus in AD patients, and will be possible to become an important leading compound, to treat these symptoms.

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Introduction

Since the initial lesions in diseases developing during childhood including atopic dermatitis (AD) can hardly be identified using clinical specimens, it is difficult to establish an effective therapy by analyzing pathological conditions. Therefore, the establishment of an animal model that is highly reproducible for similar diseases, is a requirement for the development of effective therapies. However, no perfect animal model of AD is presently available. Here, we focused on pruritus. Taguchi et al.[1] established a pruritus-induced guinea pig model enabling the quantitative measurement of general pruritus, which responded to anti-histamine, a common anti-pruritic medication; and of intractable pruritus, which did not respond to anti-histamine. We also demonstrated that apple polyphenol (AP) as a nutrition supplement also inhibited intractable pruritus. Together with a clinical study showing that AP improves skin symptoms, inhibits pruritus in AD patients[2)3)], and improves pruritus in hemodialysis patients[4], we suggested that the pruritus-induced guinea pig model of Taguchi et al.[1] might be a useful and convenient system to quantitatively analyze pruritic behavior, and predict clinical effects. Furthermore, since severe AD conditions correlate well with blood IgE levels[5], and AD is expected to increase the production of IgE antibody[6], we used an ICR mouse model of IgE-mediated inflammation (IgE-mIP-ICR mouse) that was initially developed by Kano et al.[9] as an animal model to reproduce similar diseases by using the optimal ICR mouse for the observation of pruritic behavior[8] and incorporating pruritus in dermatitis instead of the BALB/c mouse model of biphasic inflammation associated with IgE-mediated late phase reaction (LPR)[7]. Since grape seed polyphenol isolated from grape seed extract (GSE), similarly classified as AP, a proanthocyanidin (PAC) which is composed by successive condensation of monomers, and flavan-3-ol inhibited intractable pruritus and inhibited both LRP dermatitis and pruritus in the IgE-mIP-ICR mouse model, it was suggested that AP and GSE could be used as supplements for the treatment of AD.

In this study, nobiletin (Nb), a polymethoxylated flavone (PMF) that is a lipophilic single substance contained in citrus peel (Fig. 1), was investigated for the above described effects. Nb was initially chosen because it is physiologically multifunctional. In addition to its ability to inhibit the elevations of blood sugar and blood pressure, as described by Taguchi et al.[10], it has been reported to physiologically prevent the progression of chronic rheumatoid arthritis[11], have some anti-cancer actions[12], immunoregulatory actions, anti-inflammatory actions[13], antioxidative actions, neuroprotective actions[14], prevent Alzheimer's disease[15], and have many more effects. Secondly, while AP and GSE are PAC derivatives, which are water-soluble successive condensation products, Nb is a PMF, which is a lipophilic single substance. Since these compounds have very different physical properties, we examined whether the property as a single substance or a complex affects the test system. Nb was purified from wild *Citrus depressa* Rutaceae grown naturally in Okinawa, which is known to contain Nb in its peel at very high concentrations.

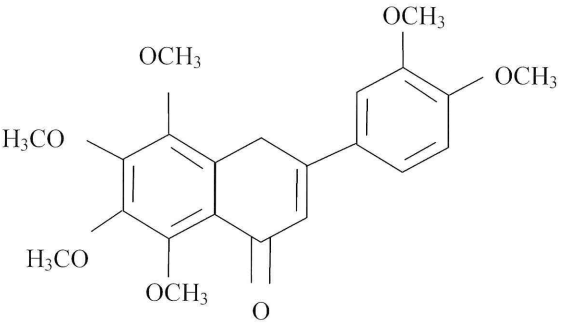

Fig. 1 Chemical structure of nobiletin (Nb)

Materials and methods

Pruritus-inducing substances and other materials

Histamine (HS) hydrochloride produced by Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and swine pancreas tissue kallikrein (KK) of low molecular weight produced by Merck Ltd. (Tokyo, Japan) were used as pruritus-inducing substances in guinea pigs. Mouse IgE monoclonal anti-dinitrophenol (DNP) antibody from Seikagaku Corporation (Tokyo, Japan) was used in IgE-mediated auricular dermatitis reactions. Dinitrofluorobenzene (DNFB), acetone, and olive oil from Nakarai Chemical Ltd. (Kyoto, Japan) were used. To measure auricular thickening, the Dial Thickness Gauge system from Mitsutoyo Corporation (Kanagawa, Japan) was used.

Experimental agents

Nb was purified from the dried peel of *Citrus depressa* Rutaceae, an Okinawan citrus, by the Laboratory of Medicinal Pharmacognosy, Tokyo University of Pharmacy and Life Sciences using the following method[16].

Purification of Nb

Reflux extraction of 500 g dried peel of *Citrus depressa* was performed twice using 2,000 mL methanol for 2 h. The extract was concentrated under reduced pressure, then loaded on a column filled with Diaion HP-20 (Mitsubishi Chemical Ltd., Tokyo, Japan), a polystyrene polymer resin, and successively eluted with 2,000 mL each of a water-methanol mixture, methanol, ethanol, and ethyl acetate. The ethyl acetate elution fraction with the lowest polarity (10 g) was loaded on a

silica gel column, and eluted with a chloroform-methanol mixture to obtain 5 fractions (I to V). Fraction III (1.63 g) was loaded on a new silica gel column, and eluted with a hexane-acetone mixture to obtain Nb (725 mg ; yield, 0.145%).

Laboratory animals

Wild-type 8-week-old male Hartley guinea pigs (Hoshino Laboratory Animals, Saitama, Japan) were used. Wild-type 4-week-old female ICR mice (Clea Japan, Inc., Tokyo, Japan) were used to examine the inflammatory reactions and pruritic behavior in the IgE-mediated auricular dermatitis model. After 1-week of preliminary rearing at room temperature (23±2°C) and at a humidity of 55±10%, the animals were assigned to specific groups. Guinea pigs and mice were fed with RC4 (Oriental Yeast Co., Ltd., Tokyo, Japan) and CE-2 (Clea Japan), respectively ; and were provided with tap water *ad libitum*.

Experimental methods

a) Pruritic behavior in guinea pigs with pruritus induced by HS or KK

The experiment was performed according to the methods of Taguchi et al.[1]. Guinea pigs were intracutaneously given 50 μL of a pruritus-inducing substance, HS hydrochloride (0.3 mg/ml) or swine pancreas KK (25 U/animal), in the middle of the right ventral region, and animal behaviors were recorded using an overhead video camera for 2 hours immediately after the injection. From the video-recorded behaviors, cumulative durations of pruritic behaviors including turning to the application site to scratch it with their teeth and scratching with a hind leg were measured (measurements were recorded in seconds within the 2 hours). For the Nb group, 10% aqueous glycerine solution containing 1% Nb (w/v) (100 μL) was applied to the pruritus-inducing substance injection site. For the control group, an equal volume of 10% glycerine aqueous solution, the solvent of the test substance, alone was applied. Each group consisted of 12 animals. Furthermore, the cumulative durations of pruritic behaviors within 2 hours in animals receiving physiological saline reported by Kano et al.[9] were compared with the results of animals receiving 10% glycerine aqueous in this report.

b) Changes in auricular skin inflammatory reactions in IgE-mIP-ICR mice over 48 hours

The experiment was performed according to the methods of Kano et al.[9]. In brief, ICR mice were passively sensitized with 0.5 mL anti-DNP IgE Ab (×7,680 as PCA titer), and 100 μL 0.15% DNFB in acetone/olive oil (4 : 1) was applied to the auricle of the animals to elicit biphasic dermatitis one hour after sensitization. At 1, 4, 24, and 48 hours after elicitation, auricular thickening (thickening based on auricular thickness before elicitation) at the DNFB application site was measured using a Dial Thickness Gauge. Immediately after application of the DNFB solution, the Nb group orally received 100 mg/kg Nb in 0.3% CMC suspension, and the control group received an equal volume of 0.3% CMC suspension alone. Each group consisted of 12 animals.

c) Cumulative durations of pruritic behavior in IgE-mIP-ICR mice within 2 hours

The DNFB solution was applied to the auricle of ICR mice sensitized with anti-DNP IgE Ab as in b) to elicit biphasic pruritus. After elicitation, pruritic behavior at the DNFB application site was video-recorded for 2 hours as in a). The Nb and control groups were orally given appropriate solutions as in b). Each group consisted of 12 animals.

d) Auricular pruritic behavior in IgE-mIP-ICR mice over 48 hours

The DNFB solution was applied to the auricle of ICR mice sensitized with anti-DNP IgE Ab as in b) to elicit biphasic skin pruritus, and pruritic behavior at the DNFB application site of the auricle was video-recorded for 1 hour at 1, 4, 24, and 48 hours after elicitation as in a). The Nb and control groups were orally given appropriate solutions as in b). Each group consisted of 12 animals.

Statistical analysis

Results were reported as means±standard deviations. Analyses of variance was performed for group comparisons, and Student's t-test was performed for pairwise comparisons with the control group. A p-value less than 0.05 was considered to indicate a stastically significant defference.

Results

1. Pruritic behavior in guinea pigs with pruritus induced by HS or KK

Figure 2 shows the total durations of pruritic behavior in the Nb group including both the turning, biting, and scratching behaviors induced by HS and KK. Both the total durations of pruritic behavior induced by HS and KK were significantly inhibited in the Nb group as follows. Pruritic behavior lasted 17.1±5.3 s/2 h ($p<0.01$) for HS-induced pruritus compared to 25.5±5.1 s/2 h in the control group ; and 39.3±6.4 s/2 h ($p<0.01$) for KK-induced pruritus compared to 52.0±7.1 s/2 h in the control group. Furthermore, almost no effect from different solvents on the total durations of pruritus behaviors was observed between water-soluble physiological saline, which is a solvent for water-soluble PAC used by Kano et al.[9] and 10% glycerine solution, which is a solvent for lipophilic Nb (PMF), used in the present study, with pruritus lasting 26.6±7.2 s/2 h in HS-induced pruritus and 45.8±9.2 s/2 h in KK-induced

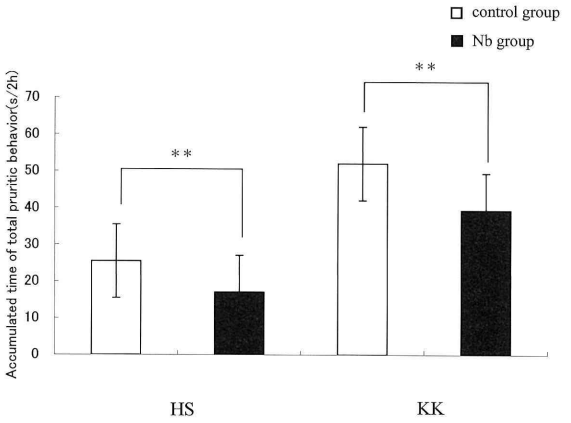

Fig. 2 Effect of topical Nb on pruritic behavior induced by histamine (HS) or kallikrein (KK) in guinea pigs. Each column represents a mean with standard deviation. Each group consisted of 12 guinea pigs. $^{**}p<0.01$ (Student's t-test)

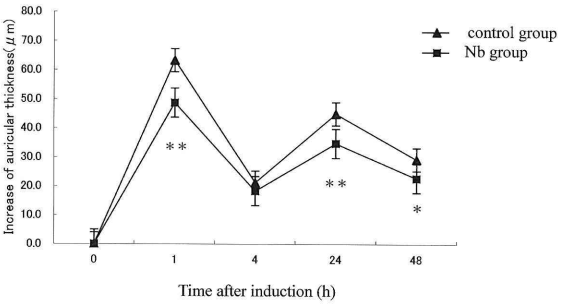

Fig. 3 Effect of oral Nb treatment on increase of auricular thickness (μm) over 48h after induction with DNFB in anti-DNP IgE Ab sensitized (PCA titer×7,680) IgE-mIP-ICR mice. Each value represents a mean with standard deviation. Each group consisted of 12 mice. $^{*}p<0.05$, $^{**}p<0.01$ (Student's t-test)

pruritus in the experiment of GSE by Kano et al.[9] Thus, this system was excellent to consistently measure pruritic behavior.

2. Changes in auricular skin inflammatory reactions in IgE-mIP-ICR mice over 48 hours

Figure 3 shows the results of changes in auricular skin inflammatory reactions over 48 hours in IgE-mIP-ICR mice prepared in b). In the control group, auricular thickening was 63.3±4.1, 21.2±4.4, 44.9±4.5, and 29.2±3.6 μm at 1, 4, 24, and 48 hours after the elicitation with DNFB, respectively, showing a clear biphasic inflammation pattern. Compared to the control group, auricular thickening was significantly inhibited at 1, 24, and 48 hours after DNFB induction, with 48.7±5.0 ($p<0.01$), 18.3±4.2, 34.7±5.5 ($p<0.01$), and 22.7±3.1 ($p<0.05$) μm, in the Nb group.

3. Cumulative durations of pruritic behavior in IgE-mIP-ICR mice within 2 hours

Figure 4 shows the results of cumulative durations of pruritic behavior within 2 hours in the IgE-mIP-ICR mice prepared in b). In the control group, clear pruritic behavior was observed of last 370.6±28.0 s/2 h. Compared to the control group, pruritic behavior was significantly inhibited, and lasted only 284.8±33.0 s/2 h ($p<0.01$) in the Nb group.

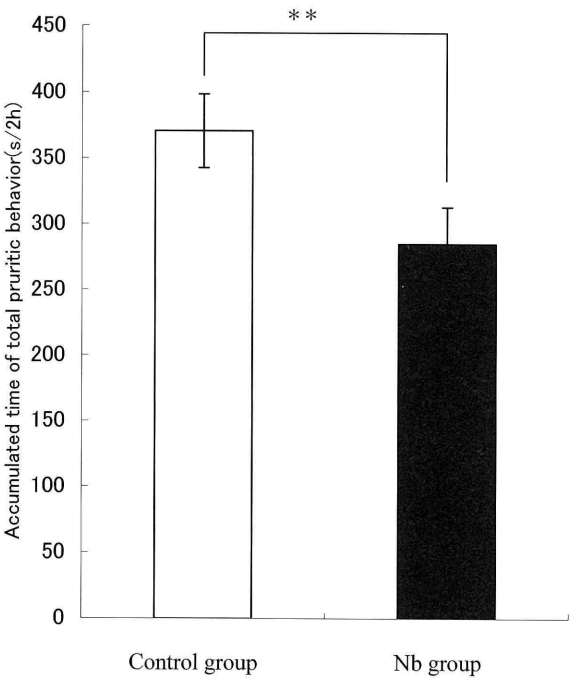

Fig. 4 Effet of oral Nb treatment on accumulated time of pruritic behaviors vs control within 2h after induction with DNFB in anti-DNP IgE Ab sensitized (PCA titer×7,680) IgE-mIP-ICR mce. Each column represents a mean with standard deviation. Each group consisted of 12 mice. $^{**}p<0.01$ (Student's t-test)

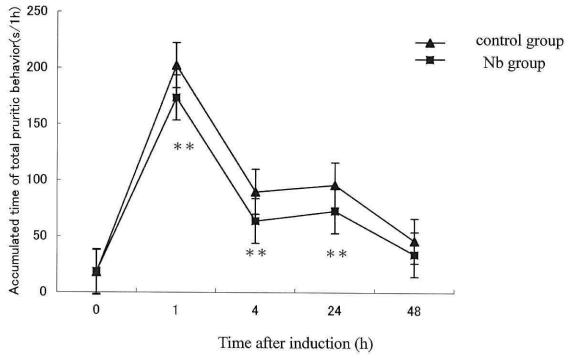

Fig. 5 Effet of oral Nb treatment on accumulated time of pruritic behaviors over 48h after induction with DNFB in anti-DNP IgE Ab sensitized (PCA titer×7,680) IgE-mIP-ICR mce. Each value represents a mean with standard deviation. Each group consisted of 12 mice. $^{**}p<0.01$ (Student's t-test)

4. Cumulative durations of pruritic behavior in IgE-mIP-ICR mice over 48 hours

The cumulative durations of pruritic behavior over 48 hours in the IgE-mIP-ICR mice prepared in b) were

compared between the Nb and the control groups. Figure 5 shows the results of pruritic behavior. In the control group, a clear biphasic pruritic behavior pattern was observed, with 202.17±21.31, 89.83±21.24, 95.92±24.54, and 46.17±19.24 s/h at 1, 4, 24, and 48 hours after the elicitation with DNFB. Compared to the control group, pruritic behavior was significantly inhibited at 1, 4, and 24 hours in the Nb group, with 173.50±22.39 ($p<0.01$), 63.83±22.48 ($p<0.01$), 72.83±12.83 ($p<0.01$), and 34.08±7.86 s/h.

Discussion

AD, an allergic disease, is not completely cured in adulthood, and the incidence of adult AD is increasing each year. In clinical practice, steroid and topical immunosuppressants such as FK506 are used as standard therapy, with the recommended use of anti-histamine with anti-allergic action as combination therapy for pruritus[6]. However, the flare of dermatitis and the associated recurrence of pruritus often fall into a vicious cycle, and dermatologists claim that, if pruritus can be suppressed, the disease will not worsen. To overcome this problem, it is critical to identify materials that can prevent intractable pruritus that cannot be suppressed by anti-histamine.

Previously, we investigated health food materials that could inhibit even intractable pruritus, i.e., AP and GSE, using the pruritus-induced guinea pig model of Taguchi et al.[1], which enable quantitative measurement of general pruritus responding to the above-mentioned anti-histamine while intractable pruritus does not respond. Studies on AP have reported the improvement of typical intractable pruritus symptoms, inhibition of pruritus in AD patients, and improvement of pruritus in hemodialysis patients[2-4]. Kano et al.[9] developed the IgE-mIP-ICR mouse model as a highly reproducible animal model for similar diseases using the optimal ICR mouse for the observation of pruritic behavior, and incorporating pruritus in dermatitis to modify the biphasic inflammation mouse (BALB/c) model with IgE-mediated late phase reaction (LPR) by Katayama[7] and Otoyama et al.[17] since AD is predisposed to produce IgE antibody[6], and severe AD conditions and blood IgE levels correlated positively[5], and reported that GSE significantly inhibited inflammation and pruritus at the second peak indicating LPR in addition to intractable pruritus in the pruritus-induced guinea pig model. However, since AP and GSE are not single compounds, but are PAC derivatives composed of successive condensations of a monomer flavan-3-ol, they are not suitable for the analysis of inhibitory mechanisms.

Accordingly, we examined whether similar effects were exhibited by Nb, a single lipophilic PMF compound contained in citrus peel, in the pruritus-induced guinea pig model and the IgE-mIP-ICR mouse model. Nb was selected as a candidate substance because it was considered that it might have inhibitory actions on both inflammation and pruritus since it is physiologically multifunctional[10-15].

First, the anti-pruritic action of Nb was examined in the pruritus-induced guinea pig model. We showed that Nb significantly inhibited both general (HS-induced) pruritus and intractable (KK-induced) pruritus (Fig. 2). The effects of water-soluble or lipophilic solvents on the assay system were examined in 12 animals per group. The results for the use of water-soluble physiological saline in the study by Kano et al.[9] and lipophilic 10% glycerine aqueous solution in this study were compared. Our study showed pruritic behavior in HS-induced pruritus in the control group and in KK-induced pruritus in the control group. Thus, 10% glycerine aqueous solution, a solvent used for lipophilic Nb (PMF), and water-soluble physiological saline used for AP and GSE, hardly affected the total durations of pruritic behavior, and the system reported in this study was excellent for the consistent and reliable measurement of pruritic behavior.

Subsequently, since severe AD conditions and blood IgE levels well correlate[5], and AD predisposes to the production of IgE antibody[6], the effects of Nb on auricular skin inflammatory reactions were examined over 48 hours in the IgE-mediated IgE-mIP-ICR mouse model by Kano et al.[9] The IgE-mIP-ICR mouse was prepared as an animal model to reproduce similar diseases using the optimal ICR mouse for the observation of pruritic behavior[8] and incorporate pruritus in dermatitis to modify the BALB/c mouse model of biphasic inflammation associated with IgE-mediated late phase reactions (LPR)[7]. In the control group, auricular thickening showed a clear biphasic inflammation pattern after elicitation with DNFB. Compared to the control group, inflammation was significantly inhibited at 1 hour (first peak ; fast phase inflammation), 24 hours (second peak ; late phase inflammation) after elicitation with DNFB, and 48 hours in the Nb group (Fig. 3).

Finally, anti-pruritic effects of Nb were confirmed by observing the cumulative durations of pruritic behavior within 2 hours in the above-described model IgE-mIP-ICR mouse (Fig. 4), and the effects of Nb on the cumulative durations of pruritic behavior over 48 hours were evaluated (Fig. 5). The cumulative durations of pruritic behavior at the DNFB application site within 2 hours after elicitation were examined to evaluate the anti-pruritic effects of Nb. In the Nb group, the cumulative durations of pruritic behavior were significantly inhibited. Thus, the anti-pruritic effects of Nb were confirmed (Fig. 4). Subsequently, the effects of Nb on

the cumulative durations of pruritic behaviors in the IgE-mIP-ICR mouse model over 48 hours were examined. As a result, a clear biphasic pruritic behavior pattern was observed after elicitation with DNFB in the control group. Nb significantly inhibited pruritus at 1 hour after elicitation with DNFB (first peak), and 24 hours (second peak) suggesting the inhibition of both fast phase pruritus and late phase pruritus (Fig. 5).

As materials that also inhibit intractable pruritus in the conventional pruritus-induced guinea pig model, and inhibit fast phase inflammation at the first peak; and general pruritus as well as late phase inflammation (LPR) at the second peak and intractable pruritus in the IgE-mIP-ICR mouse model obtained by using the pruritus ICR mouse and incorporating pruritus in dermatitis to modify the biphasic inflammation mouse with IgE-mediated LPR, we previously reported AP and GSE, some health food materials that are PAC successive condensation products, as evaluation substances. In the present study, a single substance, Nb, a lipophilic PMF purified from *Citrus depressa*, an Okinawan citrus fruit, significantly inhibited skin inflammation and pruritus in the AD animal model (IgE-mIP-ICR mouse). Epidermis in the skin is designed to protect the body from oxidation stress such as UV radiation. Vitamins with antioxidation effects and radical scavengers such as Cu, Zn-SOD play a role in this defensive system[18]. Pheripheral pruritus is suppressed by increasing Cu, Zn-SOD activity in epidermis[19]. Therefore, Nb is considered to suppress the peripheral itch receptors through potential anti-oxidative substance, the primary metabolite, 4′-hydroxy-3′, 5, 6, 7, 8-pentamethoxyflavone, of Nb demethylation, since the major metabolite was demethylated at the C-4′ position[20]. Nb may become an effective candidate substance, and leading compound as an ethical drug to treat inflammation and pruritus in AD patients.

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ICR マウスの抗-DNP モノクローナル IgE 抗体感作動物モデルにおけるノビレチンの皮膚炎および瘙痒に対する効果

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主に、沖縄県で栽培されている柑橘類のシークワーサーの果皮に多量に含まれる、ノビレチン (Nb) を精製し、化学的に同定した。この Nb のアトピー性皮膚炎 (AD) の瘙痒と炎症に対する作用を検討する目的で、田口らによる発痒モルモットモデルを用いて外用試験で瘙痒を、加納らによる IgE 介在性の皮膚炎症反応を伴う ICR 瘙痒 (IgE-mIP-ICR) マウスモデルを用いて内服試験で瘙痒と皮膚炎症を検討した。発痒モルモットはヒスタミン (HS) またはカリクレイン (KK) を皮内投与し、同部位に 1% (w/v) Nb の 10% グリセリン水液を、対照には 10% グリセリン水液を、皮内投与直後に直接 100 μl 塗布したところ、Nb は HS、KK のいずれもその発痒の発症を有意に抑制した。次に IgE-mIP-ICR マウスモデルでは惹起物質 Dinitrofluorobenzene (DNFB) を塗布後に、Nb を 100 mg/kg、0.3% CMC 懸濁液で経口投与し、対照 (0.3% CMC 懸濁) と比較したところ、2 時間累積瘙痒行動を Nb は有意に抑制した。さらに同モデルで 48 時間経時的変化を 1、4、24、48 時間で Nb の瘙痒と皮膚炎症反応に対する影響について検討したところ、いずれも 1、24 時間で二峰性にみられる瘙痒と皮膚炎症反応が、Nb 投与により 1 時間累積瘙痒行動が、1、24 時間の皮膚炎症反応が有意に抑制された。親油性の Nb は、AD 動物モデルの瘙痒と皮膚炎症反応を抑制する作用がみられることから、AD 患者に有効な健康食品素材となる可能性があり、また当該分野における医療用医薬品のリード化合物となる可能性も有している。

〈キーワード〉 ノビレチン、柑橘フラボン、AD 動物、皮膚炎、瘙痒