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Evaluation of antipruritic effect of apple polyphenols using a new animal model of pruritus

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Abstract

Patients receiving chronic hemodialysis, the elderly, and patients suffering from atopic dermatitis commonly have complaints of severe pruritus. Unfortunately, approved medications, mostly antihistamines, usually provide insufficient symptomatic control. Recent reports claimed that apple polyphenols (AP), a nutrition supplement, have antipruritic effects on patients suffering from atopic dermatitis or receiving hemodialysis. We established a new animal model to evaluate the pharmacological effectiveness of such extracts by using male Hartley guinea pigs (8 weeks of age). After intradermal injection of pruritic agents and external application of normal saline (vehicle control) or experimental agents around the injection site, accumulated times (s/120 min) of pruritic behavior were calculated by reviewing a 2 hour video taped promptly after the treatment. Pruritic agents used included pork pancreatic kallikrein (KK), and histamine (H). Antipruritic agents used included diphenhydramine HCl (DPH), AP. Pruritus induced by H (0.3 mg/ml) was significantly suppressed by 3% DPH (22.2±6.3 vs 13.6±4.6 s/120 min, $p<0.05$). 1% AP also demonstrated similar significant antipruritic effect (18.8±5.5 vs 11.9±4.3 s/120 min, $p<0.01$). Pruritus induced by KK (25 U/head) was not suppressed by 3% DPH (49.2±9.1 vs 51.3±8.1 s/120 min) while it was suppressed by 1% AP (46.8±4.2 vs 32.0±9.0 s/120 min, $p<0.05$). A minimum of 1% AP should be used in clinical trials.

Introduction

Patients receiving chronic hemodialysis, the elderly, and patients suffering from atopic dermatitis commonly have complaints of severe pruritus. Medications, mostly antihistamines, have been approved and used for symptomatic control. Unfortunately, these medications usually provide insufficient control of symptoms. Patients continue to scratch their skin and may cause physical injury resulting in secondary infection. Scratching may also cause further itchness and worsen pruritis. Effective medications are needed to control the pruritus and terminate this vicious circle. In order to study and evaluate potentially effective antipruritic medications efficiently to meet the needs of the patients, a well-established animal model is essential.

Pruritus is a symptom and it is difficult to be evaluated objectively. Furthermore, there is little recent development of basic physiology of pruritus and not to mention animal studies. Many mediators and substances causing pruritus have been studied in human subjects.[1] Tovebjörk[2] reported that pruritus was caused by the stimulation of nociceptive C-fibers through histamine (H) released from peripheral nerves in the upper extremities. This showed that C-fibers may have a specific role in transmitting the sense of itchiness.[3] On the other hand, the correlation of afferent C-fibers and the transmission of the signals of pruritus was also demonstrated in animals.[4] This indicated that the fundamental mechanism of pruritus is similar

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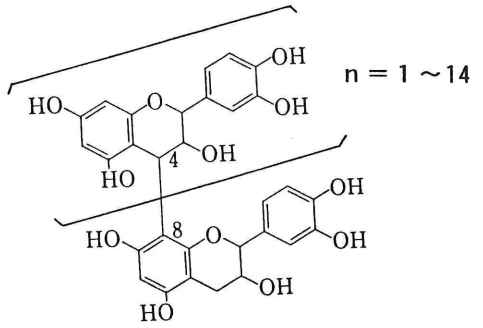

Fig. 1 Condensed chemical structure of flavane-3-ol (molecular weight ~300). Length of chain : n=1-14. Most apple polyphenols in nature exist as dimers or 3-unit complexes (molecular weight ~600-900).

between human and animals.

In 1959, Arthur and Shelley demonstrated that pruritus is caused by both physical and chemical stimuli. The latter was subsequently proved to be related to the release of H, proteases, and polypeptides. Further research showed that antihistamines such as diphenhydramine (DPH) suppressed H-induced pruritus but not kallikrein (KK)-induced ones.[6] For efficient screening of potential antipruritic drugs, small, easy-to-keep experimental animals with skin responses similar to human skin are needed. We utilized guinea pigs as an animal model and demonstrated the responses to H and KK induced pruritus treated with DPH to confirm chemical responses suggested by Arthur and Shelley in these animals.

Furthermore, by utilizing our new animal model, we screened potential drugs that may be useful for suppressing pruritus. Recent small scale clinical studies reported that apple polyphenols (AP) (Fig. 1), extracted from unripe apples used as a nutrition supplement have antipruritic effect on patients suffering from atopic dermatitis[7] or receiving hemodialysis.[8] We therefore used our newly established animal model to evaluate the effectiveness of such extract.

Materials and methods

Pruritic agents

Pruritic agents used in this experiment were histamine HCl (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and kallikrein (Merck & Co., Ltd., Tokyo, Japan). These agents were dissolved with normal saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) before use.

Experimental agents

Experimental agents used in this experiment were diphenhydramine HCl (Wako Pure Chemical Industries, Ltd.) and apple polyphenols (The Nikka Distilling Co., Ltd., Tokyo, Japan). Both agents were dissolved with normal saline (Otsuka Pharmaceutical Co., Ltd.) before use.

Subjects

The experiments were performed on male Hartley guinea pigs (8 weeks of age) purchased from Hoshino Laboratory Animals, Ltd., Saitama, Japan. The guinea pigs had been maintained three animals per cage with free access to food and water for one week and with a 12 h fluorescent lighting period before the experiment began.

Experimental design

On the day before the experiment, the hair of the experimental animals was shortened with hair clippers and was then cleanly removed by an electric razor (Matsushita Electric Works, Ltd., Osaka, Japan). Injection sites were wiped with cotton alcohol swaps. Different concentrations of each pruritic agent were prepared by dissolving the chemical in normal saline, then 0.05 ml of each of the resulted solutions was injected intradermally with a 27 G needle (Cross ; Top Corporation, Tokyo, Japan) into the right mediolateral abdominal area. 0.1 ml of experimental agents prepared (or normal saline control) was then immediately applied externally to the area of and around the injection site by the side of a syringe. Three experimental animals were then placed in a cage promptly. Their pruritic behavioral reactions were observed and recorded for 60 or 120 minutes by a video camera set up on the top of each cage. Behavior indicative of pruritus was classified as shown in Table 1 and the accumulated times (in seconds) of type 2 and type 3 were calculated respectively. About 10 guinea pigs were used in each group.

(1) Evaluation of injection sites to induce pruritus

Different areas of the guinea pigs were injected with pruritic agents intradermally (Table 2) to confirm if there were any variations in behavioral changes in response to itchiness. The mediolateral abdominal area has been used as the traditional injection site. Comparing with other sites we tested, other than injections on foot pads (front or hind limbs) and on the midline dorsal area, no differences from the traditional injection site were shown. Consequently, the mediolateral

Table 1 Classification of behavior related to pruritus in animal model

Type 1 : Turning the head around to the injection site
Type 2 : Scratching of the injection site with hind limb
Type 3 : Turning the head around and scratching the injection site with mouth
Type 4 : Jumping around
Type 5 : Scratching face with front limbs and the injection site with hind limb

Table 2 Behavioral changes with different injection sites

Mediolateral abdominal area	Traditional injection site
Anterolateral abdominal area	No behavioral differences from injecting to the mediolateral abdominal area
Posterolateral abdominal area	No behavioral differences from injecting to the mediolateral abdominal area
Midline abdominal area	No behavioral differences from injecting to the mediolateral abdominal area
Midline dorsal area (above vertebrae)	No behavioral changes
Foot pad of front limbs	Holding injected limb up with signs of pain
Foot pad of hind limbs	Holding injected limb up with signs of pain

abdominal area was used as the injection site to induce pruritus in this study.

(2) Evaluation of dosages of pruritic agents needed to induce significant pruritus

Different concentrations of DPH and KK were tested. Accumulated times of behavioral changes (type 2 and type 3) showed plateaux at about one hour after injection. (Figs. 2, 3) Thus, in this animal model, an observation time of 2 hours was adopted. This plateau was further confirmed by observing behavioral changes in guinea pigs for 2 hours at dosages of H 0.3 mg/ml and KK 25 U/head. H and KK showed similar type 2 and 3 behavior changes within 120 minutes of observation and plateaux appeared at around 70 to 80 minutes after injection (Fig. 4).

(3) Evaluation of diphenhydramine dosage needed for effectiveness study

DPH 1% (10 mg/ml) and 3% (30 mg/ml) were used as antipruritic agents. As shown in Fig. 5, 1% DPH did not demonstrate as much behavioral improvement as 3% DPH. The 3% DPH group showed a 50% lower mean accumulated time of behavioral changes comparing with the 1% DPH group and H group. Thus, 3% DPH was used to test the antipruritic effects of AP.

(4) Comparison of antipruritic effects between diphenhydramine and apple polypehnols

DPH and AP were used as antipruritic agents. As a result in (3), 3% (30 mg/ml) DPH was selected while 1% AP, the minimum concentration reported in the clinical studies was used. Each agent or normal saline was applied externally to experimental animals after intradermal injections of H 0.3 mg/ml or KK 25 U/head. Accumulated times of type 2 and type 3 behavioral changes after 120 minutes of observation were calculated and compared.

Statistical analysis

Data were presented as mean±standard deviation (SD). Statistical comparisons between data groups were performed using Student's *t*-test (paired) using StatView (version 5). A P value of <0.05 was considered to indicate a statistically significant difference.

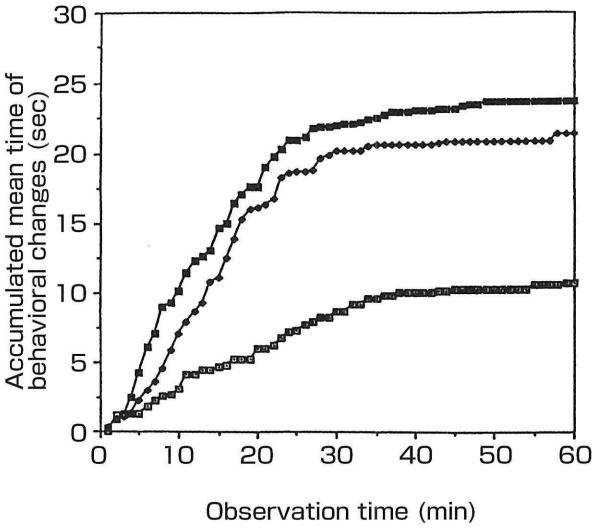

Fig. 2 Comparison of accumulated mean times of type 2 and type 3 behavioral changes caused by different concentrations of single histamine (H) intradermal injection. square with dot : H 0.03 mg/ml (n=11), solid diamond : H 0.1 mg/ml (n=10), bold square : H 0.3 mg/ml (n=10).

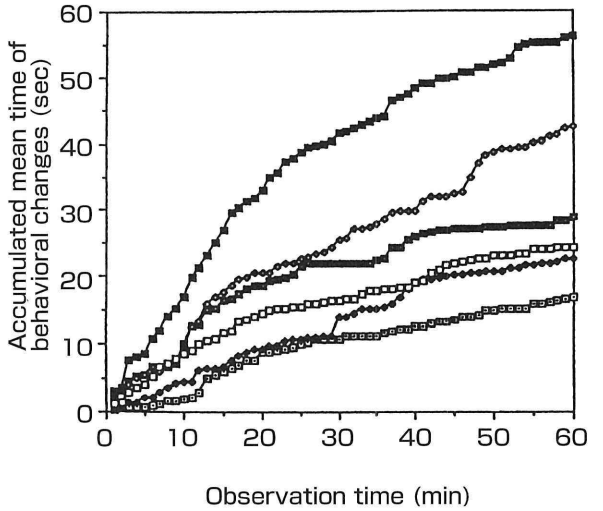

Fig. 3 Comparison of accumulated mean times of type 2 and type 3 behavioral changes caused by different concentrations of single kallikrein (KK) intradermal injection. square with dot : KK 5 U/head (n=10), solid diamond : KK 12.5 U/head (n=10), bold square : KK 18.75 U/head (n=9), diamond : KK 25 U/head (n=10), solid square : KK 37.5 U/head (n=9), square : KK 50 U/head (n=10).

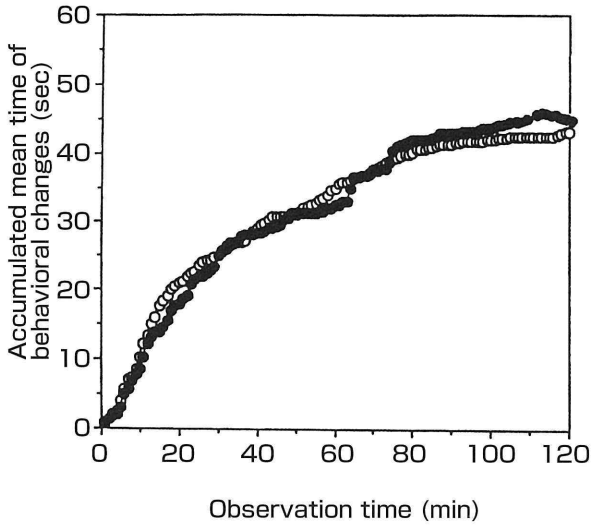

Fig. 4 Comparison of accumulated mean times of type 2 and type 3 behavioral changes caused by single histamine (H) and kallikrein (KK) intradermal injection.
circle: H 0.3 mg/ml (n=10), solid dot: KK 25 U/head (n=10).

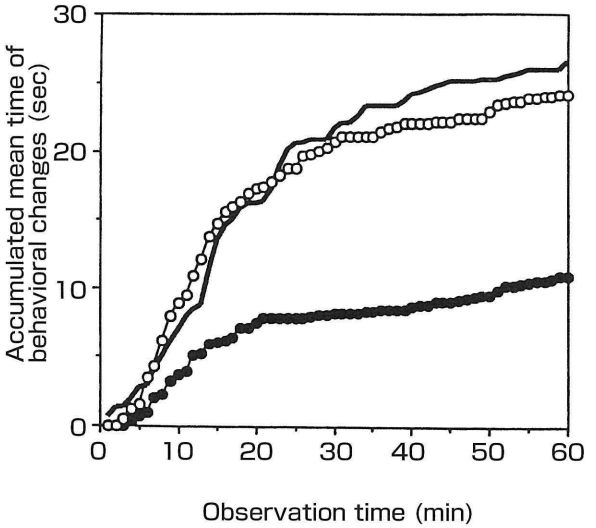

Fig. 5 Antipruritic activities of external 1% (10 mg/ml) and 3% (30 mg/ml) diphenhydramine (DPH) on itchiness induced by histamine (H) 0.3 mg/ml intradermal injection by comparing accumulated mean times of type 2 and type 3 behavioral changes.
circle: H+1% DPH (n=10), solid dot: H+3% DPH (n=11), bold line: H+normal saline (n=10).

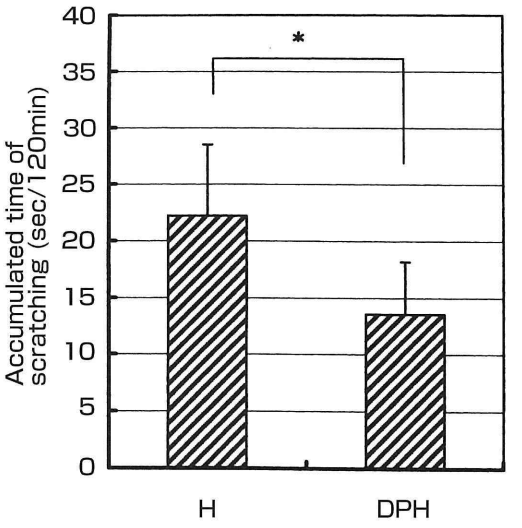

Fig. 6-a

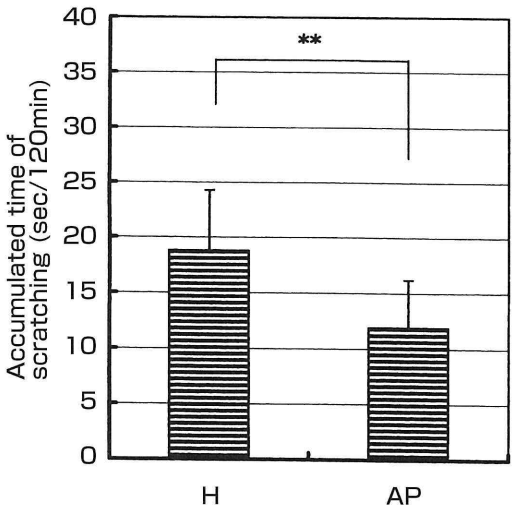

Fig. 6-b

Fig. 6 Antipruritic activity of (a) 3% (30 mg/ml) diphenhydramine (DPH) and (b) 1% apple polyphenols (AP) on itchiness induced by histamine (H) 0.3 mg/ml intradermal injection after 120 minutes. Scratching is defined as type 2 and type 3 behavior. Values represent means±standard deviations. n=12 in both experiments, $*P<0.05$, $**P<0.01$.

Results

Antipruritic effect of 3% (30 mg/ml) diphenhydramine saline solution on histamine-induced pruritus (Fig. 6a)

A single 0.3 mg/ml H intradermal injection followed by normal saline application induced pruritus with an accumulated time of scratching of 22.2±6.3 s/120 min. Comparing with control, an external application of 0.1 ml of 3% DPH saline solution significantly decreased the accumulated scratching time to 13.6±4.6 s/120 min ($P<0.05$).

Antipruritic effect of 1% apple polyphenols saline solution on histamine-induced pruritus (Fig. 6b)

A single 0.3 mg/ml H intradermal injection followed by normal saline application induced pruritus with an accumulated time of scratching of 18.8±5.5 s/120 min. Similar to the antipruritic effect of DPH, an external application of 0.1 ml of 1% AP saline solution significantly decreased the accumulated scratching time to 11.9±4.3 s/120 min ($P<0.01$) when compared with the control.

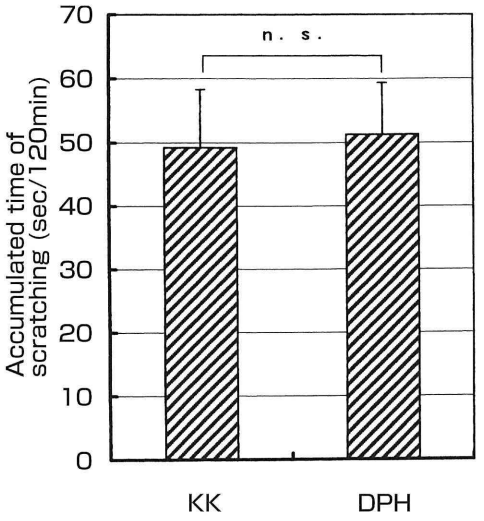

Fig. 7-a

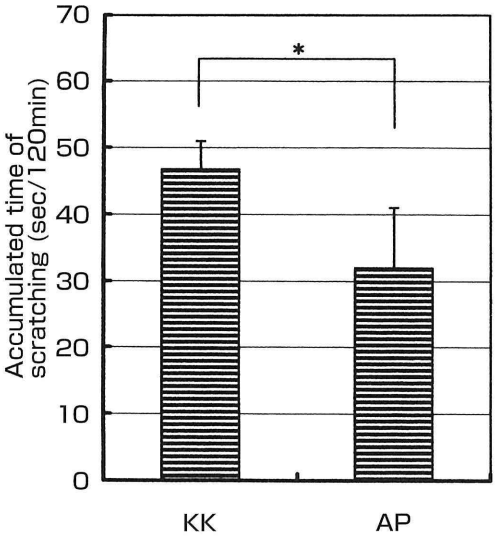

Fig. 7-b

Fig. 7 Antipruritic activity of (a) 3% (30 mg/ml) diphenhydramine (DPH) and (b) 1% apple polyphenols (AP) on itchiness induced by kallikrein (KK) 25 U/head intradermal injection after 120 minutes. Scratching is defined as type 2 and type 3 behavior. Values represent means±standard deviations. n=12 in both experiments, $*P<0.05$, n.s.=not significant.

Antipruritic effect of 3% (30 mg/ml) diphenhydramine saline solution on kallikrein-induced pruritus (Fig. 7a)

A single 25 U/head KK injection followed by normal saline application induced pruritus with an accumulated time of scratching of 49.2±9.1 s/120 min. Compared with controls, an external application of 0.1 ml of 3% DPH saline solution gave a similar accumulated scratching time of 51.3±8.1 s/120 min. This indicates that DPH cannot suppress KK-induced pruritus.

Antipruritic effect of 1% apple polyphenol saline solution on kallikrein-induced pruritus (Fig. 7b)

A single 25 U/head KK intradermal injection followed by normal saline application induced pruritus with an accumulated time of scratching of 46.8±4.2 s/120 min. Compared with controls, an external application of 0.1 ml of 1% AP saline solution significantly decreased the accumulated scratching time to 32.0±9.0 s/120 min ($P<0.05$). This indicates that AP can suppress KK-induced pruritus, which cannot be successfully treated by DPH.

Discussion

Pruritus can be caused by different mechanism including chemical mediators or physical, such as mechanical, electrical and thermal, stimuli. Chemical mediators reported included histamine, histamine-related substances (e.g. serotonin,[9] prostaglandins,[9] compound 48/80[10]), peptide (e.g. substance P[11]) and protease (e.g. bradykinin, kallikrien, and plasmin).[12] Some pruritants can directly stimulate itch receptors while some act indirectly by releasing other secondary substances that can stimulate the itch receptors.[12] The biochemical reactions after the stimulation of the receptors are still unknown. This limits the possibility of quantitative pharmacological research in pruritus therapy. Furthermore, after the landmark study done by Arthur and Shelley,[5] most of the pruritus studies were done on human subjects.[1] The desperate need of new antipruritics demands effective ways to new drug screening and development. An animal model is essential for such pre-clinical experiments. A recent report[4] on the specific role in message transmission of nociceptic C-fiber implies a similar mechanism of pruritus in animals and humans.

When establishing such an animal model, the degree and extent of locomotion of the animal cannot be too high or too small ; otherwise the behavioral reactions caused by itchness cannot be easily observed. Such a model must also be able to demonstrate pruritus induced by both H and KK, the major chemical pruritic agents reported by Arthur and Shelley.[5] In this way, antihistamines, which have been shown to be effective in pruritus induced by histamine, serotonin,[9] prostaglandins,[9] peptides from substance P,[10] and bradykinin,[12] can be evaluated while at the same time, potential medications for KK-induced pruritus can also be tested.

The method we applied in this study was to inject the mediolateral abdominal area intradermally followed by external application of 0.1 ml vehicle control (normal saline) or experimental agents (3% DPH, 1% AP) and detect any significant differences in total time of type 2 and type 3 behavior which were used as indicators of the antipruritic effect of the experimental agents. Type 2

and type 3 behaviors were used because other types of behavior could not be related to pruritus. Type 1 behavior is just turning the head while type 4 and type 5 behavior cannot be directly correlated to the itchiness induced by the injection scientifically.

Similarly to what Arthur and Shelley reported, in our model, 3% DPH significantly decreased scratching time comparing to the control when the pruritis was induced by 0.3 mg/ml of histamine. (Fig. 6a) No significant difference in scratching time was detected when induced by KK. (Fig. 7a) This proved that our guinea pig model of pruritus responded similarly to human subjects. At the same time, it also demonstrated that KK-induced pruritus is more severe than histamine-induced pruritus. External application of normal saline alone reduced the scratching time from about 40 s/120 min (Fig. 4) to 20 s/120 min (Fig. 6) while no such decrease was shown with KK-induced pruritus (Fig. 7). This animal model which can evaluate H- and KK-induced pruritus is thus of great value in screening and evaluating antipruritic drugs.

As some clinical reports showed the effectiveness of external AP in treating atopic dermatitis[7] and pruritus secondary to hemodialysis,[8] this model can be used to evaluate the efficacy of AP. Our results showed that external application of 1% AP significantly decreased the scratching time caused by H (Fig. 6b) and KK (Fig. 7b), supporting the fact that AP is potentially clinically useful. A minimum of 1% should be used in clinical trials.

Apple polyphenols exist as a dimer or a 3-unit complex (Fig. 1) in nature, having a molecular weight of 600 to 900. It thus allows external application and easy absorption. The possible mechanism of action of AP in treating intractable pruritus is not by suppressing the central nervous system but the peripheral nerve endings located between the dermis and epidermis through potential anti-oxidation stress effect.[13] 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 (superoxide dismutase) play a role in this defensive system.[14] Takeuchi et al.[15] used an immunohistochemical method to examine dermatological tissues from patients with psoriasis vulgaris. They identified decrease in the SOD enzyme protein which suggested the correlation between Cu, Zn-SOD decrease and the clinical symptoms of itchiness. Similarly, Toyama et al.[16] examined the Cu, Zn-SOD activities in the skin obtained from chronic renal failure patients receiving hemodialysis through biopsy. Using an immunohistochemical technique, the SOD activity was found to be highest in the basal cell layer. Hemodialysis patients with complaints of severe pruritus had extremely few cells with high SOD activity which also indicated the possible correlation between decrease of SOD and pruritus. Consequently, Cu, Zn-SOD activity can be used as an indicator to evaluate itchiness caused by oxidation stress.

On the other hand, low serum zinc levels and mild zinc deficiency were reported among most hemodialysis patients.[17] We demonstrated and reported that in zinc-deficient rats, nociceptive C-fibers which were shown to be related to itchiness were sensitized.[18] Prostaglandin E_2, a pruritic agent, and leukotriene B_4, an inflammation-causing agent, are increased in addition to the presence of inflammatory proteases. These chemicals and conditions could play important roles in the mechanism of pruritus.

Poblete et al.[19] also reported the presence of KK activity in psoriatic skin. This added to the possible mechanism of pruritus that in order to suppress itchiness effectively, the decrease in Cu, Zn-SOD activity in epidermis, which could be related to systemic deficiency of zinc storage, should be prevented and the formation of KK in the epidermis should also be suppressed. AP would then be a good candidate for effective antipruritic control because it can suppress KK-induced pruritus (Fig. 7b) and has strong anti-oxidation effect. It was shown to be able to restore Cu, Zn-SOD activity by 1.7×10^5 U/g measured by the xanthine-xanthine oxidase nitro-blue tetrazolium method.[13] It is a safe, natural product and has been used as a food supplement. The relatively low molecular weight of AP also allows direct external application with local absorption and distribution to target skin tissues between the dermis and epidermis and decreases oxidation stress.

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新規の発痒動物モデルによるリンゴ・ポリフェノールの鎮痒効果

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【要約】 慢性腎不全の血液透析患者の皮膚掻痒症，老人性皮膚掻痒症およびアトピー性皮膚炎の患者は，抗ヒスタミン剤を主体とする市販鎮痒剤で抑えられない難治性掻痒で悩まされている。近年，健康食材のリンゴ・ポリフェノール (AP) がアトピー性皮膚炎や血液透析患者の皮膚掻痒症に対して鎮痒効果のあることが報告されている。我々は，この難治性掻痒について薬理学的評価できる新規の動物モデルをハートレー系雄性モルモット (8 週齢) で作成した。発痒剤を皮内投与し，その投与部位に溶剤対照 (生理食塩液) あるいは被験液を外用塗布後，2 時間に亘りビデオカメラで連続撮影し，掻痒行動の累積時間 (s/120 min) を測定した。発痒剤には，ブタ膵臓カリクレイン (KK) およびヒスタミン塩酸塩 (H) を用い，鎮痒剤は塩酸ジフェンヒドラミン (DPH) および AP を用いた。H (0.3 mg/ml) 発痒に対して，3% DPH は有意な鎮痒効果 (22.2±6.3 vs 13.6±4.6 s/120 min, $P<0.05$) を示し，1% AP も同様に有意な鎮痒効果 (18.8±5.5 vs 11.9±4.3 s/120 min, $P<0.01$) を示した。KK (25 U/head) 発痒に対しては，3% DPH は効果はなく (49.2±9.1 vs 51.3±8.1 s/120 min)，一方，1% AP は KK 発痒でも 46.8±4.2 vs 32.0±9.0 s/120 min ($P<0.05$) と有意な鎮痒効果を示した。また，AP の濃度，1% は臨床試験で使用された下限の用量である。

〈**Key words**〉難治性掻痒症，動物モデル，カリクレイン