Yokukansan Appears to Regulate Epidermal Glutamate Signaling, Notably NMDA Receptors, in the Epidermis

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Yokukansan (YKS) has been used in Japan as a remedy for neurosis, insomnia, and children with night crying. Recently, many studies on the central nervous system (CNS) in terms of the effect of YKS have been reported in Japan. Here, we introduce our studies of YKS effects in the dermatological field. Our first study showed that YKS controls scratching behaviors and inhibits the development of AD-like lesions in isolated NC/Nga mice. In the second study, we compared the efficacy of YKS and fexofenadine (anti-allergic drug) using the same experimental system. Both YKS and fexofenadine inhibit aggravation of AD-like symptoms in socially isolated NC/Nga mice with respect to TEWL and dermatitis scores. However, YKS decreases the scratching and grooming behaviors in socially isolated NC/Nga mice. Thus, we speculate that YKS inhibits the aggravation of AD-like skin lesions in isolated NC/Nga mice due to mechanisms different from fexofenadine. From the results for the central nervous system, we focused on glutamate signaling to evaluate the effect of YKS in the epidermis. Immunohistochemistry and RT-PCR revealed that N-methyl-D-aspartate (NMDA) receptor expression was increased in the skin of conventional control mice and was decreased in YKS-treated mice. Glutamate transporter-1 (GLT-1) mRNA levels were decreased in the skin of conventional control mice and were increased in YKS-treated mice. The results indicate that YKS ameliorates AD-like skin lesions in NC/Nga mice through a mechanism distinct from that of fexofenadine. Our latest experiment showed that the extracellular concentrations of glutamate increased as the cell density increased in cultured keratinocytes. We speculate that this increase originated from an outflow of glutamate from the keratinocytes. Furthermore, the effects of YKS are suggested to regulate epidermal glutamate signaling, notably NMDA receptors, in the epidermis.

Key words: yokukansan, atopic dermatitis, NC/Nga mice, NMDA receptor, epidermal glutamate signaling

Introduction

Atopic dermatitis (AD) is a chronic relapsing eczematous skin disease characterized by pruritus and inflammation with cutaneous physiological dysfunction. Long–lasting itching is an intolerable sensation for patients with severe AD. Psychosocial stresses such as human relationships, pressures of work, worries about career, and anxieties for independence affect the skin condition of patients with AD. Such patients tend to have habitual scratching behavior called “addictive scratching”
or "scratch dependence"; this behavior worsens their eruptions gradually and forms a vicious cycle called the 'itch-scratch cycle'. Therefore, treatments are necessary for both the physical and the mental conditions of the patients. We term these adverse conditions of AD as "behavioral and psychological symptoms of atopic dermatitis (BPSA)" (Figure-1) and it has become an issue of public concern. Effective anti-pruritic and anti-BPSA drugs are needed by intractable AD patients. We introduce the knowledge of yokukansan in dermatological field, at the center of our research results to explore the possibility of treatment of severe AD.

NC/Nga mice have been regarded as an excellent model for AD since they develop AD-like skin lesions spontaneously under conventional conditions, but not under specific pathogen free (SPF) conditions. Before starting our study, we assessed the effects of the environment and stress on the skin of NC/Nga mice by comparing the mice bred in groups with those that were kept isolated under conventional conditions and under SPF conditions, respectively. It is well known that individually housed mice undergo strong social stress because mice are social animals that live in groups in the natural environment, measurements were made of dermatitis scores, transepidermal water loss (TEWL), scratching behaviors as well as grooming behaviors. Under SPF conditions, dermatitis was not observed either in the group housed or the individually housed animals. In that study, we used
data derived from the isolated mice kept under SPF conditions as a SPF control, which had no skin lesions. The results showed that isolated NC/Nga mice kept under conventional conditions manifested the most severe dermatitis with increased scratching behaviors and pathological grooming behaviors (data not shown). We concluded that socially isolated NC/Nga mice kept under conventional conditions exhibit skin lesions similar to those of human AD patients with BPSA under stressful conditions (Figure-2).

Previously, our group reported that Paroxetine, a selective serotonin re-uptake inhibitor (SSRI), inhibits the development of AD-like lesions in NC/Nga mice. We expected that Paroxetine might be an alternative or complementary therapeutic option for the treatment of AD. Previous studies have indicated that centrally acting therapeutic drugs are useful for anti-pruritic therapy.

Yokukansan (YKS) (Figure-3) is a traditional Japanese medicine called Kampo medicine. In Japan, Kampo medicines are often used for AD patients who do not respond to existing drugs or want to avoid using topical steroid ointment. Although Kampo medicines have long been used for many AD patients, there is no sufficient evidence about their efficacy so far. The reason is that Kampo medicines are usually prescribed individually for patients according to their symptoms, called “shou”, and therefore it is difficult to perform large-scale clinical studies using Kampo medicines. Recently, however, several studies have been performed using Kampo medicines and evidence-based data are now available. For example, Kobayashi et al. reported that Hochu-ekki-to is effective and safe for AD patients using multicenter, double-blind, randomized, placebo-controlled studies. Gao et al. reported that Juzen-taiho-to, Hochu-ekki-to, Shofu-san and Oren-gedoku-to might correct the Th1/Th2 balance skewed to Th2, and thus inhibit dermatitis in NC/Nga mice. With this background, we examined the efficacy of YKS in the treatment of skin lesions in NC/Nga mice to obtain basic information regarding the treatment of AD in humans.

YKS has been used as an anti-anxiety agent to treat neurosis, insomnia, and children with night crying. Clinically, it has been reported that YKS ameliorates excitement, anger, and hallucinations in behavioral and psychological symptoms of dementia (BPSD), and in patients with Alzheimer’s disease. BPSD treatment with YKS has been successful in clinical cases of dementia with Lewy bodies, Parkinsonian dementia, other forms of senile dementia, and also is successful in treating cases of neuropathic pain, schizophrenia, and restless legs syndrome. Recently, Yamamura et al. reported clinically case that YKS was effective in controlling refractory chronic urticaria. Thus, YKS is a notable Kampo medicine in various fields.

In our previous study using NC/Nga mice, YKS-controlled scratching behaviors inhibited the

Figure-3 YKS is a vacuum-concentrated extract of seven herbs in the following ratio: 4.0 g Atractylodes lancea rhizome, 4.0 g Hoelen, 3.0 g Cnidium rhizome, 3.0 g Japanese Angelica root, 2.0 g Bupleurum root, 1.5 g Glycyrrhiza root, and 3.0 g Uncaria thorn.
development of AD-like lesions and showed a "preventive effect" as well as a "therapeutic effect" on dermatitis\(^{16}\). However, the mechanism by which YKS controls scratching behaviors and inhibits the development of AD-like lesions in NC/Nga mice remains unexplored. In second study\(^{37}\), we investigated the effects of YKS on the development of AD-like lesions in socially isolated NC/Nga mice and compared its effects with those of fexofenadine, a histamine H1-receptor antagonist.

**Time courses of dermatitis scores, TEWL and the numbers of scratching behaviors and grooming behaviors**

Dermatitis scores of the conventional control group were aggravated as time goes on. Both the YKS- and the fexofenadine-treated groups significantly inhibited the aggravation of skin lesions in NC/Nga mice from 3 weeks after the start of the experiments. The dermatitis score of the SPF control group was not more than a minor increase (Figure-4A). The TEWL of the conventional control group increased as time goes on. The YKS- and the fexofenadine-treated groups significantly inhibited the increase of TEWL compared with the conventional control mice from 3 weeks. The TEWL of the SPF group, which had no skin lesions, was not increased (Figure-4B). Scratching behaviors of the conventional control and the fexofenadine-treated groups increased as time goes on. The YKS-treated group significantly decreased the scratching behaviors compared with the conventional control group after 6 weeks. The scratching behaviors of the SPF group were not increased (Figure-4C). Grooming behaviors of the SPF control: unfilled circle, conventional control: filled circle, YKS: filled square, fexofenadine: filled circle.

**Figure-4** The effects of YKS and fexofenadine on dermatitis score (A), the numbers of scratching behaviors (B) and grooming behaviors (C), TEWL (D), for AD-like skin lesions in NC/Nga mice.

A. The skin lesions of the conventional control group were aggravated as time goes on. Both the YKS- and the fexofenadine–treated groups significantly inhibited the aggravation of skin lesions in NC/Nga mice from 3 weeks after the start of the experiments. The dermatitis score of the SPF control group was not more than a minor increase.

B. Scratching behaviors of the conventional control and the fexofenadine–treated groups increased as time goes on. The YKS–treated group significantly decreased the scratching behaviors compared with the conventional control group after 6 weeks. The scratching behaviors of the SPF group were not increased.

C. Grooming behaviors of the conventional control group and also the fexofenadine–treated group were increased under social isolated conditions. The YKS–treated group significantly decreased the grooming behaviors compared with the conventional control mice from 3 weeks. The grooming behaviors of the SPF group increased under social isolated conditions.

D. The TEWL of the conventional control group was increased as time goes on. The YKS– and the fexofenadine–treated groups significantly inhibited the increase of TEWL compared with the conventional control mice from 3 weeks. The TEWL of the SPF group, which had no skin lesions, was not increased.
conventional control group and the fexofenadine-treated group were increased under social isolated conditions. The YKS-treated group significantly decreased the grooming behaviors compared to the conventional control mice from 3 weeks. The grooming behaviors of the SPF group increased under social isolated conditions (Figure-4D).

**Assessment of the number of infiltrating mast cells in the skin of NC/Nga mice**

Using histological examination, the numbers of mast cells infiltrating the skin were enumerated. The numbers of infiltrating mast cells in 0.25 × 0.25 mm (0.0625 mm²) squares were counted using a microscope. The numbers of infiltrating mast cells in the YKS-treated or the fexofenadine-treated mice were significantly decreased compared with the conventional control mice (Figure-5A).

**RT-PCR analyses of NMDA receptor, glutamate aspartate transporter, excitatory amino-acid carrier 1 and glutamate transporter-1 in the skin of NC/Nga mice**

The level of NMDA receptor mRNA in the skin was significantly increased in the conventional control mice compared with the SPF control mice, and was significantly decreased in the YKS-treated mice compared with the conventional control mice (Figure-5B). The level of glutamate transporter-1 (GLT-1) mRNA in the skin was decreased in the conventional control mice compared with the SPF control mice and the YKS-treated mice. The levels of glutamate aspartate transporter (GLAST) and excitatory amino-acid carrier 1 (EAAC1) mRNAs were not significantly different among the four groups (data not shown).

**Article consideration**

The results of our study demonstrate that the traditional Japanese medicine, YKS, ameliorates the development of AD-like lesions and the increase in TEWL and also decreases the number of scratching
behaviors and pathological grooming behaviors in socially isolated NC/Nga mice. It is also shown that NMDA receptors and GLT-1 in the skin are involved in the AD-like skin lesions of NC/Nga mice. YKS ameliorated the AD-like skin lesions as well as fexofenadine as shown in the present study. In our previous study, we demonstrated that YKS ameliorates the AD-like skin lesions in a dose-dependent manner in NC/Nga mice. In the present study, we utilized a Microact® to count the scratching behaviors of the animals, which provides a more objective and accurate evaluation of scratching behaviors as compared with visual counting by the investigators. Long-lasting ([1.5 s] scratching behaviors were counted, so that scratching behaviors which are equivalent to human “addictive scratching” were evaluated in NC/Nga mice. YKS significantly inhibited scratching behaviors compared with the conventional control mice and the fexofenadine-treated mice in which the numbers of scratching behaviors gradually increased. Although grooming behaviors are intrinsically essential in many animal species, anxiety and/or stress induce (s) the increase in involuntary grooming behaviors, which leads to inflammation, erosion, and/or ulceration in the skin. Such behaviors are also known to increase in mice that are models for obsessive compulsive disorder. Obsessive-compulsive disorder is an anxiety disorder and is characterized by persistent intrusive thoughts (obsessions) and repetitive behaviors (compulsions). Our experimental results show that YKS reduces the numbers of aberrant grooming behaviors of mice compared with the control mice and the fexofenadine–treated mice. It was shown that YKS alleviates excessive anxiety and/or stress in NC/Nga mice. Our experimental results suggest that YKS acts on the central nervous system (CNS) inducing a tranquilizing effect.

Itching is one of the major diagnostic criteria of AD and is also one of the most troublesome symptoms in AD. The discomfort of itching causes anger and irritation in AD patients and in family members. The itching sensation disturbs sleep, work, school and social life in AD patients for a long period of time, and eventually their quality of life (QOL) will be debased. The effective control of itching will contribute to controlling the skin lesions and to improving the patients’ QOL. The purpose of our study was to suppress the intolerable itching sensations which are not readily cured.

YKS, a traditional Japanese medicine, has been used as a remedy for neurosis, insomnia, and children with night crying. Since Iwasaki et al. 14 reported that YKS is effective for treating BPSD in a randomized, observer-blind, controlled trial in 2005, many basic and clinical studies using YKS have been reported. Clinically, it has been reported that YKS ameliorates excitement, anger, and hallucinations in BPSD, and in patients with Alzheimer’s disease. Treatment of BPSD with YKS has been successful in clinical cases of dementia with Lewy bodies, Parkinsonian dementia and other forms of senile dementia, and also treatments of neuropathic pain, schizophrenia and restless legs syndrome with YKS have been successful.

Basic research has shown that YKS regulates both serotonin signaling and glutamate signaling. Serotonin is made from tryptophan and suppresses the excitement of neuronal cells. Serotonin receptors now number more than 14, one of them being the 5-HT1A receptor. YKS is a partial agonist of the 5-HT1A receptor, and down-regulates the 5-HT2A receptor. Terawaki et al. 21 stated that YKS is a partial agonist of 5-HT1A receptors. Egashira et al. 22 stated that YKS is regulate to suppress the 5-HT2A receptor. Kawana et al. 23 reported that 5-HT1A receptors agonist is effective for AD patients. This report suggests that YKS which has partial agonist effect of 5-HT1A receptors is effective for AD patients. Glutamate is a major excitatory neurotransmitter, and accounts for 70–80% of neurotransmitters in the CNS. Glutamate is related to cognition, memory, study, motion control and so forth. Excess glutamate itself has excitotoxicity and is related to several psychoses. Ikarashi et al. 24 reported that YKS inhibits glutamate-mediated excitotoxicity as one of its mechanisms of action. Kawakami et al. 22 25 26 reported that YKS binds antagonistically to NMDA receptors and exerts a neuroprotective effect against glutamate-induced excitotoxicity, using cultured rat cortical astrocytes. Hiratsuka et al. 27 explained clearly that YKS inhibits neuronal death during ER stress by regulating the unfolded protein response. Accordingly, YKS controls extracellular glutamate concentrations by suppressing NMDA receptors.
and activating glutamate transport, and by suppressing glutamate-mediated excitotoxicity and ER stress, and ultimately inhibiting neuronal death in the CNS. Uchida et al.29 reported that the effects of YKS might be mediated by inhibiting the activity of the dopaminergic system. Thus, YKS has beneficial effects on BPSD and on various psychoses.

Furthermore, it has become clear that glutamate signaling also functions in non-neuronal tissues and occurs in sites as diverse as bone, pancreas, and skin30 31. It was reported by Lagerström et al.32 that vesicular glutamate transporters (VGLUT2) regulate the chronic itch sensation in mice. Intriguingly, Skerry and Genever31 reported that keratinocytes, dermal fibroblasts, melanocytes and Merkel cells also express glutamate transporters and NMDA receptors. They also reported that NMDA receptors are expressed on keratinocytes, and GLAST and GLT-1 are expressed by fibroblasts. Our experimental results indicate that YKS might affect glutamate transport and NMDA receptors in the skin, and thus might ameliorate skin homeostasis. YKS activates glutamate transport and reduces free glutamate among neurons. Thus, we examined the effects of YKS on dermatitis in a mouse model for AD, NC/Nga mice. In addition, Fuziwara et al. and others31-36 reported that glutamate plays an important role as a signal in cutaneous barrier homeostasis and in epidermal hyperplasia induced by barrier disruption. We postulate that YKS might affect glutamate transport and NMDA receptors in the skin, and thus might ameliorate AD-like lesions in NC/Nga mice.

Our first study16 showed that YKS controls scratching behaviors and inhibits the development of AD-like lesions in isolated NC/Nga mice. In the second study37, we compared the efficacy of YKS and fexofenadine using the same experimental system. Both YKS and fexofenadine inhibit aggravation of AD-like symptoms in socially isolated NC/Nga mice with respect to TEWL and dermatitis scores. However, YKS decreases the scratching and grooming behaviors in socially isolated NC/Nga mice. Thus, we speculate that YKS inhibits the aggravation of AD-like skin lesions in isolated NC/Nga mice due to mechanisms different from fexofenadine. According to the RT-PCR for NMDA receptors and GLT-1, YKS has a tendency to decrease the mRNA levels of NMDA receptors and to increase the mRNA levels of GLT-1. Thus, YKS is an excellent cell excitement modulator due to its regulation of intracellular glutamate concentrations in the skin.

Several studies analyzed the function of NMDA receptors in the skin. NMDA receptors reside on keratinocytes and are known to be involved in regulating their proliferation and differentiation and in skin barrier repair30-33. The role of GLT-1 which resides on keratinocytes and fibroblasts in the skin is not well understood. From literature on the function of NMDA receptor in the skin, when aggravating AD-like lesions occur in NC/Nga mice, the NMDA receptors may be activated and stimulate the keratinocytes, so that skin barrier repair is delayed. YKS inhibits NMDA receptor activity, thus skin barrier repair might be normalized. Further, YKS ameliorates the development of AD-like lesions in NC/Nga mice.

The second study37 suggests that YKS ameliorates the development of AD-like skin lesions and scratching behaviors in NC/Nga mice due to a mechanism different from fexofenadine. YKS suppresses the activity of NMDA receptors and enhances the activity of GLT-1 in the skin. We expect that YKS inhibits NMDA receptors and activates GLT-1 by adjusting the extracellular concentration of glutamate in the skin of NC/Nga mice. Further study is necessary to characterize the mechanism(s) of glutamate signaling and the relationship between the itch sensation and glutamate signaling function in the skin of NC/Nga mice. The authors plan to investigate the role of glutamate in the induction of itch sensation in future experiments. This study showed that YKS was effective for treating AD-like skin lesions with NC/Nga mice, and might be a useful therapeutic strategy for AD patients with BPSA under stressful conditions. In addition, we disclosed the glutamate signaling pathway involved in the AD-like skin
lesions with NC/Nga mice.

Our latest study ⁵⁰ is the experiments in vitro. In the cultured keratinocytes, the extra-cellular concentrations of glutamate increased as the cell density increased. We observed increased glutamate concentrations in the AD-like lesion of conventional control mice. However, we observed that the glutamate concentrations were decreased in the skin of YKS-treated mice. The glutamate concentration and dermatitis score were correlated. Dermatitis lesions can produce and release excess glutamate. These results suggested that YKS reduced the excessive glutamate production in NC/Nga mice skin. We speculate that this increase originated from an outflow of glutamate from the keratinocytes. We demonstrated that YKS suppresses the activity of NMDAR2D-type receptors in cultured keratinocytes. NMDA receptors reside on keratinocytes and are known to be involved in the process of skin barrier repair. YKS, which is also an NMDA antagonist, might improve skin barrier function.

**Conclusion remarks**

In conclusion, we suggest that YKS affects both the epidermis and the CNS. YKS might be an alternative or a complementary therapeutic option for the treatment of patients with severe pruritus and dermatitis, such as BPSA. Further more detailed experiments are necessary to clarify the precise functional nature of epidermal glutamate signaling and its medication by YKS.

**References**

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