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# Anti-pruritic Effect of Botulinum Toxin Type A against Histamine-induced Pruritus on Canine Skin

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**Abstract :** Botulinum toxin type A (BoNT/A) has been proven to be a safe and effective treatment for wrinkles in human. As well as the anti-wrinkle effects, the anti-pruritic effect of BoNT/A has been revealed from several researches for new therapy. The aim of this study was to investigate the anti-pruritic effect of BoNT/A against histamine-induced pruritus on canine skin. Five clinically healthy beagles were included in the study. All dogs were received 0.05 ml (5 Unit) of BoNT/A on the right dorsal thoracic region as an experiment and the same volume of saline solution was injected on the left dorsal thoracic region as a control, respectively. Intradermal histamine injections were performed four times (before treatment and days 1, 3 and 7 after BoNT/A injection). The severity of pruritus, the diameter and thickness of wheal, the erythema index and cutaneous temperature were assessed. The severity of pruritus was reduced on BoNT/A treated sides, compared with saline treated sides ($p < 0.05$). BoNT/A decreased wheal size, in both diameter and thickness ($p < 0.05$). Although, erythema index of both sides were increased after first histamine injection, BoNT/A treated sides showed the low-value as compared with saline treated sides. Cutaneous temperature was decreased significantly on BoNT/A treated sides. These results indicates that BoNT/A reduce histamine-induced pruritus on canine skin and suggested a possibility of application of BoNT/A for local intractable dermatologic problem in dogs.

***Key words*** **:** Botulinum toxin, histamine, pruritus, dog.

## Introduction

Pruritus in dogs is an extremely frustrating problem to owner and veterinary clinicians. Many kinds of skin diseases (e.g. atopic dermatitis, food hypersensitivity, flea allergic dermatitis, ectoparasitic skin infestation) manifest with erythema and mild to severe pruritus which increase predisposition to skin infection in canine skin (28). Also, chronic pruritus induces a significant discomfort effect on patients' quality of life and stress, which accelerates the ongoing alopecia on canine dermatologic patients (7,19). Such condition is difficult to manage, and traditional therapy with glucocorticoid is partly ineffective with undesirable side effects (39).

Many endogenous agents are considered to be mediator of pruritus, such as histamine, serotonin, tryptase, prostaglandin, leukotriene, substance P and interleukin-2. These substances directly stimulate and sensitize the itch either by mediating sensory nerve endings or by acting on mast cells (16,23).

Histamine is released by mast cells in response to various stimuli and is a potent biogenic amine with multiple activities in various physiological and pathological conditions. It is well known mediator in allergic diseases and has long recognized as a classical inducer of pruritus in dogs (21). It causes a wheal and erythema when injected intradermally. It also causes an intense pruritus and is often used as positive control in pruritus studies (17).

Botulinum toxin is manufactured by *Clostridium botulinum* as a complex mixture of neurotoxic polypeptides and non-toxic protein components. There are seven serologically distinct toxin types - A, B, C, D, E, F and G. The toxin is a two-chain polypeptide with a 100 kDa heavy enzyme that against the fusion (SNAP-25, syntaxin or synaptobrevin) at a neuromuscular junction, preventing vesicle from releasing acetylcholine. Acetylcholine is stored from binding to the membrane where neurotransmitter can be released. By blocking release of acetylcholine, the toxin interferes with nerve impulse and cause paralysis of muscles. It can hold the muscle unable to contract for three to four months. Even though botulinum toxin is originally a lethal substance, it can be used as an effective and powerful medication, when carefully isolated and purified. The most characterized serotype toxin is botulinum toxin A (BoNT/A) for both therapeutic and cosmetic use (3).

BoNT/A has been used to treat various disorders, including dermatological problem such as hyperhidrosis (34,42). Several studies have shown that BoNT/A injection reduce pruritus in human skin.

An anti-pruritic effect of BoNT/A originate from clinical investigation in lichen simplex (14) and dyshidrotic hand eczema (41,34). The precise mechanism of anti-pruritic effect

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of BoNT/A was still poorly understood - BoNT/A inhibit the release of acetylcholine from presynaptic vesicle (12,18) and the release of substance P, which may be involved in pruritus (5,20).

Several research showed that BoNT/A was adapted to the dog. Injection BoNT/A into dog prostate reduces contractile function while maintaining relaxation response of the prostates (22). Intraglandular injections of botulinum toxin type A and D significantly reduced the production of saliva from canine submandibular gland (31). BoNT/A appeared to be effective in the treatment of essential blepharospasm in this dog (24).

Recently, botulinum toxin has been widely used for its effects of hyperkinetic facial lines in cosmetic dermatology. Studies on the effects of this potent toxin can lead to another possible application for dermatologist or specialist. To the author's knowledge there are little report about BoNT/A application in canine skin for anti-pruritic effect. Among the many effects of botulinum toxin, this study focuses on anti-pruritic action.

The purpose of the present study was to investigate the anti-pruritic effect of BoNT/A against histamine-induced pruritus on canine skin based on various factors including pruritus severity, change in wheal size and thickness, erythema index and cutaneous temperature.

## Materials and Methods

### Experimental animals

Five healthy Beagle dogs (3 male and 2 female; age 3.7 ± 0.4 years, body weight 9.1 ± 2.4 kg) were used in the study. Each dog was housed separately in different cages, fed commercial diet. None of dogs had a previous history of pruritus or skin disease, and they had not received any medications for at least 4 weeks prior to experiment.

### Experimental design

The study was shown schematically in Fig 1. Twenty-four hours prior to the experiment, all dogs were examined by the investigator to ensure that they did not have any dermatological or systemic disease. Bilateral dorsal thoracic region (5 cm apart from 3 to 4th thoracic spinous process) was shaved by clipper (#10, Oster, USA) for injections. The BoNT/A was injected to right side and saline was injected to left side. Histamine was injected four times (before treatment, day 1, 3 and 7 after histamine application). All of tests were carried out by same investigator to minimize variability in the application technique. Test site was marked with TLS™ Surgical Skin Markerpen (Portex Surgical Inc.). The intradermal histamine injection and related measurement were executed in a quite laboratory (room temperature was maintained 20 to 25°C humidity was 30 to 45%). Before each experiment, the population rested for at least 30 min in the room; the tests were performed between 10:00 and 18:00.

### Botulinum toxin type A

A vial of BoNT/A (MEDITOXIN®, 100U of *Clostridium botulinum* Type A Neurotoxin Complex, Pacificpharma Co., Korea) was diluted with 1 ml of saline as recommended by manufacturer. Five units of BoNT/A, 0.05 ml of reconstituted BoNT/A were injected into the skin using insulin syringes, fitted with 29 gauge needles. The same volume of sterile saline was injected to contra lateral dorsal region in canine skin. Prior to BoNT/A injection, the skin was washed with shampoo and water, and dried.

### Intradermal histamine injection

Histamine dihydrochloride (assay ≥ 99%, powder, Sigma-Aldrich Co., USA) was injected into the skin to induce pruritic response. The histamine was injected to the test sites of the dorsal thoracic regions as described above. Pre-experiment was performed to identify optimal concentration of histamine. Three different concentrations of histamine (0.01%, 0.1% and 1%) were tested to determine the concentration which could yield the most potent reaction. According to the result, 0.1% concentration of histamine was shown to be the most potent pruritus inducing concentration. In this study, 0.1% of histamine dihydrochloride was injected into the skin. Prior to the histamine test, the skin was cleaned with alcohol.

### Assessments

#### Pruritus severity

Pruritus severity was presented as a visual analogue scales (VAS) and a numerical rating scales (NRS), manifest levels of severity, and various behavior changes. In order to identify accurate pruritus severity, each behavior was scored. Severe pruritus (eg. chewing and scratching) scored two and moderate pruritus (eg. shaking and licking) scored one. All scores were added for comparison of pruritic level. BoNT/A treated sides were compared with saline treated sides for assessing pruritus. Pruritus severity was calculated during 15 min. All processing of records were done by video camera (Handycam, HDR-SR12 Sony, Japan).

#### Wheal measurement

The wheal was measured by an electronic digital caliper (Digimatic caliper, Mitutoyo Corp., Japan). By folding the skin, thickness of wheal was measured.


**Fig 1.** Simplified experimental design.
*intradermal histamine injection and assessment. **BoNT/A or Saline injection

### Erythema index

To evaluate skin redness, a Mexameter (MX18, Courage and Khazaka, Germany) was used. It measures the stimulation of microcirculation before and after histamine application with measuring of hemoglobin value. Skin redness manifests numerically. Histamine injection site and surrounding skin were measured by Mexameter. The difference of value presents to erythema index.

### Cutaneous temperature

Skin temperature was measured by using an infrared thermometer (Raytek Co., USA). Comparative temperature degree was measured through difference between histamine injected area and surrounding area. The values were expressed as the mean of three times to each site.

### Statistical analysis

Statistical analysis was executed at four time points, data were analyzed with non-parametric statistics for two factors of treatment (BoNT/A *vs.* Saline) and trials (repeated factor: pretreatment, day 1, day 3, day 7). The Willcoxon's rank sum test was used for comparison of treatment (BoNT/A *vs.* Saline). The Friedman test (Friedman two-way analysis of variance by ranks) was used for comparison of trials (repeated factor: pre-treatment, day 1, day 3, day 7). The statistical evaluation was performed using SAS (Ver. 9.1.3) and PASW statistic 18 (SPSS Inc.)

## Results

### Pruritus severity

In all experimental groups, itch was begun within 1 min after histamine injection and lasted for 5 - 10 min. Ten minutes after histamine application, pruritus was gradually decreased. Pruritus was severely decreased with a BoNT/A treated sides compared with saline treated sides at day 3 and 7 ($p < 0.05$). Prior to BoNT/A and saline treatment, BoNT/A treated sides showed severe pruritus compared with saline treated sides. After-treatment of BoNT/A, however, pruritus was severely reduced in BoNT/A treated sides compared with saline treated sides. In day 3 and 7, BoNT/A induced remarkably lower pruritus level compared with pretreatment (day 3 decreased 44.4% and day 7 decreased 61.1%, $p < 0.05$) (Fig 2).

### Wheal measurement

In general, wheal became visible about 1 min after histamine application. Wheal formation lasted for about 20 min, and disappeared about 30 min later. BoNT/A treated sides showed thinner thickness as compared with saline treated sides at day 3 and 7 ($p < 0.05$). In day 1 and 7, BoNT/A induced significantly thinner thickness compared with pretreatment (day 1 decreased 9.5% and day 7 decreased 22.2%, $p < 0.05$) (Fig 3). The change of saline treated wheal diameter was stable during a week. As time goes by, however, the wheal diameter of BoNT/A treated sides were slowly decreased. Especially, BoNT/A induced significantly lower wheal diameter at day 7, as compared with pre-

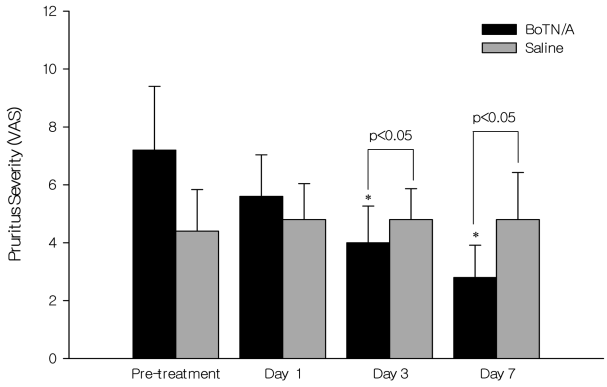


**Fig 2.** Pruritus severity following histamine injection in skin treated with botulinum toxin type A and saline. BoNT/A showed lower pruritus severity as compared with saline treatment at day 3 and 7 ($p < 0.05$). * BoNT/A induced remarkably lower pruritus level compared with pretreatment at day 3 and 7, respectively ($p < 0.05$).

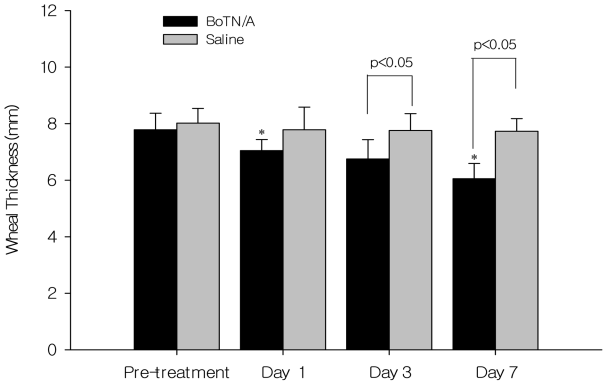


**Fig 3.** Wheal thickness following intradermal histamine injection in skin treated with botulinum toxin type A and saline, respectively. BoNT/A showed thinner thickness as compared with saline treatment at day 3 and 7, respectively ($p < 0.05$). *indicates that BoNT/A induced significantly lower thickness compared with pretreatment at day 1 and 7, respectively ($p < 0.05$).

treatment (day 7 decreased 19.9%, $p < 0.05$) (Fig 4).

### Erythema index

After the first histamine injection, erythema value was suddenly raised at day 1. Most of dogs scratched their injection site, between pretreatment and day 1. Saline treated sides showed significant increase at day 1 as compared with pretreatment (324.4% increased, $p < 0.05$). On the other hand, BoNT/A treated sides showed slight increase at day 1 as compared with pretreatment (77.5% increased). In BoNT/A treated sides, erythema value decreased in time- dependant manner, in proximity to pretreatment value on day 7. Additionally, BoNT/A treated sides showed lower erythema value at day 7 as compared with pretreatment (68% decreased, $p < 0.05$) (Fig 5).

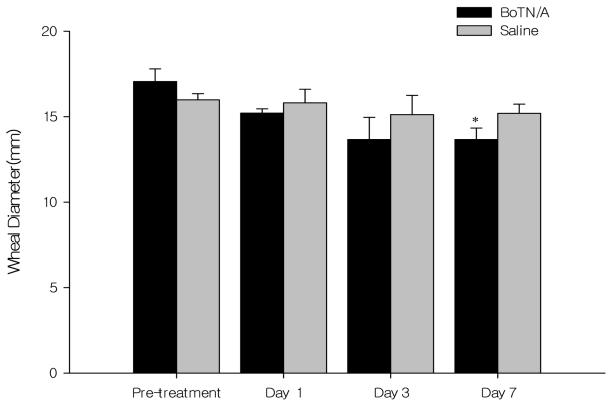


**Fig 4.** Wheal diameter following intradermal histamine injection in skin treated with botulinum toxin type A and saline. *indicates that BoNT/A significantly lower diameter of wheal at day 7 as compared with pretreatment ($p < 0.05$).

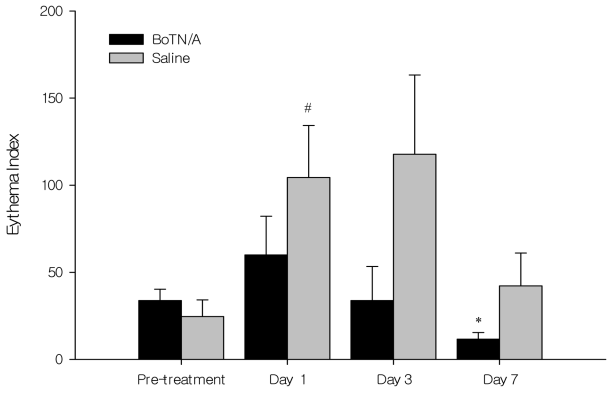


**Fig 5.** Erythema index following intradermal histamine injection in skin treated with botulinum toxin type A and saline. # indicates that at day 1, the significant induction of erythema confirmed in saline treatment as compared with pretreatment ($p < 0.05$). *indicates that BoNT/A lowered erythema value at day 7, as compared with pretreatment ($p < 0.05$).

### Cutaneous temperature

After first histamine treatment, cutaneous temperature decreased in a time-dependant manner. BoNT/A treated sides showed significantly low temperature of skin at day 3 and 7, as compared with saline treated sides ($p < 0.05$). (Fig 6)

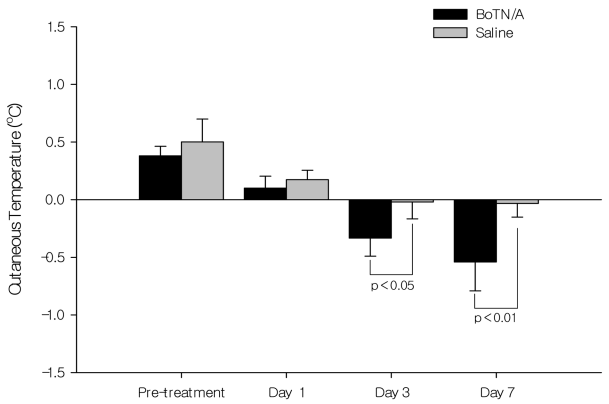


**Fig 6.** Cutaneous temperature following intradermal histamine injection in skin treated with botulinum toxin type A and saline. BoNT/A showed significantly low temperature of skin at day 3 and 7, as compared with saline.

## Discussion

In this study, BoNT/A was shown to reduce histamine induced pruritus based on various factors including pruritus severity, change in wheal size and thickness, erythema index and cutaneous temperature. Similar to those reported in human skin, histamine reaction was reduced by BoNT/A treatment.

There were various previous studies on the application of BoNT/A in human which confirmed the other effects of BoNT/A such as pain relieving effect, causing muscular atrophy, inactivation of exocrine gland and anti-pruritic effect on skin (9,26,34,36). To the author's knowledge, very few studies have been published on the application of BoNT/A in dogs. These studies used BoNT/A on submandibular gland to reduce saliva and prostate to reinforce cystic sphincter (22,31). There is no research to treat with canine skin on BoNT/A until now. The present study based on human research; the anti-pruritic effect of BoNT/A on histamine induced itch (8). Compared with human research, evaluation factors were similar but fitted with canine model, such as pruritus severity (27,29). The results of canine studies were similar to those of humans until day 3. In human studies, the data was decreased until day 3. However, in canine studies, the data was decreased until day 7. It is considered that the activity of BoNT/A lasts longer in canine skin compared with human skin.

Pruritus severity was decreased immediately in BoNT/A treated sides. It lasted until 7 day. It means that the most potent reaction of BoNT/A treatment can be seen at day 7 or after. Weal size was decreased as pruritus severity. As for the erythema, blood flow raised more in saline treated sides than in BoNT/A treated sides. After the first histamine injection, dogs began to scratch in histamine treated site. As result, erythema index rose in both sides. However, erythema values were different in each side; BoNT/A treatment showed low erythema index compared with saline treatment. It means that BoNT/A injection suppress scratching behaviors and inhibit pruritus in canine skin. Cutaneous temperature was decreased at day 3 and 7. It is considered that repeated histamine injection made skin cool compared with normal skin.

Pruritus is defined as a discomfort sensation that causes the desire to scratch. Pruritus is classified into four categories: 1.pruritoceptive pruritus; derived from skin inflammation (e.g. scabies, urticaria) 2.neurogenic pruritus; originated from disease of afferent pruritic pathway (e.g. post-herpetic neuralgia, multiple sclerosis) 3.neurogenic originated from the central nerve system disorder due to circulating metabolites (e.g. pruritus cholestasis) 4.psychogenic pruritus from delusional disorder (11,38,43).

The present study is concerned with pruritoceptive pruritus which peripherally induced pruritus in canine skin because pruritoceptive pruritus is the most common in veterinary patients (4). The sensation is generated when a subset of C afferent nerve fibres (C-fibres) conduct sensory information from the epidermis through the spinothalamic tract to thalamus, where bundle of neurons are activated (6). The stimulation translated into pruritic sensation in the sensory cerebral cortex (10). These neurons end in the dermoepidermal junction of the skin, occasionally penetrating the epidermis as free endings. Pruritogenic substances (e.g. histamine) activate these neurons in epidermis and outer layer of dermis followed by release of substances considered as pruritic mediators (substance P, somatostatin, vasoactive intestinal polypeptide, neuropeptide Y, acetylcholine, bradykinin, serotonin and prostaglandin E). These pruritic mediators can stimulate mast cells to release histamine and neurotransmitters (40).

The present study used histamine as a pruritus stimulation agent in canine skin. As histamine is unable to penetrate the intact skin barrier, it is applied by intradermal injection. This method delivers histamine to the dermoepidermal junction of skin, and induces pruritic sensation (32). In intradermal skin test, histamine is used as a positive control (15). The mechanism that histamine induces pruritus associated with neurotransmitter is still unknown. However, phospholipase CB3, a molecule that links G protein-coupled receptors to an intracellular signaling network, mediates the scratching respond activated by the histamine $H_1$ receptor on C-fibres (13).

This study shows that BoNT/A can remarkably reduce the pruritus severity in canine skin. There are several possible reasons for the reduced pruritus by BoNT/A. One of the early theories is that BoNT/A inhibits release of acetylcholine from presynaptic vesicles (18). Acetylcholine in particular has been reported to evoke pruritus in patients with atopic dermatitis (12). Our observation that the pruritus was relieved after BoNT/A treatment further supports this. In recent microdialysis study in human skin, although BoNT/A prevents the release of neuronal acetylcholine, it does not prevent the release of non-neuronal acetylcholine (30). It means that another mediator is associated with inhibition of pruritus.

Previous studies showed that BoNT/A influences neurons involved in pain perception and blocks the neurotransmitter (1,2). BoNT/A has been shown the effect of reducing the release of glutamate, substance P, calcitonin gene-related peptide (CGRP), vasopressin (20). In rat formalin test, BoNT/A is able to inhibit pain and neuropeptide release (5). In injection of capsaicin intradermally, BoNT/A reduced the pain and neurogenic vasodilatation in human skin (37). Based on these studies, it is proposed that BoNT/A is associated with substances in pain perception and other neurogenic mediators such as substance P and glutamate, which may contribute to anti-pruritic effects (5,20). Substance P activates mast cells for histamine and tryptase release and induces pruritus (33). BoNT/A could inhibit the release of such mediator and decrease itch signaling pathway. The anti-pruritic effect of BoNT/A in lichen simplex and dyshidrotic hand eczema can explain the inhibition of substance P and glutamate release.

Our result showed that the BoNT/A treatment reduced values; wheal size, eythema index and cutaneous temperature. When histamine was injected into skin, specific unmyelinated C-fibres release vasodilatory neuropeptides such as substance P and CGRP, which induce erythema surrounding the application site (43). Substance P induces erythema by releasing more histamine from cutaneous mast cell and induces plasma extravasation on small blood vessel in skin (16). In atopic dermatitis patient, substance P and CGRP was abnormally increased in the density of peptidergic afferent staining (35). Other neurotransmitter possibly associated with axon reflex, and may also be affected BoNT/A. For instance, BoNT/A has been reported to reduce norepinephrine release from sympathetic nerves (25).

In conclusion, although mechanism underlying the inhibitory effects remains unknown, it was proven that BoNT/A play a role in reducing skin pruritus. The present findings reinforce the hypothesis that BoNT/A represents an interesting opportunity to treat pruritic conditions characterized by intractable dermatologic problem.

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# 개에서 Histamine으로 유발한 피부소양증에 대한 보툴리늄 톡신의 항소양 효과

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**요 약** : 보툴리늄 톡신(BoNT/A)은 사람에서 안전하고 효과적인 주름치료제로 적용되고 있으며 최근에는 주름치료효과 이외의 효능에 관한 연구가 활발히 진행되고 있다. 본 연구의 목적은 개 피부에 히스타민을 피하 주입하여 소양증을 유발한 다음 보툴리늄 톡신의 항소양 효과를 평가하는 것이다. 총 5 마리의 비글을 이용하여 우측 배측 흉부 피부에 보툴리늄 톡신 0.05 ml (5unit)를 주입한 처치부위와 좌측 배측 흉부 피부에 0.05 ml의 생리식염수를 주입한 대조부위를 비교하였다. 보툴리늄 톡신 투여 전, 투여 후 1, 3, 7일에 Histamine 을 피내주입하여 소양증을 유발하였다. 소양증의 정도, 팽진의 지름과 두께, 홍반수치 및 피부표면 온도를 측정하여 보툴리늄 톡신 주입 효과를 평가하였다. 소양증의 정도는 처치부위에서 유의하게 감소하였으며($p < 0.05$) 팽진의 지름과 두께도 처치부위에서 유의하게 감소했다($p < 0.05$). 홍반수치는 최초 히스타민을 피내투여한 직후에 처치부위와 대조부위에서 모두 증가했으나 대조부위에 비해서 처치부위에서 적게 증가하였다. 피부표면온도는 처치부위에서 유의하게 감소하였다($p < 0.05$). 본 연구결과 보툴리늄 톡신은 개 피부에서 히스타민에 의한 소양증에 대한 항소양 효과를 보였으며 임상적으로 극심한 국소 소양증을 보이는 피부질환에 대해 적용할 수 있을 것으로 사료된다.

**주요어** : 보툴리늄 톡신, 히스타민, 소양증, 개