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NOTE

Toxicology

Effect of clothianidin exposure at the no-observed-adverse-effect level (NOAEL) in a mouse model of atopic dermatitis

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ABSTRACT. The effects of exposure to clothianidin (CLO), a neonicotinoid pesticide (NN), on the thymus and intestinal microbiota were recently revealed. Immune cells express nicotinic acetylcholine receptors (nAChRs), an NN target, suggesting CLO may disrupt the immune system. However, the relationship between CLO and atopic dermatitis (AD) is unknown. We administered a no-adverse-effect-level (NOAEL) dose of CLO to male NC/Nga mice with induced AD and measured, at three time points, key AD symptom indicators: epidermal thickening, mast cell number, total plasma IgE, and histamine levels. CLO increased total plasma IgE levels but reduced epidermal thickening, mast cell number, and plasma histamine levels in the early stages of AD. This demonstrates for the first time that CLO exposure inhibits AD's early symptoms.

KEYWORDS: α7 nAChR, atopic dermatitis, clothianidin, IgE, NC/Nga mice

Atopic dermatitis (AD) is a pruritic eczematous dermatitis. Its symptoms chronically fluctuate, alternating between remissions and relapses. Most individuals with AD exhibit atopic diathesis, characterized by either a personal or family history of asthma, allergic rhinitis, conjunctivitis, or AD, or by a predisposition to overproduce IgE [17]. The pathophysiology of AD includes characteristics such as dryness, susceptibility to infection, abnormal sweating, a low itch threshold, and various non-allergic mechanisms, including stress. Additionally, allergic inflammation, triggered by environmental and food antigens, is a specific factor [14]. Abnormalities in skin barrier function and immune response are believed to play significant roles in its pathogenesis [2]. Exposure to environmental chemicals is also known to affect AD symptoms [9], highlighting the importance of investigating the connection between AD and chemicals in the environment around us.

Neonicotinoid pesticides (NNs) began to be used in Japan in 1993, after which their usage rapidly increased. NNs are compounds with structures similar to that of nicotine. They were originally believed to selectively agonize nicotinic acetylcholine receptors (nAChRs) in insects. However, they were also found to act on mammalian nAChRs [10]. nAChRs are also expressed on immune cells [8] and regulate antigen presentation, cytokine production, and anti-inflammatory responses [16, 25]. Numerous reports indicate the involvement of α7 nAChR in the regulation of anti-inflammatory responses, with its expression observed in macrophages, T cells, B

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cells, mast cells, and so on [See Review 19]. Previous studies showed that exposure to clothianidin (CLO), a class of NNs, causes a decrease in rat thymus weight [15], an increase in Hassall bodies in mouse thymus [13], and dysbiosis of the intestinal microbiota [13, 15, 24]. It has been shown that an imbalance of the intestinal microbiota causes allergies [See Review 18], raising the possibility that NNs may disrupt the immune system and contribute to the development of allergic diseases. Therefore, NN exposure may affect AD symptoms by disrupting the immune system via immune cells and gut microbiota. However, no studies have examined the relationship between NNs and AD. Thus, we examined the effects of CLO on AD in a mouse model (NC/Nga).

Male NC/Nga mice (8 weeks old) were purchased from the Jackson Laboratory Japan (Yokohama, Japan) and maintained as described elsewhere [7]. The present study was approved by the Institutional Animal Care and Use Committee (Permission #30-01-01) and conducted in accordance with the Kobe University Animal Experiment Regulations. Mice were divided into two groups: the AD group, which received AD-induced treatment, and the AD·CLO group, which received AD-induced treatment along with CLO administration. These animals underwent sampling on Days (D) 14, 21, and 28, with the day of the first AD-induced treatment day set as D0 (Fig. 1).

CLO was purified according to a previous study [6], and the concentrations of CLO in the AD group (vehicle: 1% dimethyl sulfoxide) and the AD·CLO group (CLO: 47.2 mg/kg/day) were set based on a no-adverse-effect-level (NOAEL) dose (47.2 mg/kg/day [4, 23]). To eliminate the risk of adverse effects associated with forced oral administration and to establish sufficient CLO exposure, the animals were given rehydration gel (MediGel® Sucrarose; ClearH_2O, Portland, ME, USA) with or without CLO from D–11 (11 days prior to the initiation of treatment) to the sacrifice day.

Atopic dermatitis-like skin lesions were induced in 10-week-old NC/Nga Tnd Crlj mice using Biostir-AD (Biostir Inc., Osaka, Japan), an ointment reagent containing allergens from *Dermatophagoides farinae*, according to the manufacturer's protocol. In brief, the hair on the upper back was shaved off with an electric clipper, and depilatory cream (Veet; Reckitt Benckiser, Slough, UK) was applied to remove the residual hairs. To disrupt the skin barrier, 150 µL of 4% w/v sodium dodecyl sulfate (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) was applied to the shaved back skin 2 hr before topical application of 100 mg Biostir-AD. Biostir-AD was applied twice a week for a total of 6 times (4 times for the D14 specimens) (Fig. 1).

Animals were euthanized under isoflurane anesthesia by making an incision in the thoracic cavity to expose the heart, from which whole blood samples were taken. The back skin was then removed. The skins were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C for 24 hr. They were embedded in paraffin according to the established method. Plasma collected from blood was frozen at −80°C until use.

Paraffin-embedded blocks were cut into 5-µm-thick sections using a sliding microtome (SM2000R, Leica Microsystems, Wetzlar, Germany) and mounted on glass slides precoated with 2% 3'-aminopropyltriethoxysilane (Shin-Etsu Chemical Co., Tokyo, Japan).

For the general histological analyses, tissue sections were stained with hematoxylin–eosin (HE) and toluidine blue (TB) after deparaffinization and hydration, and then were examined under an optical microscope. The stained images were analyzed quantitatively for epidermal thickening based on the HE-stained images and for number of mast cells based on the TB-stained images using the free software ImageJ (National Institutes of Health, Bethesda, MD, USA). Epidermal thickening was calculated as the mean value of epidermal thickening (defined as the granular to basal layers) at 10 locations on the tissue sections. The number of mast cells was calculated as the average count in three areas (1.0×10^5 µm^2 each) within a tissue section.

To measure plasma histamine levels, we performed the following procedure. In a 1.5 mL Eppendorf tube, 50 µL of plasma, 30 µL of IS-mix solution, and 170 µL of 1% acetonitrile formate were added, vortexed, and centrifuged at 25°C, 10,000 × *g*, for 10 min. After centrifugation, 30 µL of the supernatant and 20 µL of Py-Tag (Taiyo Nippon Sanso Corp., Tokyo, Japan) were added to the Eppendorf tube, and a derivatization reaction was performed for 15 min at 50°C using a thermal cycler. Following termination of the derivatization reaction with the addition of 1 µL of 50% formic acid water, 50 µL of 1% acetonitrile formate was added to each tube, and the mixture was then centrifuged in a desktop centrifuge. After the purification and concentration of a 100 µL sample using MonoSpin Phospholipid (GL Sciences, Inc., Tokyo, Japan) through centrifugation at 5,000 × *g* for 3 min, an additional 100 µL of DW was added to dilute it. Histamine levels were then assessed using LC-ESI/MS/MS and quantified using the internal standard method. Total plasma IgE levels were determined from collected plasma using an ELISA kit (FUJIFILM Wako Pure Chemical Corp.) according to the manufacturer's instructions.

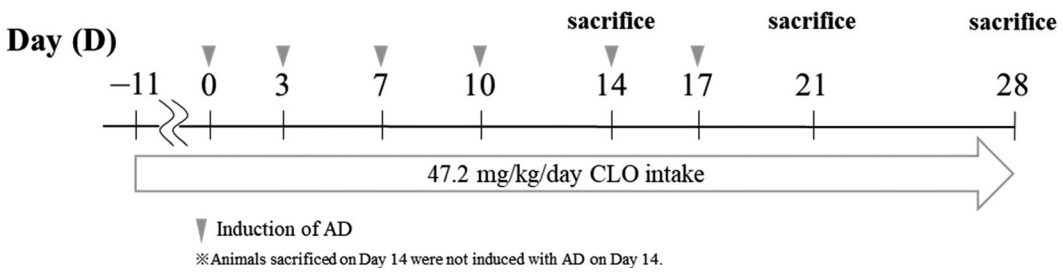

Fig. 1. Timeline of the overall experimental design. To induce atopic dermatitis (AD), ointment reagent Biostir-AD (Biostir Inc., Osaka, Japan) containing allergens from *Dermatophagoides farinae* was applied twice a week, until Day (D) 17, according to the manufacturer's protocol. Clothianidin (CLO) (47.2 mg/kg/day) was administered to male NC/Nga mice from D–11 (11 days prior to the initiation of treatment) to the sacrifice day, with the day of the first AD-induced treatment day set as D0.

Statistical analysis was performed with BellCurve for Excel (Version 4.04; SSRI, Tokyo, Japan). The Shapiro–Wilk test was used to test for normality, and the *F* test was used to test for homogeneity of variance. Welch's *t*-test, Student's *t*-test, and Mann–Whitney's *U*-test were used to analyze each measurement result. The results of analysis were considered significant when the *P*-value was less than 0.05.

AD-like symptoms, represented by erythema, crust formation (Fig. 2A), epidermal thickening, and mast cell mobilization in inflammatory skin areas (Fig. 2B), were observed in untreated NC/Nga mice after AD-induced treatment (on D28).

Epidermal thickening tended to be lower in the AD·CLO group than in the AD group on D14 (P=0.067) (Fig. 3A) and lower in the AD·CLO group than in the AD group on D21 (P=0.050) (Fig. 3B). There was no statistically significant difference on D28 (Fig. 3C). Since epidermal keratinocytes express nAChR and α7 nAChR signaling has been reported to regulate keratinocyte cell cycle progression [5], it is possible that CLO had a direct effect on α7 nAChR in epidermal keratinocytes. It is also possible that CLO may have affected the inflammatory response. Epidermal thickening is induced by inflammatory reactions in the skin. Type 2 inflammation, triggered by allergen penetration into skin tissues, leads to the production of IgE. Mast cells, which express the high-affinity IgE receptor (FcεRI), release cytokines and chemical transmitters like histamine through the binding of allergen-specific IgE, thus inducing inflammation. In the presence of such inflammation, thymus and activation-regulated chemokine (TARC) is produced in the lesional skin, facilitating the infiltration of Th2 cells into the lesion [17]. The present study considers the involvement of mast cells and their

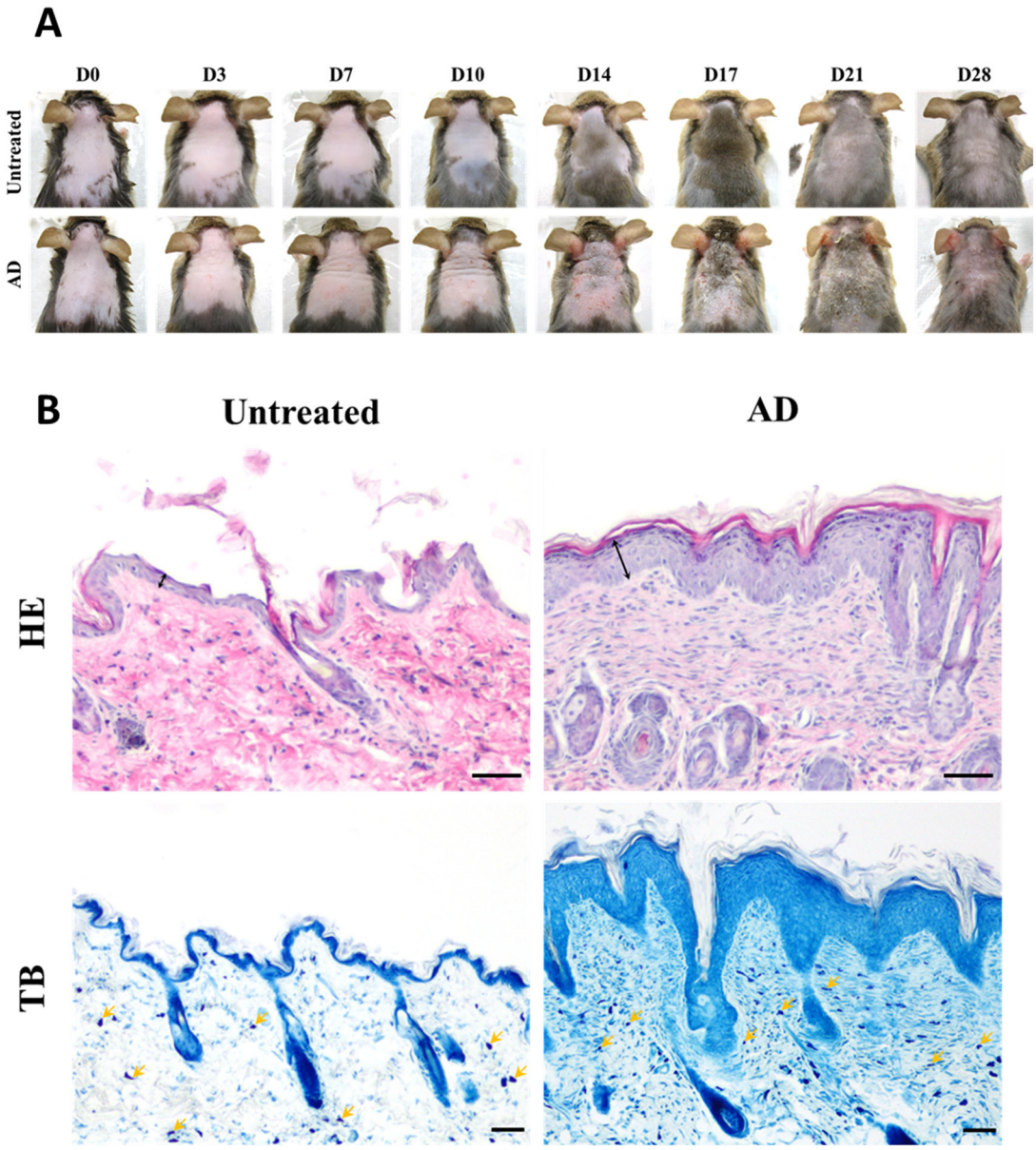

Fig. 2. Results of atopic dermatitis induction in this study. **A**: Change in mouse dorsal skin (top: untreated NC/Nga mice, bottom: AD group). **B**: Comparison of hematoxylin–eosin (HE)- and toluidine blue (TB)-stained images of untreated NC/Nga mice and AD group on D28. Bidirectional arrows indicate epidermal thickening. Arrows indicate mast cells. AD: atopic dermatitis, Scale bars=50 μm.

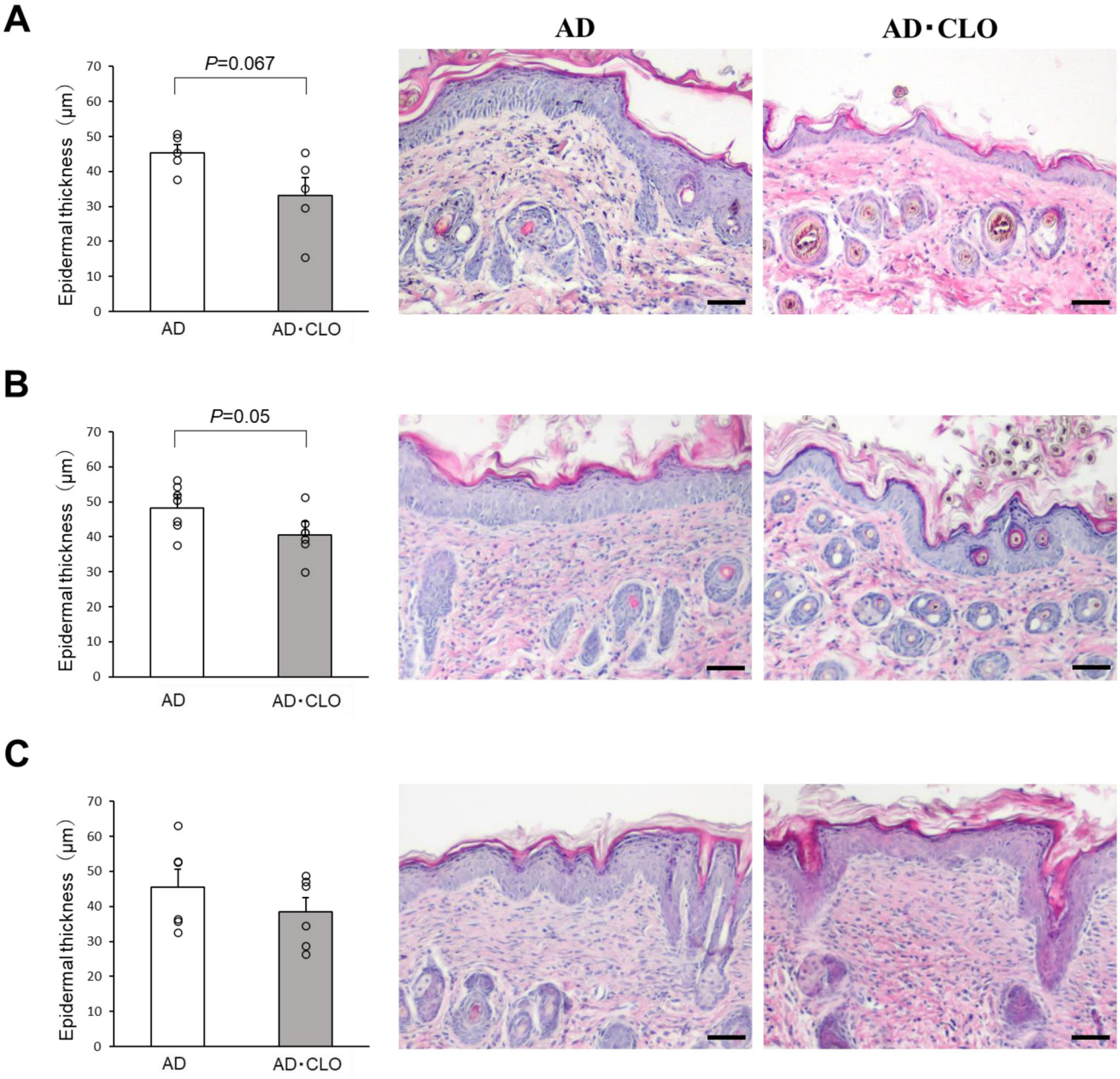

Fig. 3. Epidermal thickening on D14 (**A**), D21 (**B**), and D28 (**C**). **A**: Epidermal thickening showed a decreasing trend after clothianidin (CLO) exposure on D14 (AD: n=5; AD·CLO: n=5). **B**: Epidermal thickening showed a decreasing trend after CLO exposure on D21 (AD: n=7; AD·CLO: n=7). **C**: CLO had no effect on epidermal thickening on D28 (AD: n=6; AD·CLO: n=6). These results were analyzed by Student's *t*-test. All data represent the mean + SE of each group, and circles show the values for individual mice. AD: atopic dermatitis, Scale bars=50 μm.

release of mediators in the mechanisms of inflammation as described above. Mast cell degranulation has been observed in AD, along with mast cell mobilization in inflammatory skin areas; this correlation with the severity of AD has been demonstrated [11]. The muscarinic acetylcholine receptor and nAChR are expressed in mast cells. The former is reported to be involved in promoting degranulation and the latter in inhibiting it, suggesting that NN may act directly on mast cells [3]. We therefore counted mast cells and measured plasma histamine levels in this study. The AD·CLO group had significantly fewer mast cells than the AD group on D14 ($P<0.05$) (Fig. 4A), and there were no statistically significant differences between the groups on D21 and D28 (Fig. 4B and 4C). Plasma histamine levels were significantly lower in the AD·CLO group than in the AD group on D14 ($P<0.01$) (Fig. 5A), and there were no statistically significant differences between them on D21 and D28 (Fig. 5B and 5C). These results suggested that CLO exposure may be involved in the reduction of the number of mast cells and histamine release on D14. Since mast cells are a major source of histamine, it is quite possible that a decrease in their number affected plasma histamine. In addition, previous studies on plasma histamine have shown that imidacloprid, a class of NN, inhibits IgE-mediated degranulation in rat mast cell model cells (RBL-2H3), mouse bone marrow-derived mast cells, and human basophilic cells (KU812). This inhibition of degranulation has been associated

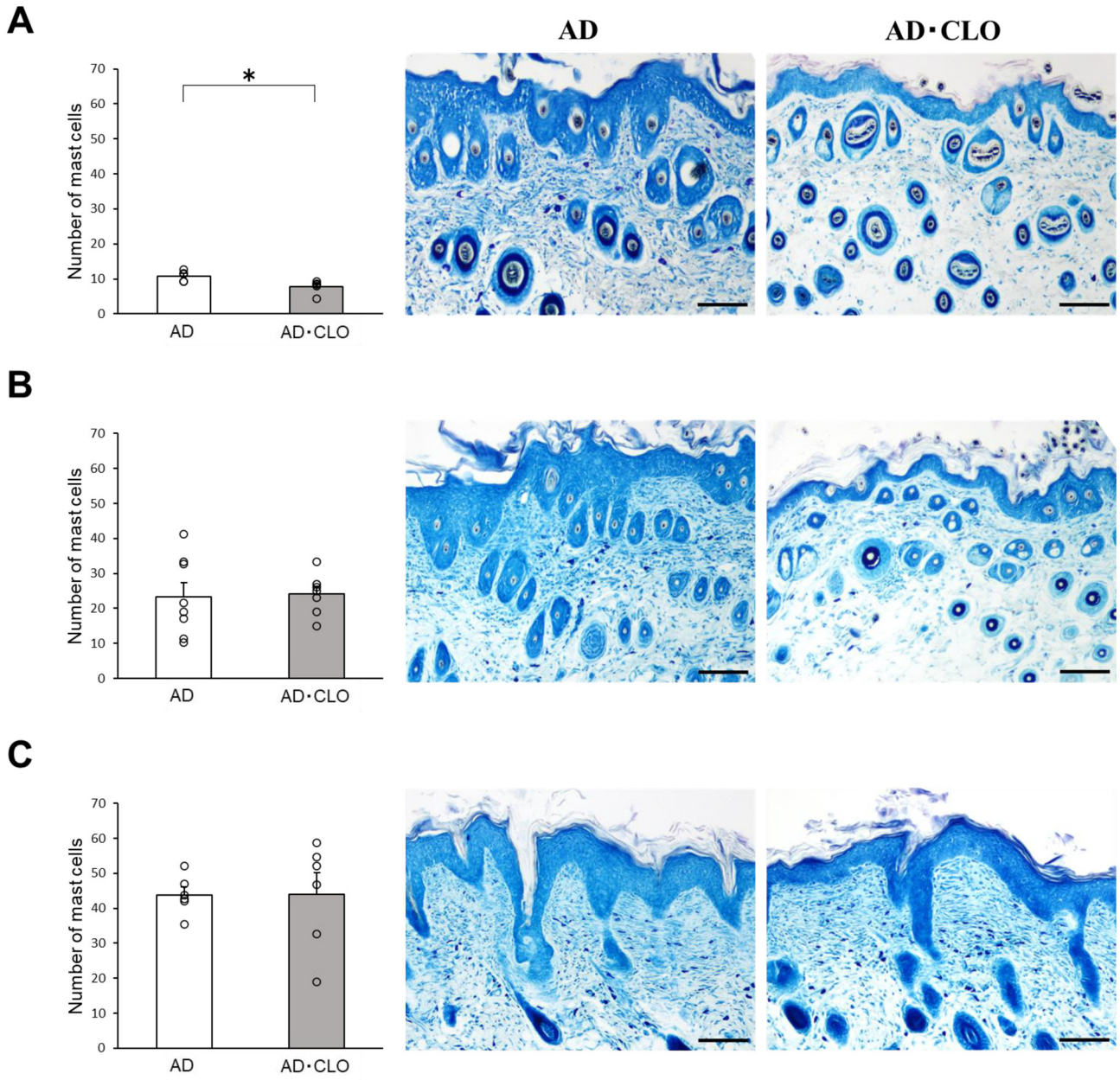

Fig. 4. Numbers of mast cells on D14 (**A**), D21 (**B**), and D28 (**C**). **A**: The numbers were significantly reduced by clothianidin (CLO) exposure on D14 (AD: n=5; AD·CLO: n=5). **B**: CLO had no effect on the number of mast cells on D21 (AD: n=8; AD·CLO: n=7). **C**: CLO had no effect on the number of mast cells on D28 (AD: n=6; AD·CLO: n=6). These results were analyzed by Student's *t*-test (**A**, **B**) and Welch's *t*-test (**C**). All data represent the mean + SE of each group, and circles show the values for individual mice. **P*<0.05 vs. the AD group. AD: atopic dermatitis, Scale bars=100 µm.

with α7 nAChR [20–22]. Therefore, in the present study, CLO exposure may have inhibited mast cell degranulation via α7 nAChR, thereby suppressing histamine secretion and the induction of inflammation. It is also known that IL-22 produced by Th22 is involved in epidermal thickening. Further investigation of the amount of IL-22 produced will provide a better understanding of the decrease in epidermal thickening observed in D14 and D21 in this experiment.

Mast cells release various cytokines and chemokines in addition to histamine. Hence, it remains unclear whether mast cell recruitment from other sites or the proliferation of resident mast cells was suppressed. However, the observed suppression of degranulation in this experiment may have played a role in the reduction of mast cell numbers. Further studies are needed to clarify this issue.

Finally, total plasma IgE levels tended to be higher in the AD·CLO group than in the AD group on D14 (P=0.095) (Fig. 6A), and there were no statistically significant differences on D21 and D28 (Fig. 6B and 6C). α7 nAChR is expressed on antigen-presenting cells such as macrophages and dendritic cells [12]. It has been reported that α7 nAChR is also expressed on T cells and B cells and is involved in their differentiation and proliferation [See Review 19]. Activation of α7 nAChR on antigen-presenting cells is expected

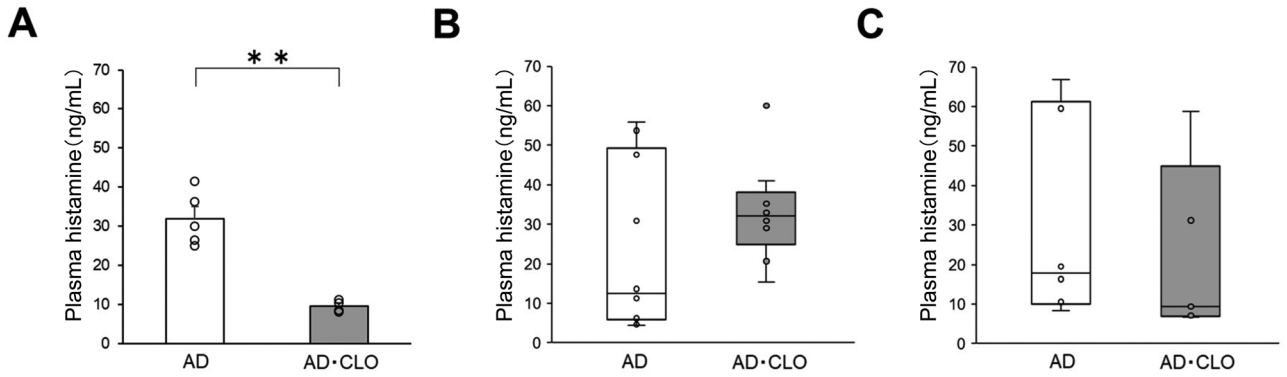

Fig. 5. Plasma histamine levels on D14 (**A**), D21 (**B**), and D28 (**C**). **A**: Plasma histamine levels were significantly decreased by clothianidin (CLO) exposure on D14 (AD: n=5; AD·CLO: n=4). **B**: CLO had no effect on plasma histamine levels on D21 (AD: n=10; AD·CLO: n=9). **C**: CLO had no effect on plasma histamine levels on D28 (AD: n=6; AD·CLO: n=5). These results were analyzed by Student's *t*-test (**A**: Data represent the mean + SE) and Mann–Whitney's *U*-test (**B**, **C**). Circles show the values for individual mice. ***P*<0.01 vs. the AD group. AD: atopic dermatitis.

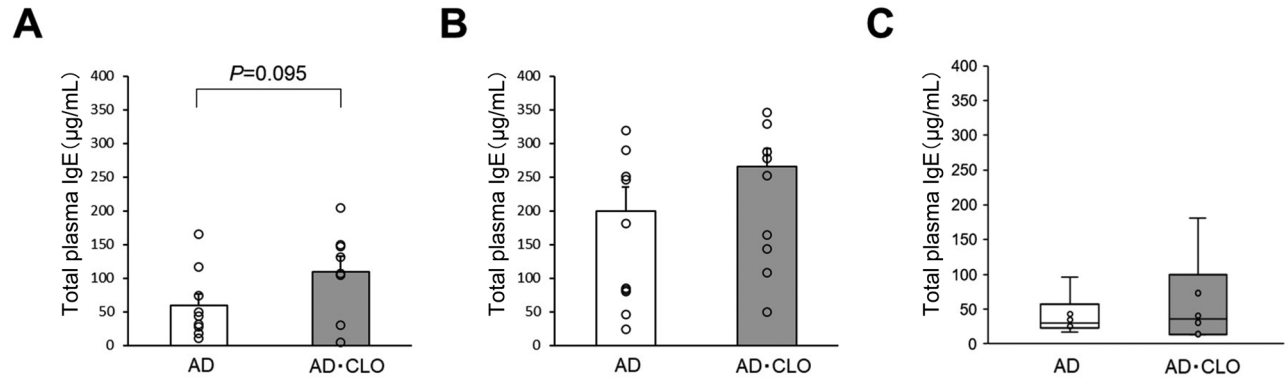

Fig. 6. Plasma total IgE levels on D14 (**A**), D21 (**B**), and D28 (**C**). **A**: Total plasma IgE levels tended to increase with clothianidin (CLO) exposure on D14 (AD: n=9; AD·CLO: n=8). **B**: CLO had no effect on total plasma IgE levels on D21 (AD: n=10; AD·CLO: n=8). **C**: CLO had no effect on total plasma IgE levels on D28 (AD: n=6; AD·CLO: n=6). These results were analyzed by Student's *t*-test (**A**, **B**: Data represent the mean + SE) and Mann–Whitney's *U*-test (**C**). Circles show the values for individual mice. AD: atopic dermatitis.

to have an inhibitory effect on IgE production because its signal inhibits antigen processing [12]. In contrast to the results of this culture experiment, an increase in plasma total IgE was observed in the present study using a mouse model of atopic dermatitis. This suggests that CLO exposure does not interfere with antigen processing.

Regarding the increase in total plasma IgE levels in the AD·CLO group of D14 in the present study, a previous study reported that transdermal sensitization of mast cell–deficient (W/W^v) mice with ovalbumin increased total serum IgE production compared to wild-type mice [1]. In the present study, CLO exposure reduced the number of mast cells in the dermis, and this reduction may have contributed to the increase in total IgE production. It is also possible that the lack of mast cells, which are the recipients of IgE, may have led T and B cells to recognize that signals were not being transmitted downstream, thereby further enhancing IgE production. Alternatively, it is plausible that CLO had a direct effect on T and B cells. Further studies are needed to elucidate these possibilities. In addition, we measured total plasma IgE levels in the present study. Further analysis of antigen-specific IgE levels may provide new findings.

The differences between the AD group and the AD·CLO group were observed mainly on D14, suggesting that CLO exposure delays the onset of epidermal thickening, mast cell infiltration and plasma histamine level in the early stages of AD. On the other hand, total plasma IgE levels may be increased by CLO exposure on D14. The effect of CLO was observed in epidermal thickening only on D21, after D14. Differences in conditions, such as the frequency of atopic dermatitis induction and the number of days elapsed after the final AD induction, could be influencing these results. Although AD is formed by various environmental factors, this study revealed for the first time a direct relationship between AD symptoms and CLO, a type of NN. This suggests that exposure to CLO suppresses early symptoms of AD under the present conditions.

CONFLICT OF INTEREST. The authors declare that there are no conflicts of interest.

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