

The immunological and oxidative stress regulation of non-thermal plasma-aided water on atopic dermatitis-like lesion in dinitrochlorobenzene-induced SKH-1 hairless mice

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Abstract

Backgrounds: While non-thermal plasma aided water (NTP-AW) has been currently used as a novel therapeutic against wide array of diseases, it remains unknown on therapeutic effect against atopic dermatitis (AD). To answer this, we evaluated the effect of NTP-AW on the regulation of immune and antioxidant markers in dinitrochlorobenzene (DNCB)-induced AD in hairless mice.

Methods: The skin damage in mice was induced by 1% DNCB for the first wk and 0.5% every alternate day for 3 wks followed by 4 wks NTP-AW treatment through spraying and bathing method.

Results: Treatment with NTP-AW reduced the severity of dermatitis symptoms and skin scratching behavior. In line, immunological profiling showed the improved parameters such as decrease in the number of neutrophils, lymphocyte, eosinophil as well as Th2-mediated IgE overproduction, and the reduced level of pro-inflammatory cytokine such as interleukin (IL)-1 β , and tumor necrosis factor (TNF)- α . Redox profiling showed

that NTP-AW reduced the production of total reactive oxygen species (ROS), and enhanced the antioxidant enzyme activity.

Conclusion: Collectively, this study clearly indicates the therapeutic effect of NTP-AW on DNCB-induced AD in hairless mice via immuno-redox regulation, clinically implying that NTP-AW treatment might be a safe and promising therapeutic candidate against AD.

Keywords: Atopic dermatitis, Non-thermal plasma, DNCB, Redox profiling, Pro-inflammatory cytokines

Introduction

Atopic dermatitis (AD) is the most common and chronic relapsing skin allergic inflammatory disorder with a prevalence of 10–20% of people worldwide^{1,2}. Pathogenesis of AD is complex process and can be caused by the interaction of environmental allergens, genetic and pharmacological factors, and psychological disturbances, resulting in an imbalance within the immune system³. Therefore, inflammation may induce a skin barrier dysfunction and aggravate the eczematous reaction in AD^{4–6}. Various innate and adaptive immune and inflammatory cells participate in the immunological and cutaneous inflammatory response of AD. Approximately 80% of AD patients are manifested with IgE sensitization whereas other 20% with non-IgE-mediated reaction⁴. AD is classified into acute and chronic phase according to the chronicity of inflammation. The acute phase is mainly driven by T helper 2 (Th2) cells that actively secretes various cytokines including interleukin (IL)-4, IL-5 and IL-13⁵. These cytokines stimulate IgE

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production by B cells and the development of eosinophils⁶. There have been several treatment methods used for treating AD including the use of topical or systemic steroids, immune suppressive drugs, anti-inflammatory drugs and skin creams for enhancement of the skin barrier function^{7–9}. Topical or systemic steroids and immune suppressive drugs have a lot of side effects¹⁰. Therefore, an effective and safer therapeutic strategy to control the inflammation is strongly necessary for AD treatment.

Currently, plasma medicine has been emerging worldwide as a new therapeutic medicine, and cumulative evidences support its biological responses to treat various diseases¹¹. With this, continuous research in plasma field led to innovate non-thermal plasma aided water (NTP-AW). NTP-AW is electrolyzed water treated by non-thermal plasma (NTP) generating device. This device partially ionizes gas through a direct current glow discharge in water where energy is stored in the electron without any change of temperature. Recent studies in NTP has found its promising effects in different biological application because of its enriched properties of H_2O_2 , OH^- , HOCl , O_3 , H_2 and other ions^{11,12}. Thus, many promising results have been yielded by research into NTP as a potential tool for sterilization¹³, wound healing and anti-aging of skin^{14,15}. Studies showed that NTP would be effective for sterilization of medical equipment as well as infected wound when heat inactivation is not suitable^{16–19}. Otherwise in several skin diseases, it remains unknown on therapeutic effect against AD. To answer this, we evaluated the effect of NTP-AW on the regulation of immune and antioxidant markers in dinitrochlorobenzene (DNCB)-induced AD in hairless mice.

Materials & Methods

Animals

Five-wk old female SKH-1 hairless mice with mean weight of 25 ± 4.2 g were purchased from Orient Bio Inc. (Seongnam, Republic of Korea). The mice were kept in spacious plastic cages ($390 \times 275 \times 175$ mm) with wood shaving bedding. Mice were acclimatized for 7 days prior to experiment and were maintained in a controlled atmospheric environment with temperature of $22 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ humidity in a pathogen free condition under a 12:12 hrs light-dark cycle. Mice were fed with standard rodent chow food (5L79, PMI Nutrition®, LAND O'LAKES, INC, Minnesota, USA) and primary filtered water with free to access. Forty mice were randomized into four different groups, five mice each cage ($n = 10$ respectively) as follows: Normal control (NC);

Negative control (DNCB only); Positive control (PC): DNCB + 0.1% tacrolimus ointment; Non-thermal plasma aided water (NTP-AW): DNCB + NTP-AW. All the experiments were conducted between 9 a.m and 6 p.m to minimize environmental changes.

Induction of allergic dermatitis in hairless mice

Six-wk old female SKH-1 hairless mice was induced with AD-like lesional inflammation with 1% DNCB with the volume of $200 \mu\text{L}/\text{mouse}/\text{d}$ (dissolved in a 3:1 mixture of acetone and olive oil) for 1 wk and boosted by 0.5% DNCB with the volume of $150 \mu\text{L}/\text{mouse}$, every alternate day for 3 wks. After 3 wks boosting with 0.5% DNCB along with 3 times spraying and 1 time bathing with NTP-AW in a day, it was followed by one more wk of spraying and bathing with NTP-AW only. DNCB solution was applied at dorsal back skin (approximately 4 cm^2) on each mouse except NC group mice. In PC mice, tacrolimus ointment (0.1% Protopic Co. Ltd. Osaka, Japan) was applied on the dorsal skin, seven times a week for 4 wks.

Preparation of experimental water

NTP-AW was generated from non-thermal plasma device (AB-50, PLUSE Co., Ltd., Chuncheon, Korea) where 24 V direct current electric source and 12 W electric power was applied and 6 mesh type of platinumized titanium electrodes were arranged at a distance of 0.5 mm. NTP-AW was generated from heated tap water ($34 \pm 1^\circ\text{C}$) and was characterized by pH 6.3 ± 0.3 , dissolved hydrogen concentration (DH) 400 ± 50 ppb and active chlorine concentration (ACC) $2.8 \sim 3.0$ ppm. Control water was prepared by purification of tap water through sedimentation filter, activated carbon filter and UF hollow fiber membrane filter, which was pH 7.0 ± 0.3 , DH 0 ppm and ACC 0 ppm. Control water also was heated to $34 \pm 1^\circ\text{C}$ before using.

Bathing method for treatment after DNCB induction

Five mice freely bathed in each plastic cage ($390 \times 275 \times 175$ mm) containing approximately 4 cm depth NTP-AW water for 15 min/day for 4 wks. The mice of PC and DNCB only (negative control) groups were bathed in control water to provide the same bathing condition. Both experimental and control water was heated to $34 \pm 1^\circ\text{C}$ before bathing.

Evaluation of dermatitis severity

The relative dermatitis severity was assessed macroscopically by conducting skin scoring procedure, counting and observing scratching behaviour and taking photographs. The severity of dermatitis was assessed

macroscopically according to the eczema area and severity index scoring system: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms. The dermatitis score was defined as the sum of scores for erythema/haemorrhage, edema, excoriation/erosion, and scaling/dryness. The skin scoring was assessed once a week during 4 wks of treatment. Simultaneously, scratching acts for mouse rubbing dorsal skin with their hind paws, their nose and ears were observed on day 14 and 28 per cage for 15 min in triplicate observations. Lastly, we took photographs of mice once weekly for 4 wks.

Sample preparation

After 4 wks of treatment with experimental and control materials, mice were anesthetized with isoflurane (Hana Pharm. Co., Hwaseong, Republic of Korea) and blood samples were collected by retro-orbital venous plexus and put in sterilized EDTA vacutainer tubes. Serum samples were prepared by centrifugation at 14,000 rpm for 5 min at 4°C. Thereafter, we prepared skin lysate samples by cutting skin tissue (1 × 1 cm) from the dorsal back area of the mice after sacrifice and were placed in ice-cold RIPA buffer (Pierce Biotechnology Inc., IL, U.S.A.) with protease inhibitor cocktail (Sigma Chemical Co., St Louis, U.S.A.) and was homogenized at 25 rpm for 15 min. Thereafter, the skin lysate was centrifuge at 14,000 rpm for 10 min at 4°C and the supernatant. Both collected serum and lysate samples were stored at –80°C until use.

White blood cell (WBC) and its differential count analysis

Blood was collected from the retro-orbital plexus in tubes coated with anticoagulant and was immediately mixed with an automatic mixer for 5 min with roller. Thereafter, WBC and its differential members such as total WBC, lymphocytes, monocytes, neutrophil, eosinophil and basophil were counted by using an automatic blood analyser by (HEMAVET HV950 FS, Drew Scientific, Erba Diagnostics, Inc., Dallas, Texas, USA).

Intracellular total reactive oxygen species (ROS) detection

To determine oxidative stress and superoxide in blood serum and skin lysate ROS detection kit by Enzo Life Sciences Inc. (Farmingdale, NY, USA) was used according to the manufacturer's instructions. Briefly, 25 µL sample was loaded and detection solution was added to each well. The plate was measured in the DTX-800 multimode microplate reader by Beckman Counter Inc. (Fullerton, CA, USA) using a filter set of 485/20 excitation and 528/20 emission.

Determination of nitric oxide (NO) level

To determine the nitrite (NO_2^-) present in the blood serum and skin lysate of mice, the Griess reagent by Promega Corp. (Madison, USA) was used according to the manufacturer's instructions. Briefly, 50 µL of the serum was mixed with an equal volume of Griess reagent in a 96-well microtiter plate and incubated at room temperature for 15 min. The absorbance was measured at 540 nm using a DTX-880 multimode microplate reader (Beckman Counter Inc., Fullerton, CA, USA).

Measurement of antioxidant endogenous enzyme activities

To check the antioxidant levels in serum and skin lysate the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were measured using Biovision kit (Milpitas, CA, USA). The normalized concentrations of cell lysate were used to measure the activities of different antioxidant enzymes (SOD, GPx and CAT) according to the manufacturer's instruction.

Measurement of total IgE level

In order to check the amount of total IgE in serum and skin lysate the mouse IgE ELISA kit (BD Biosciences, San Diego, CA, USA) was used by a sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions. Determined reaction product was measured calorimetrically at 450 nm with a microplate reader (BioTek Instrument, Winooski, VT, USA).

Measurement of serum inflammatory cytokines

Serum inflammatory cytokines including IL-1 β , IL-4, IL-5, IL-6, IL-13 and TNF- α were analyzed using Bio-Plex Cytokine Assay by Bio-Rad (CA, USA) according to manufacturer's instructions. Standard curves for each cytokine were generated by using the reference concentrations provided in the kits. Mean fluorescent intensity was acquired on a Luminex technology by Biorad's Bio-Plex 200 system Multiplex Bead Array SystemTM (CA, USA) and analyzed with associated software using a 5-parameter logistic method.

Measurement of intracellular calcium levels

In order to check the calcium concentration in serum and skin lysate Cayman Calcium Assay Kit (Ann Arbor, USA), was used following manufacturer's instructions. Briefly, samples were added into the wells followed by the working detector reagent. The plate was incubated for 5 min and was analyzed at 590 nm (Biotech Instrument Inc, VT, USA).

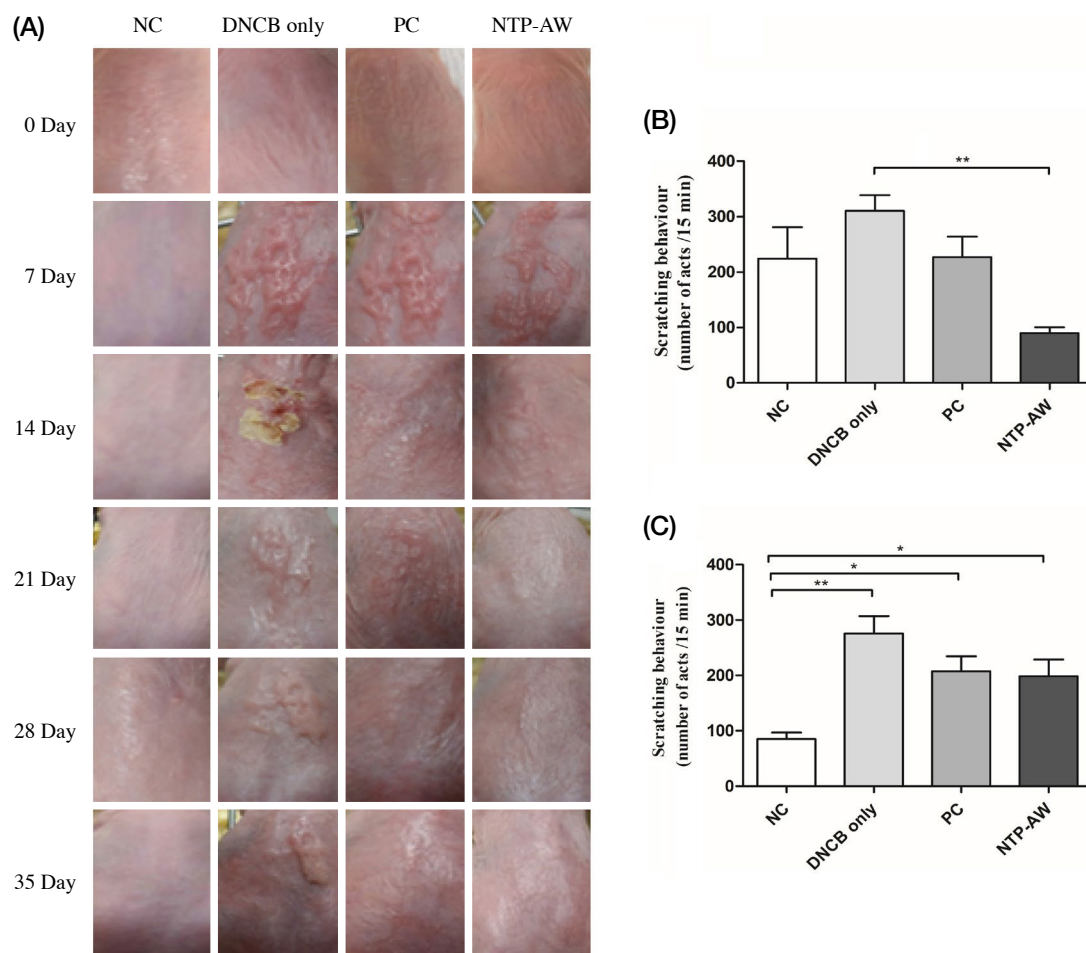


Figure 1. Effects of NTP-AW treatment on skin severity in DNCB-induced hairless mice. (A) Representative of dorsal skin photographs of each treatment groups showing comparison of AD-like skin lesions. (B) Scratching frequency counted on the 2nd wk after DNCB-induction in each groups. (C) Scratching frequency counted on the 4th wk after DNCB-induction in each groups. Data are the means $\pm$ SD for 10 mice. Significant differences were analyzed with ANOVA Tukey's test (* $P < 0.05$, ** $P < 0.01$).

Statistical analysis

Statistical analysis was carried out using analysis of variance (ANOVA) followed by subsequent multiple comparison test (Tukey's post hoc test) using GraphPad Prism version 5.0 software packages (GraphPad Software, La Jolla, CA, USA). Differences were considered significant at $P < 0.05$.

Results

Effects of NTP-AW treatment in the AD-like skin lesion by improving AD-like symptoms, suppressing scratching behavior and total IgE level in DNCB-induced hairless mice

To investigate the effects of NTP-AW in the atopic dermatitis-like skin lesion, we observed the morphological

skin condition of mice for 4 wks. We found that topical application of DNCB to dorsal surface of hairless mice induces AD-like lesions and symptoms including erythema, erosion, scaling and edema. However, topical bathing and spraying with NTP-AW group showed improved recovery in skin condition faster than other groups (Figure 1A). Next, we examined scratching behavior of mice. Our results showed that scratching behavior was reduced in 2nd wk of treatment in the NTP-AW group ($P < 0.01$) compared to DNCB only group (Figure 1B). However, in 4th wk of NTP-AW treatment, mice showed the lowest trend of scratching behavior compared to NC ($P < 0.05$) (Figure 1C). IgE is one of the most important diagnostic and prognostic indicators in AD. Therefore, we evaluated the total IgE level in serum and skin lysate. Our results revealed that NTP-AW treated group showed the lowest IgE levels compared to all other groups whereas DNCB only group showed the

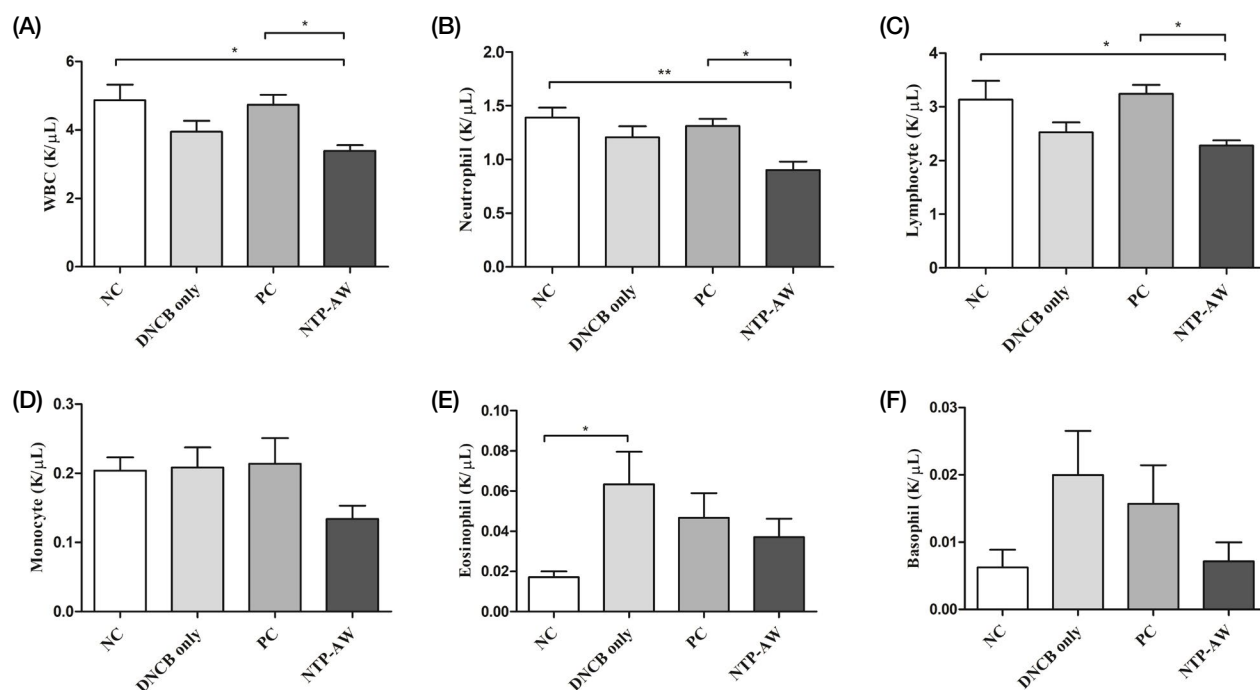


Figure 2. Effects of NTP-AW treatment on the number of total and differential WBC count in DNCB-induced hairless mice. (A) Total WBC (B) Neutrophil (C) Lymphocyte (D) Monocytes (E) Eosinophil and (F) Basophil. Data are the means $\pm$ SD for 10 mice. Significant differences were analyzed with ANOVA Tukey's test (* $P < 0.05$, ** $P < 0.01$).

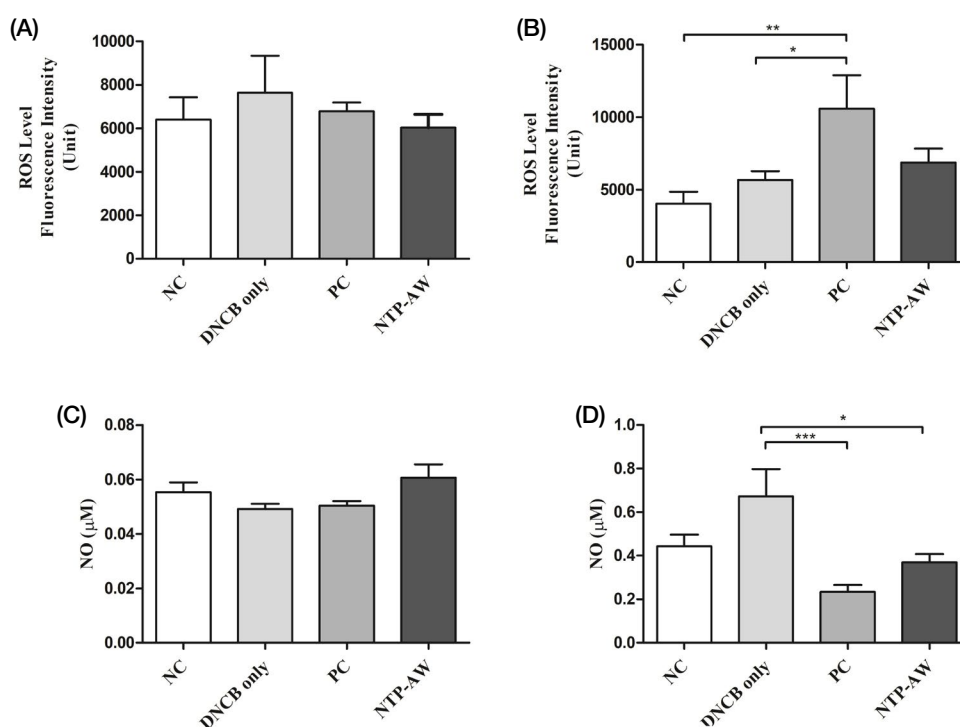


Figure 3. Effects of NTP-AW treatment on total ROS and NO level in DNCB-induced hairless mice. (A) ROS level in serum (B) ROS level in skin lysate (C) NO level in serum (D) NO level in skin lysate. Data are the means $\pm$ SD for 10 mice. Significant differences were analyzed with ANOVA Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

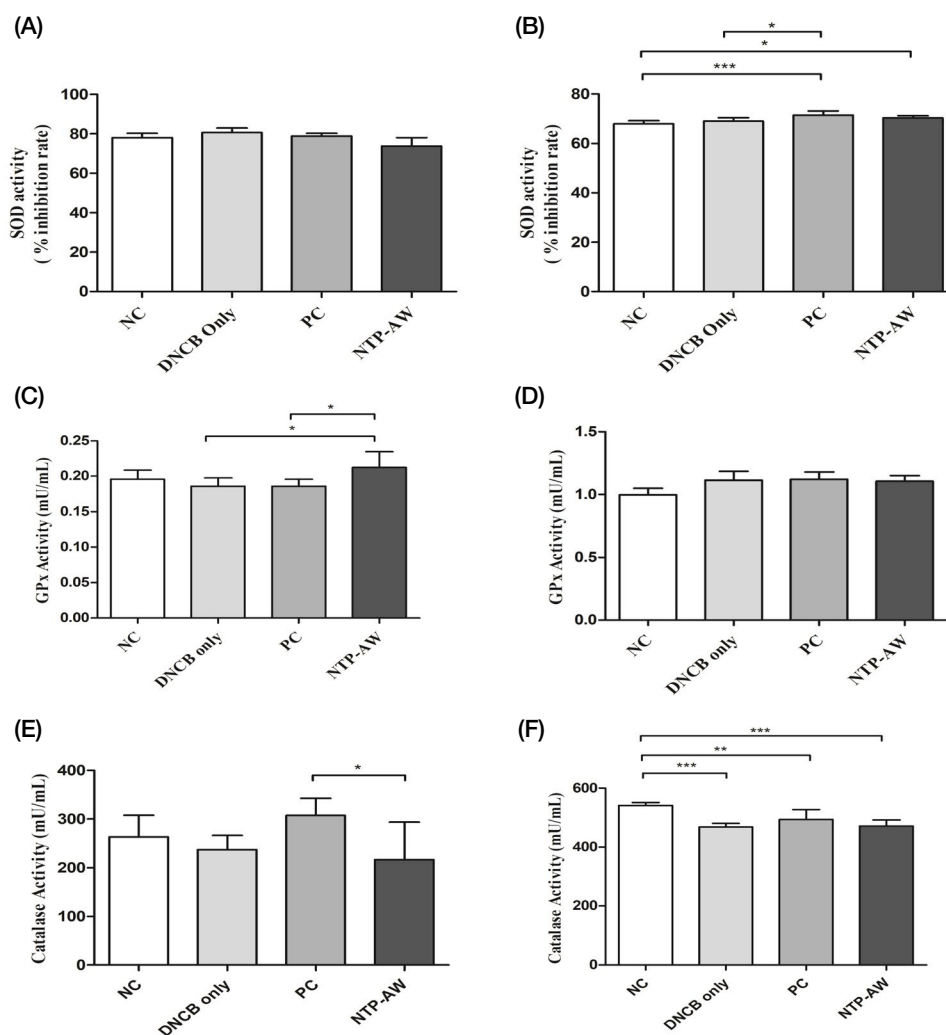


Figure 4. Effects of NTP-AW treatment on anti-oxidant enzymes balance in DNCB-induced hairless mice. (A) SOD activity in serum. (B) SOD activity in skin lysate. (C) GPx activity in serum. (D) GPx activity in skin lysate. (E) Catalase activity in serum. (F) Catalase activity in skin lysate. Data are the means $\pm$ SD for 10 mice. Significant differences were analyzed with ANOVA Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

highest concentration of IgE levels both in serum and lysate (Figure 6A and B).

NTP-AW treatment suppressed serum leukocytes and inflammatory cells infiltration in the AD-like skin lesion in DNCB-induced hairless mice

WBCs have a major influence on both innate and adaptive immunity. Any invasion into the body by foreign particles triggers the production of WBC, which leads to the recruitment function of specific white blood cells. Thus, we evaluated the total WBC and their differential counts. Our results revealed that NTP-AW treatment significantly lowered the WBC, neutrophil and lymphocyte counts compared to PC and NC groups (Figure 2A,

B and C). Eosinophil, monocytes and basophil levels also showed reduced pattern with NTP-AW treatment but without statistical significance (Figure 2D, E and F).

Effects of NTP-AW on redox-balance activities in the AD-like skin lesion

To evaluate the bathing effects of NTP-AW in DNCB-induced oxidative stress, we examined redox parameters. Our results showed that the total ROS generation was decreased in NTP-AW group as compared to DNCB only group in serum level (Figure 3A). Skin lysate result showed the increase ROS generation in PC group compared to all other groups (Figure 3B). In contrast NO result in skin lysate showed significant

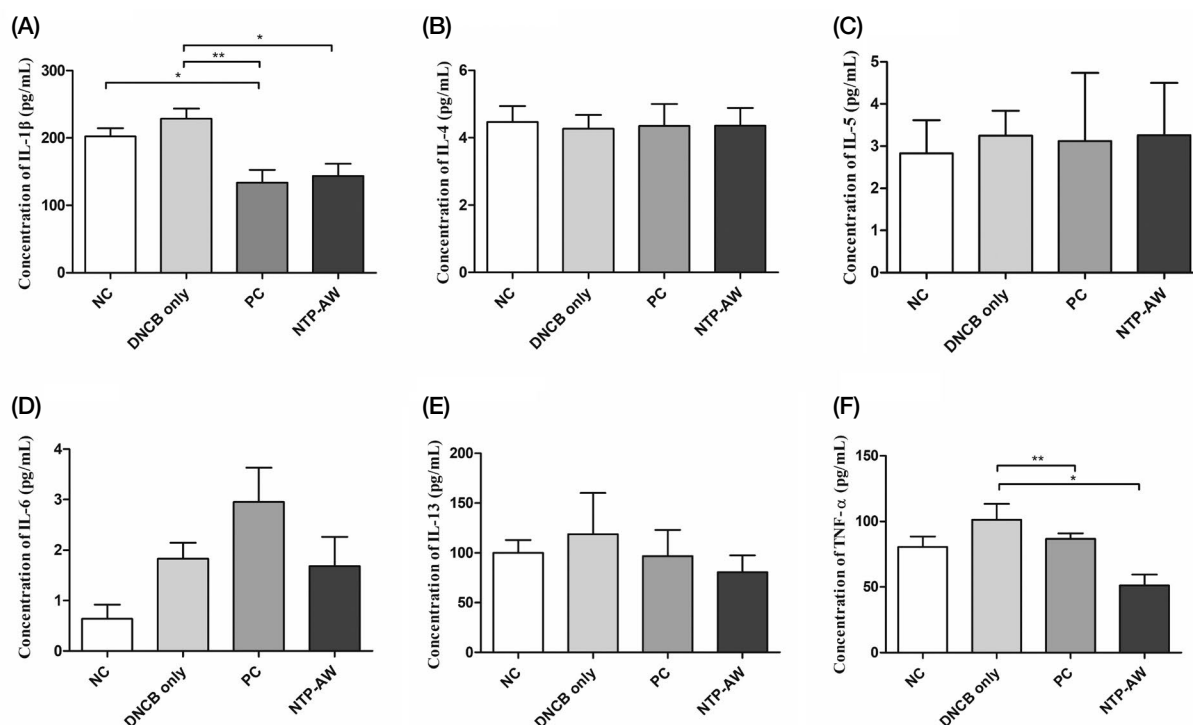


Figure 5. Effects of NTP-AW treatment on pro and anti-inflammatory cytokines level in DNCB-induced hairless mice. (A) IL-1 β , (B) IL-4, (C) IL-5, (D) IL-6, (E) IL-13, (F) TNF- α . Data are the means $\pm$ SD for 10 mice. Significant differences were analyzed with ANOVA Tukey's test (* $P < 0.05$, ** $P < 0.01$).

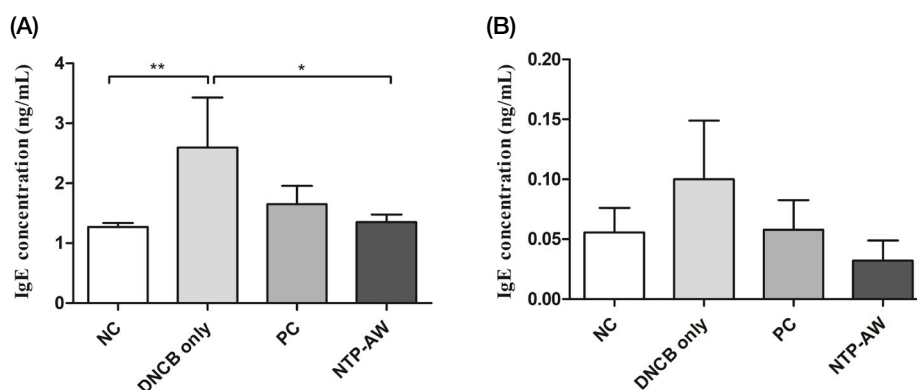


Figure 6. Effects of NTP-AW treatment on total IgE level in DNCB-induced hairless mice. (A) IgE level in serum. (B) IgE level in skin lysate. Data are the means $\pm$ SD for 10 mice. Significant differences were analyzed with ANOVA Tukey's test (* $P < 0.05$, ** $P < 0.01$).

reduction ($P < 0.05$) in NTP-AW group compared to DNCB only group (Figure 3D). Next, we assessed the antioxidant effect of NTP-AW treatment. We found that the SOD enzyme in skin lysate showed significantly increased activity in NTP-AW group compared to NC group whereas we did not see any significant change in serum (Figure 4A and 4B). Likewise, catalase activity was significantly decreased ($P < 0.05$) in NTP-AW

group as compared to PC group whereas we did not see any significant change with NTP-AW treatment in lysate (Figure 4E and F). Furthermore, GPx activity was significantly increased in NTP-AW treated group compared to PC and DNCB only groups whereas no significant difference was observed in skin lysate (Figure 4C and D).

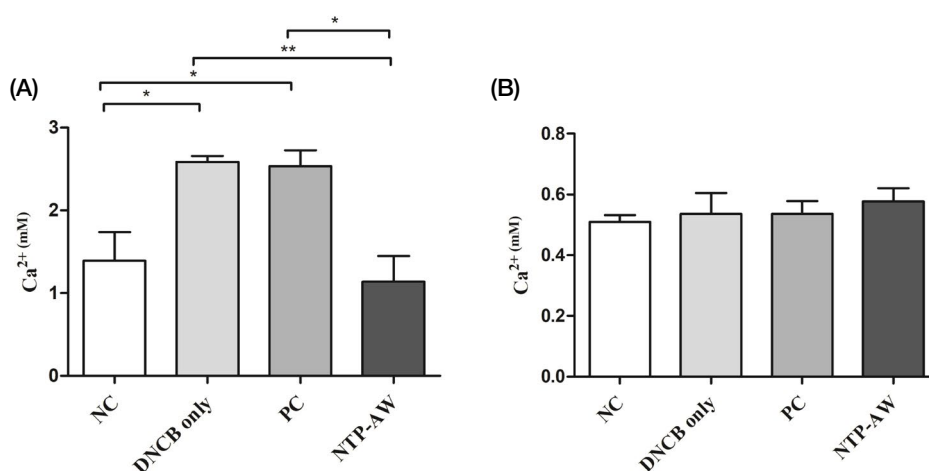


Figure 7. Effects of NTP-AW treatment on calcium level (Ca^{2+}) in DNCB-induced hairless mice. (A) Ca^{2+} level in serum. (B) Ca^{2+} level in skin lysate. Data are the means $\pm$ SD for 10 mice. Significant differences were analyzed with ANOVA Tukey's test (* $P < 0.05$, ** $P < 0.01$).

NTP-AW treatment reduces the pro-inflammatory cytokines levels in the AD-like skin lesion in DNCB-induced hairless mice

To investigate immunomodulatory function of NTP-AW in the AD-like skin lesion, we measured the production of pro-inflammatory cytokines levels. We found that NTP-AW treatment significantly decreased ($P < 0.05$) IL-1 β and TNF- α levels compared to DNCB only group in the serum of AD-like lesional skin of DNCB-induced AD mice (Figure 5A and 5F). However, IL-13 also showed the decreased level with NTP-AW treatment compared with other treatment groups but with no significant difference (Figure 5E). Likewise, other cytokines such as IL-4, IL-5 and IL-6 did not show any difference among normal and treated groups (Figure 5B–D).

Effects of NTP-AW on intracellular calcium activity in the AD-like skin lesion

To investigate whether NTP-AW could have effect on AD-like skin lesion, intracellular calcium level was assayed in mice. We found that intracellular calcium level in serum showed significant reduction ($P < 0.01$) in NTP-AW group (Figure 7A) whereas we did not see any significant difference in skin lysate (Figure 7B).

Discussion

This study clearly indicates the therapeutic effect of NTP-AW on DNCB-induced AD in hairless mice via immuno-redox regulation. To prove this, first we evaluated skin morphology and inflammatory state mac-

roscopically. NTP-AW group showed rapid recovery when compared to other groups. Consistently, the clinical score or AD-like skin lesions gradually reduced in PC and NTP-AW groups, and the degree of inflammation was markedly decreased in NTP-AW group when compared to PC group. Scratching behavior is one of the important manifestations of IgE sensitized AD^{20,21}. Scratching behavior showed a significant reduction in 2nd wk of treatment in the NTP-AW group compared to negative ($P < 0.01$) and positive control groups. While the 4th wk of treatment also showed similar trend, there was no significance between groups. These results might partially support that NTP-AW treatment suppresses the IgE mediated allergic manifestation on AD-like lesions.

To identify how NTP-AW mediated the immune response which might contribute in its rapid healing process on DNCB induced AD-like skin lesions, WBC and its differential counts were analyzed. We found that NTP-AW group revealed the lowest number of WBC and differential counts as compared to all other control groups. In particular, total WBC of NTP-AW group significantly reduced as compared to PC ($P < 0.05$). The significant reduction of neutrophils ($P < 0.05$) and lymphocytes ($P < 0.05$) might reflect the anti-inflammatory action of NTP-AW in the disease progression of AD. Monocyte, eosinophil and basophil also showed the lowest trend in NTP-AW group as compared to all other control groups. Meanwhile eosinophil and basophil are responsible for central pathogenesis of AD²². Overall, the decreased count of WBC, specifically neutrophil, lymphocyte, eosinophil, and basophil might contribute to a decrement of immunological dysfunction of AD. Further, NTP-AW treatment showed the lowest concen-

tration of IgE level in both serum and skin lysate. Here the results of IgE were also correlated with total and differential WBC count. About 70–80% patients of AD has involvement with IgE sensitization which is also associated with eosinophilia in all cases²³. Clamp down of IgE and eosinophil uttered the IgE mediated allergic reaction/sensitization on AD.

Many evidences indicate that allergic skin inflammatory diseases are mediated by oxidative stress. Likewise, ROS and NO plays a pivotal role in healing process from allergic inflammation and damaging effects²⁴. To identify whether these would be involved in anti-inflammatory effect of NTP-AW, analysis of ROS and NO were done. Our results showed that, DNCB group tends to have the highest ROS level as compared to NTP-AW group. Whereas there was a significantly lower NO level in PC and NTP-AW as compared to DNCB group ($P < 0.05$). The significant reduction of ROS and NO in NTP-AW treatment might reflect the potential defense against oxidative stress as evidenced by ROS and NO, which had been considered as one of the essential biomarkers related to oxidative stress, and could provoke AD-like inflammation as second messenger of pro-inflammatory and Th2 cytokines^{25,26}. Consistent with ROS and NO results, the antioxidant enzyme level increased in NTP-AW group. Higher level of GPx might imply a protective role against oxidative stress via peroxidase scavenging coupled with lowering ROS²⁷.

As a final immunological evidence, we already hypothesized that NTP-AW might instigate cytokine homeostasis. To support this notion, we checked the crucial cytokines (IL-1 β , IL-4, IL-5, IL-6, IL-13 and TNF- α) deeply linked to AD pathogenesis. In acute phase of AD the activated CD4⁺ Th2 cells produce abundant level of IL-4, IL-5 and IL-13²⁸, which could support the reduction of these serum cytokines by NTP-AW treatment. Similarly, the reduction of IL-1 β and TNF- α might be supported by the facts that epidermal cell-derived cytokines such as IL-1 β and TNF- α play a major role in the sensitization and elicitation phase of AD progression²⁹. Given this, NTP-AW therapy would fairly reduce all pro- and anti-inflammatory cytokines, consequently leading to correcting cytokine imbalance of AD. These results might justify the other immuno-modulatory effects of NTP-AW.

Calcium act as a central regulator of skin homeostasis and is also involved in inflammatory reaction through ROS production^{30,31}. The significant reduction of intracellular calcium in NTP-AP group then showed that NTP-AW could have an effect on its role in AD-like skin lesions.

With the evidences, our hypotheses that NTP-AW has immuno-modulatory and redox homeostasis effects on DNCB induced AD-like skin lesions can be supported.

Firstly, NTP-AW showed evidence on the reduction of morphologic skin severity. It is also correlated with the intense reduction of scratching behavior, which could correlate with the lowest level of IgE-mediated allergic manifestation. On the other hand, IgE has a close relation with the reduction trend in total WBC and its differential count especially eosinophil and basophil. Consistently, pro-inflammatory markers such as IL-1 β , IL-4, IL-5, IL-6, IL-13 and TNF- α level also showed the reduction trend in NTP-AW group, which implied the attenuation of acute inflammatory response. Importantly, NTP-AW showed the counter balance between redox molecules (ROS and NO) and antioxidant enzymes (SOD, GPx and CAT) which evidenced an excellent redox homeostasis.

Conclusion

Collectively, this study clearly indicates the therapeutic effect of NTP-AW on DNCB-induced AD-like skin lesions in hairless mice via immuno-redox regulation, clinically implying that NTP-AW treatment might be a safe and promising therapeutic candidate against AD. However, further study to unravel the molecular mechanism of NTP-AW in immunological and oxidative stress regulation in AD is underway. To our knowledge, this might be the first note on NTP-AW treatment evidenced with immuno-redox balance.

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Conflict of Interest Jesmin Ara, Johny Bajgai, Ma. Easter Joy Sajo, Ailyn Fadriquela, Cheol-Su Kim, Soo-Ki Kim & Kyu-Jae Lee declare that they have no conflict of interest.

Human and animal rights All animal experiments were conducted in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent experiment provided by the Institutional Animal Care and Use Committee (IACUC) at Wonju campus, Yonsei University, Gangwon, Wonju, and Republic of Korea (approval number: YWC-180508-1).

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