

Positive Effects of Hydrogen Water on 2,4-Dinitrochlorobenzene-Induced Atopic Dermatitis in NC/Nga Mice

Yang-Suk Yoon,^{a,#} Ma. Easter Joy Villarosa Sajo,^{a,#} Rosa Mistica Coles Ignacio,^a Soo-Ki Kim,^b Cheol-Su Kim,^b and Kyu-Jae Lee^{*a,c}

^aDepartment of Environmental Medical Biology, Wonju College of Medicine, Yonsei University; ^bDepartment of Microbiology, Wonju College of Medicine, Yonsei University; Wonju, Gangwon 220–701, Republic of Korea; and ^cInstitute for Poverty Alleviation and International Development, Yonsei University; Wonju, Gangwon 220–710, Republic of Korea.

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Atopic dermatitis (AD) is a chronically relapsing, pruritic, eczematous skin disorder accompanying allergic inflammation. AD is triggered by oxidative stress and immune imbalance. In the present study, we investigated the effect of drinking hydrogen water (HW) on 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis in NC/Nga mice and found that HW ameliorated DNCB-induced AD-like clinical symptoms. In line with this, the level of reactive oxygen species in the HW group was significantly inhibited compared with that in the purified water (PW) group. In parallel, HW enhanced glutathione peroxidase activity in DNCB-induced AD as compared with the PW group. Accordingly, the levels of thymus and activation-regulated chemokine and cytokines were significantly decreased in the HW group compared with the PW group. Notably, the levels of Th₂ cytokine, interleukin-5 (IL-5), and proinflammatory cytokines such as tumor necrosis factor- α and IL-6 in HW-fed mice were significantly lower than in control and PW-fed mice. The total serum immunoglobulin E level was also markedly reduced in the HW group. The collective results indicate that HW suppresses DNCB-induced AD in NC/Nga mice *via* redox balance and immune modulation and could be a safe clinical fluid treatment for AD.

Key words hydrogen water; atopic dermatitis; oxidative stress; immune modulation

Atopic dermatitis (AD) is a chronically relapsing, pruritic, eczematous skin disorder accompanying allergic inflammation.^{1,2)} AD is characterized by an impairment of the skin-barrier function, increased oxidative stress, and dysfunctional immune system. Intense infiltration of inflammatory cells release bioactive substance such as cytokines, chemokines, and reactive oxygen species (ROS).³⁾ Of these AD's etiologic factors, inflammatory cytokines are known to regulate skin barrier, thereby aggravating the eczematous reaction in AD.⁴⁾ Most acute skin lesions in AD are exhibited by Th₂ inflammatory cytokines, whereas the chronic phase is characterized by Th₁ immune responses.⁵⁾ The imbalance of cytokine network would play a critical role in the development of AD.^{6,7)} Together, oxidative stress such as increased ROS and lipid peroxidation is evident in any stage of AD.^{8–10)} An imbalance between ROS and antioxidants can lead to an elevated oxidative stress level.¹¹⁾

Hydrogen molecule (H₂) has been widely used in medical applications as a safe and effective antioxidant and immunomodulator with minimal side effects.^{12–15)} Unlike other antioxidants, which are unable to target organelles, H₂ can penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus.¹³⁾ Moreover, H₂ has been reported to have \cdot OH-scavenging activity in culture medium.¹⁴⁾ Oral administration of hydrogen water (HW), inhalation of hydrogen gas, and injecting H₂-dissolved saline is widely accepted to incorporate H₂ in the body. Recently, we reported that HW is effective in house mite-induced dermatitis *via* immunomodulation.¹⁵⁾ However, this study has a limitation owing to the lack of the evidence such as redox balance, immunoglobulin (Ig) E level

consistent with clinical sign and systemic manifestation. To date, there has been poorly documented regarding the immunologic and antioxidant effects of drinking HW against AD. To clarify this, we investigated the HW would be effective on 2,4-dinitrochlorobenzene (DNCB)-induced AD in NC/Nga mice through clinico-laboratory measurements, redox balance assay including ROS production, glutathione peroxidase (GPx) activity and malondialdehyde (MDA) level, and immunologic parameter assay including chemokine, cytokines, and IgE levels.

MATERIALS AND METHODS

Animal Experiment Male NC/Nga mice (8 weeks old) were purchased from the Orient Bio company (Seongnam, Korea). The mice were maintained in a controlled environment with a temperature of 22 \pm 2°C and 40–60% humidity under a 12h light-dark cycle. At the start of the experiment, mice were randomized into three groups ($n=9$): the control group was not treated with DNCB and orally administered with purified water (PW), the PW group was treated with DNCB and orally administered with PW, and the HW group was treated with DNCB and orally administered with HW. All the mice were administered with experimental waters accordingly for 12 weeks. All experimental procedures involving the mice use and care protocols were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at Wonju Campus, Yonsei University, Wonju, Gangwon, Korea.

Induction of Allergic Dermatitis in NC/Nga Mice AD-like skin lesions were induced in NC/Nga mice using DNCB. Briefly, the dorsal hair of Nc/Nga mice was removed by a hair removal cream for sensitive skin (Veet, Reckitt Benckiser, France). After 24h, 200 μ L of 1% DNCB solution (ace-

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[#]These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: medbio9@gmail.com

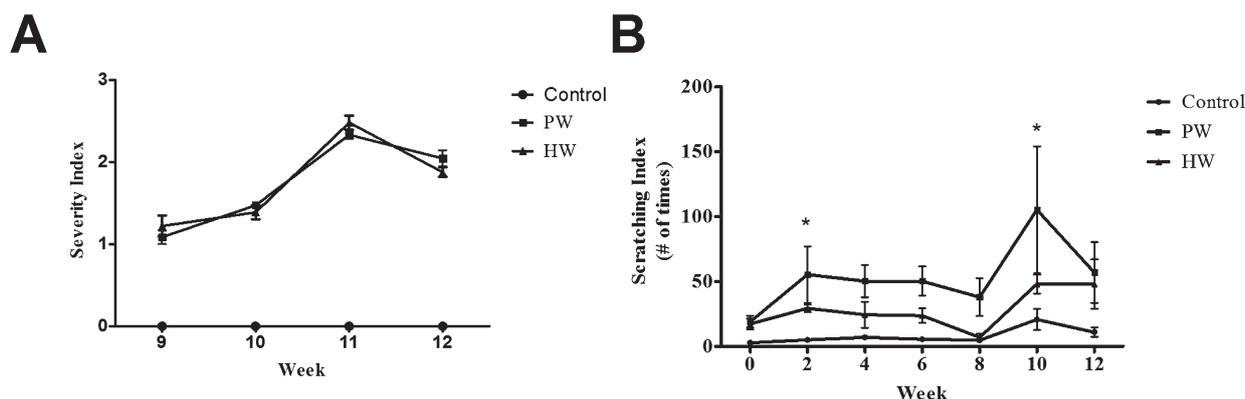


Fig. 1. Effects of HW on the Severity of AD and Scratching Behavior in DNCB-Induced NC/Nga Mice

HW showed slight improvement on the severity of AD (A) in DNCB-induced AD mice during the last four weeks (Weeks 9, 10, 11, 12) of the experiment. A significant reduction of scratching behavior (B) was observed in HW group DNCB-induced AD mice in Week 2 and Week 10. Control group (PW, DNCB⁻), PW group (PW, DNCB⁺), HW group (HW, DNCB⁺). Data were expressed as mean \pm S.D., $n=9$. * $p<0.05$ indicates significant differences with ANOVA. Tukey's test was used for *post hoc* tests.

tone:olive oil, 3:1) was applied on the back skin once a day for 3d for sensitization. After 3d of sensitization, 150 μ L of 0.2% DNCB were treated on the back skin at an interval of 3d during the last 4 weeks (weeks 9–12) of the experiment to induce AD-like skin lesion.

Preparation of Experimental Water PW was prepared through 4 filtration processes: sediment, pre-carbon, reverse-osmosis membrane, and post-carbon filtration using tap water. PW was used as control water given in control and PW group. It had a pH value of 7.0 ± 0.2 (TOA HM-21P, Japan), an ORP value of 270 ± 20 mV (TOA DH-35A, Japan) and a dissolved hydrogen (DH) content of less than 0.001 ppm. On the other hand, HW was produced by High-Concentrated Hydrogen Water Generator (CWP-2184, Chunwoo Co., Seoul, Korea). PW was electrolyzed in the electrolytic cell with platinum-coated titanium electrode and nafion membrane (N-117). Hydrogen gas produced from the cathode was collected and dissolved in water passing through hydraulic system under a pressure of 4 kgf/cm². HW has a pH value of 7.3 ± 0.2 (TOA HM-21P, Japan), an ORP value of -500 ± 30 mV (TOA DH-35A, Japan) and a DH content of 1.5 ± 0.2 ppm. Glass bottles were used for water feeding and were changed two times a day in order to keep the constant water conditions.

Evaluation of Dermatitis Severity The relative dermatitis severity was assessed macroscopically using skin scoring procedure, frequency of scratching, and skin test after triggering AD *via* DNCB. 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.¹⁶⁾ The dermatitis score was defined as the sum of scores for erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness. The skin scoring was assessed once per week in 12 weeks. Scratching frequency was observed per cage within 20 min in triplicate observation. Scratching count was assessed every other week for 12 weeks.

Determination of ROS Generation The level of ROS production in serum was assessed by measuring the oxidation of 2,4-dichlorodihydrofluorescein diacetate (DCFH-DA) using DTX-880 Multimode microplate reader (Beckman Coulter Inc., Fullerton, CA, U.S.A.). All treatments were performed in triplicate and data expressed were averaged values of all cells

counted in each condition.

Measuring GPx Activity GPx activity in serum was measured for H₂O₂ scavenging capacity by modified Cayman's GPx assay kit (Cayman Chemical Co., Ann Arbor, MI, U.S.A.) according to the manufacturer's instruction. Oxidized glutathione, produced upon reduction of hydroperoxide by GPx, was recycled in its reduced state by glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The oxidation of NADPH to NADP⁺ was accompanied by a decrease in absorbance at 340 nm.

MDA Assay Urine samples from each mouse were collected using metabolic cage (Techniplast Co., U.S.A.) every two weeks. After centrifugation, supernatant of the urine samples were used to analyze MDA. The level of MDA, a marker of oxidative stress, was measured using TBARS Assay Kit MDA Quantification (Cell Biolabs, Inc., San Diego, CA, U.S.A.). The assay was performed according to the manufacturer's instructions. Reaction product was measured colorimetrically at 532 nm with a microplate reader (BioTek Instruments, Winooski, VT, U.S.A.). The content of MDA in the samples was expressed as micromolar of MDA produced per gram of protein.

WBC Differential Counting Mice were sacrificed and blood samples were collected for the analysis of total WBC and its differential count (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). It was measured by an automatic blood analyzer (HEMAVET HV950 FS, Drew Scientific Inc., Dallas, TX, U.S.A.).

Measurement of Chemokine and Cytokine Concentration Serum chemokine and cytokines of sacrificed mice were measured. The collected blood was centrifuged for 10 min at 16000 $\times g$, and serum sample was stored in -70°C until analysis. Serum concentrations of thymus and activation-regulated chemokine (TARC), interleukin-1 beta (IL-1 β), IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, GM-CSF, tumor necrosis factor-alpha (TNF- α), and interferon-gamma (IFN- γ) were measured using Multiplex kit (Bio-Rad, San Diego, CA, U.S.A.) and run on Luminex technology (Bio-Plex Multiplex Bead Array SystemTM, Bio-Rad Hercules, CA, U.S.A.) according to the manufacturer's instruction. Raw fluorescence data were analyzed by the software using a 5-parameter logistic method.

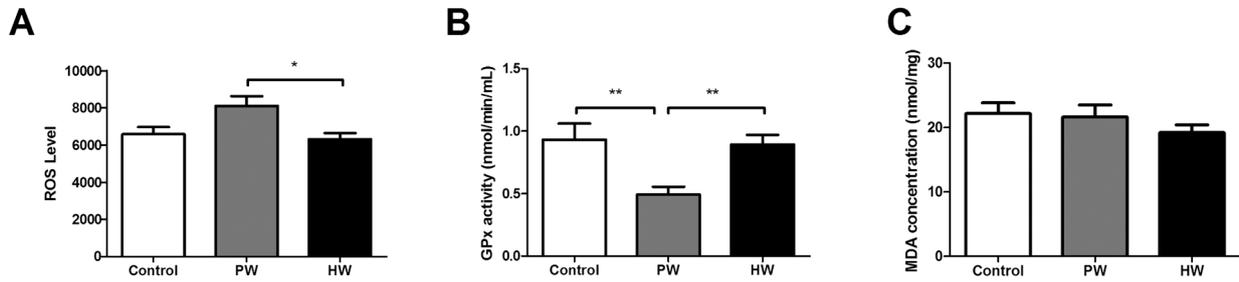


Fig. 2. Effects of HW on Redox Balance Markers in DNCB-Induced NC/Nga Mice

HW group showed a significantly reduced endpoint serum ROS (A) and elevated serum GPx activity (B) in DNCB-induced NC/Nga mice. Urine MDA concentration (C) was measured every-two-week for 12 weeks, resulted in lower level in HW group than PW group, but no statistical significance. Control group (PW, DNCB-), PW group (PW, DNCB+), HW group (HW, DNCB+). Results were expressed as mean±S.D., n=9. Significant differences were indicated as **p*<0.05 and ***p*<0.01 with ANOVA. Tukey's test was used for *post hoc* tests.

Table 1. Total White Blood Cell (WBC) and WBC Differential Counts

	Control	PW	HW
Total WBC (K/ μ L)	5.88±1.45	4.99±1.34	4.08±0.89
Neutrophil (K/ μ L)	1.04±0.27	1.38±0.37	0.66±0.30
Lymphocyte (K/ μ L)	4.31±1.38	3.33±1.09	2.64±0.71*
Monocyte (K/ μ L)	0.22±0.06	0.25±0.07	0.28±0.09
Eosinophil (K/ μ L)	0.015±0.005	0.012±0.010	0.010±0.013
Basophil (K/ μ L)	0.005±0.005	0.008±0.008	0.009±0.008

Control group (PW, DNCB-), PW group (PW, DNCB+), HW group (HW, DNCB+). Data were expressed as mean±S.D., n=9. **p*<0.05 versus PW group, indicates significant differences with ANOVA. Tukey's test was used for *post hoc* tests.

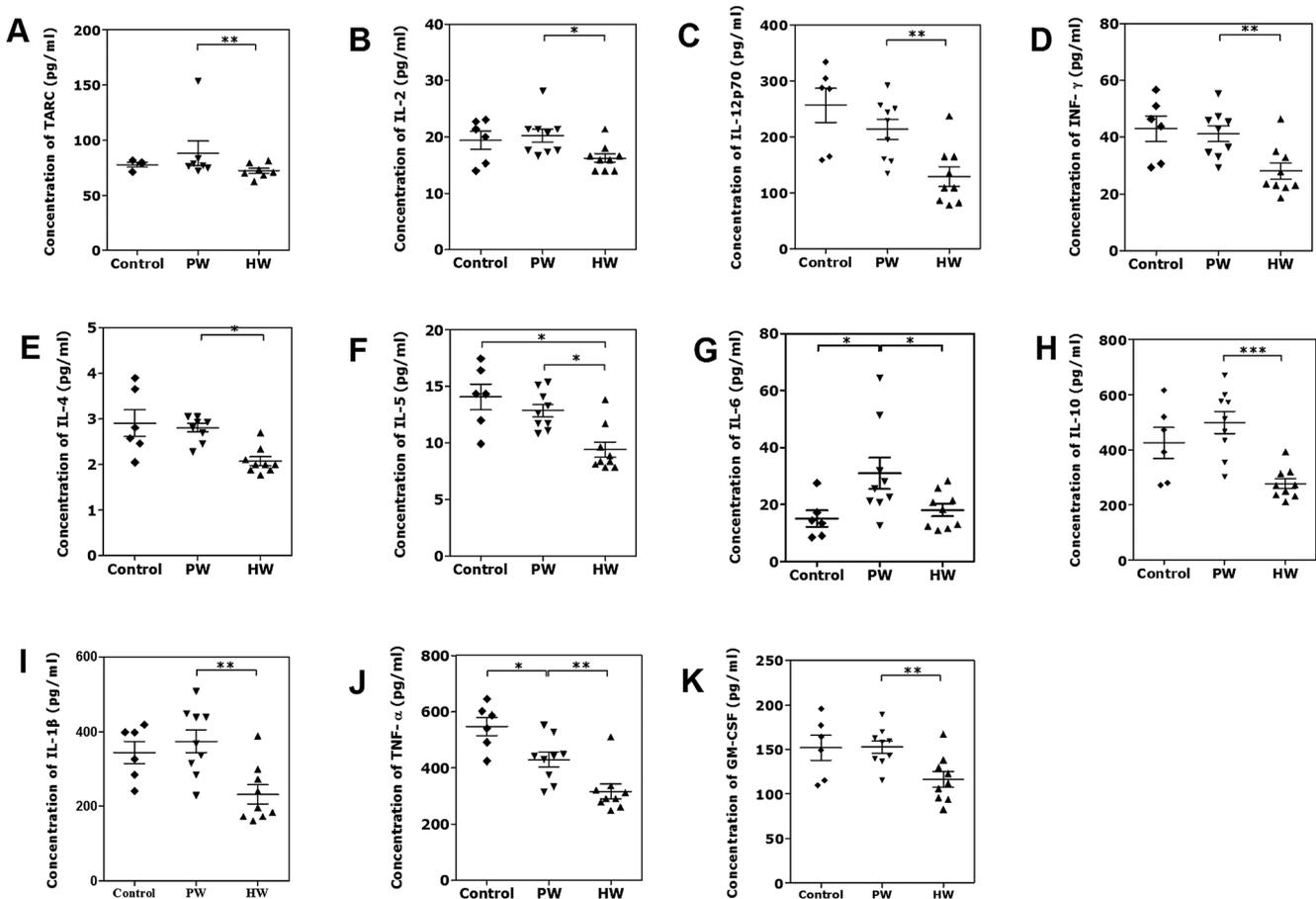


Fig. 3. Effects of HW on Serum Chemokine and Cytokine in DNCB-Induced NC/Nga Mice

Hydrogen water in HW group showed lowered levels of (A) TARC, (B) IL-2, (C) IL-12p70, (D) IFN- γ , (E) IL-4, (F) IL-5, (G) IL-6, (H) IL-10, (I) IL-1 β , (J) TNF- α , and (K) GM-CSF than in PW group. Control group (PW, DNCB-), PW group (PW, DNCB+), HW group (HW, DNCB+). Data were expressed as mean±S.D., n=9. **p*<0.05, ***p*<0.01, and ****p*<0.001 indicate significant differences with ANOVA. Tukey's test was used for *post hoc* tests.

Measurement of IgE Levels by Enzyme-Linked Immunosorbent Assay (ELISA) Serum IgE levels were measured by Mouse IgE ELISA Kit (Shibayagi Co., Ltd.) according to the manufacturer's instructions. Reaction product was measured colorimetrically at 450nm with a microplate reader (BioTek Instruments, Winooski, VT, U.S.A.).

Statistical Analysis Data values were expressed as the mean±S.D. The mean values among groups were analyzed and compared using one-way ANOVA followed by subsequent multiple comparison test (Tukey) with Prism version 5.0 software packages (GraphPad Software Inc., U.S.A.). Differences were considered statistically significant at * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

RESULTS

HW Ameliorates DNCB-Induced AD-Like Symptoms in NC/Nga Mice First, we evaluated the effects of DNCB severity of AD by skin scoring following eczema area, severity index, and scratching tendency. The repetitive application of DNCB induced AD-like skin lesions involving severe clinical symptoms in NC/Nga mice. Our results revealed that the severity induced by DNCB was slightly improved but without significant difference (Fig. 1A). Similarly, to investigate the clinical symptoms of allergic reaction, we measured the scratching tendency of three groups. HW group showed significantly less scratching tendency than PW group for 12 weeks (Fig. 1B). The oral administration of HW decreased the clinical symptoms of dermatitis evidenced by ameliorating the pruritic condition in DNCB-induced NC/Nga mice. However, eczematous skin lesion did not showed significant difference.

Effect of HW in Oxidative Stress in AD In the result presented, ROS generation was significantly decreased in HW group compared to PW group ($p<0.05$) (Fig. 2A). Additionally, GPx was enhanced in HW group ($p<0.01$) (Fig. 2B). The urine MDA content in HW group showed a lower value than PW group, however not statistical significance compared to PW group (Fig. 2C).

Effect of HW in WBC Differential Count The total WBC and the individual subtypes levels just showed a slightly lower pattern but not significantly reduced with some exception for monocytes, which showed an increase but not significant (Table 1). On the other hand, lymphocyte showed significantly lower levels in HW group than the PW group ($p>0.05$).

Effect of HW in Chemokine and Cytokines Serum concentrations of chemokine and cytokines that has been known to regulate AD were analyzed. We found that HW group showed the reduced levels of TARC as compared to PW group (Fig. 3A). Overall, the cytokine levels in HW group were more reduced than those in PW group. Of note, the levels of Th₂ cytokine, IL-5 and pro-inflammatory cytokines such as TNF- α and, IL-6 in HW-fed mice were significantly lower than in control and PW-fed mice.

Effects of HW on IgE Levels in NC/Nga Mice AD is frequently mediated by IgE. Thus, we measured the IgE levels in three experimental groups. HW group revealed the significant reduced IgE levels in NC/Nga mice compared to the PW group (Fig. 4).

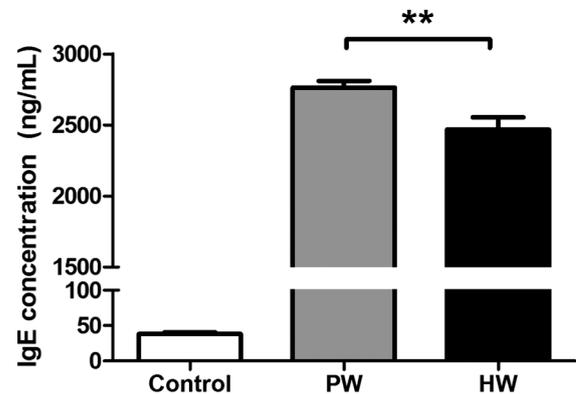


Fig. 4. Effects of HW on IgE Levels in DNCB-Induced NC/Nga Mice

Mice administered with HW slightly reduced IgE levels in DNCB-induced NC/Nga mice. Control group (PW, DNCB-), PW group (PW, DNCB+), HW group (HW, DNCB+). Data were indicated as mean±S.D., $n=9$. ** $p<0.01$ indicates significant differences with ANOVA. Tukey's test was used for *post hoc* tests.

DISCUSSION

Our study indicates that HW could suppress DNCB-induced AD in NC/Nga mice *via* redox balance and immune modulation. To testify our hypothesis, adopting an *in vivo* AD model induced by topical application of DNCB on NC/Nga mice model, we attempted oral PW and HW intake consecutively for 12 weeks.

Cumulative data demonstrate that H₂ has anti-oxidant, anti-inflammatory, anti-apoptotic and other protective effects.^{12,17)} While there are several methods to deliver H₂ into the body including inhalation of H₂ gas,¹⁸⁾ injection of hydrogen-saturated saline,¹⁹⁾ and drinking hydrogen water,²⁰⁾ we utilized HW produced by electrolysis wherein H₂ was highly dissolved in PW passing through the hydraulic system of the generator, thereby producing characteristics such as neutral pH, extremely low ORP, and high DH.

Prior to the redox and immunological study, we first examined the clinical severity score and scratching tendency of the mice groups. We found that the time-dependent severity score in HW group was slightly lower than PW group but not statistically significant (Fig. 1A). Additionally, the scratching tendency of HW group was significantly lower than PW group. These data show that HW could ameliorate the AD-like symptoms such as pruritic condition in DNCB-induced NC/Nga mice. However it might need long-term supply of HW to improve eczematous skin lesion.

Assuming the close linkage of redox imbalance in the pathophysiology of AD,^{3,11)} we measured different oxidative stress markers. First, we found that there was higher activity of GPx in HW group than that of PW group. Similarly, in our previous study, it had been revealed that bathing with HW had increased activity of glutathione peroxidase suggesting a protective role in UV-skin damage in mice.²¹⁾ GPx enzyme is a major peroxide scavenging enzyme important for protecting us from oxidative stress.^{22,23)} In that context, this result implies that HW supplementation would induce ROS scavenging ability. To further explore the clue for the regulation of redox imbalance, we measured ROS levels by DCFH-DA. Figure 2A clearly shows that the ROS level of HW group is significantly suppressed as compared to that of PW group. Since ROS is considered as a secondary messenger that can induce the generation of Th₂ and proinflammatory cytokines,²⁴⁾ this re-

sult could influence the inflammatory response in host such as DNCB-induced AD mice. Besides, oxidative stress in AD can result in oxidative damage to lipids, proteins, and DNA.³⁾ Escalating level of ROS can induce lipid peroxidation.²⁵⁾ Further to support the measurement of ROS, we measured urine MDA, one of the most commonly used markers of overall lipid peroxidation level and oxidative stress. In our study, HW group showed a lower level of MDA than the PW group but there was no significant difference. Given our redox data, we speculate that the high dissolved hydrogen in HW might be a plausible anti-oxidant molecule against DNCB-induced AD mice. In the study by Ohsawa *et al.*,¹⁴⁾ H₂ owed its antioxidant mechanism to its ability to rapidly diffuse across membranes, react with cytotoxic ROS, and to scavenge hydroxyl and peroxynitrate radicals. Taken together, the enhanced GPx enzyme, as well as reduced ROS and MDA might support the anti-oxidant effect of HW in DNCB-induced NC/Nga mice.

In relation to this redox balance, in cellular level, we determined inflammatory markers closely implicated in AD. WBC differential counting showed that HW further reduced the number of total WBC, neutrophils, lymphocytes, eosinophils, and basophils in the blood (Table 1) as compared to PW. Eosinophils have a critical role in AD development, as lymphocytes participate in the inflammatory cascade events.²⁶⁾ Given these, these cellular data might be the direct evidence that HW is able to affect inflammation response in AD. For non-cellular assay to prove this notion, we quantified the serum chemokine, cytokines, and IgE levels. Surprisingly, drinking HW reduced the production of TARC and IgE levels in DNCB-induced NC/Nga mice. According to other studies, chemokine production can be observed in various immune cells, including lymphocytes, dendritic cells, keratinocytes, mast cells, and eosinophils, which are activated by various stimuli.²⁷⁾ The reduction of TARC might affect other immune cells, finally blocking the production of pro-inflammatory cytokines. Last, to check immune modulation in host, cytokine profiling showed a consistent decrement in cytokine levels of HW group as compared to PW group. Of note, there were significantly lower levels of Th₂ cytokine such as IL-5, and pro-inflammatory cytokines such as TNF- α and IL-6 in HW-fed mice than in control or PW-fed mice. HW might influence cytokine production but the mechanism is unclear yet. Other studies reported that H₂ can downregulate the expressions of pro-inflammatory cytokines such as TNF- α , IL-6, IL-1 β , CCL2, *etc.*¹⁸⁾ Our results suggest that HW would improve the immunopathogenesis of AD by way of modulating the production of Th₂ and pro-inflammatory cytokines, similar to our previous results.¹⁵⁾ Since IL-10 level is strongly correlated to IgE titer,²⁸⁾ and AD is often involved in the infiltration IgE, the significant reduction of the IL-10 level could lead to inhibition of IgE.^{29,30)} Interestingly, our result revealed a consistent decrement pattern of IL-10 and IgE level in HW group as compared to PW group. Viewed together, HW might affect the immune balance through the suppression of TARC, inflammatory cytokines, and IgE levels in DNCB-induced AD mice.

Hydrogen molecule was proven to have no toxicity even at high concentrations as it regulates the immune components in one's body. Thus, many researchers have focused on the oral administration of HW in a wide array of diseases.^{13,31)} However, the effect of HW on allergic skin disease such as AD is veiled. This research might shed light on positive application

of HW for the alternative and complementary management of AD and related allergic disease. Further study to unravel the molecular mechanism of HW in immune modulation in AD is underway. Collectively, our study indicates that HW could suppress DNCB-induced AD in NC/Nga mice *via* redox balance and immune modulation, clinically signifying HW could be a safe fluid remedy against AD.

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REFERENCES

- 1) Kay AB. Allergy and allergic diseases. First of two parts. *N. Engl. J. Med.*, **344**, 30–37 (2001).
- 2) Novak N. New insights into the mechanism and management of allergic diseases: atopic dermatitis. *Allergy*, **64**, 265–275 (2009).
- 3) Briganti S, Picardo M. Antioxidant activity, lipid peroxidation and skin diseases. What's new. *J. Eur. Acad. Dermatol. Venereol.*, **17**, 663–669 (2003).
- 4) Vestergaard C, Hvid M, Johansen C, Kemp K, Deleuran B, Deleuran M. Inflammation-induced alterations in the skin barrier function: implications in atopic dermatitis. *Chem. Immunol. Allergy*, **96**, 77–80 (2012).
- 5) Brandt EB, Sivaprasad U. Th2 cytokines and atopic dermatitis. *J. Clin. Cell. Immunol.*, **2** (2011).
- 6) Hattori K, Nishikawa M, Watcharanurak K, Ikoma A, Kabashima K, Toyota H, Takahashi Y, Takahashi R, Watanabe Y, Takakura Y. Sustained exogenous expression of therapeutic levels of IFN-gamma ameliorates atopic dermatitis in NC/Nga mice *via* Th1 polarization. *J. Immunol.*, **184**, 2729–2735 (2010).
- 7) Grassegger A, Hopfl R. Significance of the cytokine interferon gamma in clinical dermatology. *Clin. Exp. Dermatol.*, **29**, 584–588 (2004).
- 8) Sivarajani N, Rao SV, Rajeev G. Role of reactive oxygen species and antioxidants in atopic dermatitis. *J. Clin. Diagn. Res.*, **7**, 2683–2685 (2013).
- 9) Tsukahara H. Current status of redox markers in immunological and inflammatory diseases. *Rinsho Byori (Japanese J. Clin. Pathol.)*, **53**, 759–767 (2005).
- 10) Tsukahara H, Shibata R, Ohshima Y, Todoroki Y, Sato S, Ohta N, Hiraoka M, Yoshida A, Nishima S, Mayumi M. Oxidative stress and altered antioxidant defenses in children with acute exacerbation of atopic dermatitis. *Life Sci.*, **72**, 2509–2516 (2003).
- 11) Bowler RP, Crapo JD. Oxidative stress in allergic respiratory diseases. *J. Allergy Clin. Immunol.*, **110**, 349–356 (2002).
- 12) Qian L, Shen J, Chuai Y, Cai J. Hydrogen as a new class of radioprotective agent. *Int. J. Biol. Sci.*, **9**, 887–894 (2013).
- 13) Ohta S. Recent progress toward hydrogen medicine: potential of molecular hydrogen for preventive and therapeutic applications. *Curr. Pharm. Des.*, **17**, 2241–2252 (2011).
- 14) Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat. Med.*, **13**, 688–694 (2007).
- 15) Ignacio RMC, Kwak H-S, Yun Y-U, Sajo MEJV, Yoon Y-S, Kim C-S, Kim S-K, Lee K-J. The drinking effect of hydrogen water on atopic dermatitis induced by *Dermatophagoides farinae* allergen in NC/Nga mice. *Evd. Based Complement. Alternat. Med.*, **2013**, 538673 (2013).
- 16) Hanifin JM, Cooper KD, Ho VC, Kang S, Krafchik BR, Margolis DJ, Schachner LA, Sidbury R, Whitmore SE, Sieck CK, Van Voorhees AS. Guidelines of care for atopic dermatitis. *J. Am. Acad. Dermatol.*, **50**, 391–404 (2004).

- 17) Zhang JY, Liu C, Zhou L, Qu K, Wang RT, Tai MH, Lei Lei JC, Wu QF, Wang ZX. A review of hydrogen as a new medical therapy. *Hepatogastroenterology*, **59**, 1026–1032 (2012).
- 18) Buchholz BM, Kaczorowski DJ, Sugimoto R, Yang R, Wang Y, Billiar TR, McCurry KR, Bauer AJ, Nakao A. Hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury. *Am. J. Transplant.*, **8**, 2015–2024 (2008).
- 19) Sun Q, Cai J, Liu S, Liu Y, Xu W, Tao H, Sun X. Hydrogen-rich saline provides protection against hyperoxic lung injury. *J. Surg. Res.*, **165**, e43–e49 (2011).
- 20) Cardinal JS, Zhan J, Wang Y, Sugimoto R, Tsung A, McCurry KR, Billiar TR, Nakao A. Oral hydrogen water prevents chronic allograft nephropathy in rats. *Kidney Int.*, **77**, 101–109 (2010).
- 21) Ignacio RM, Yoon Y-S, Sajo MEJ, Kim C-S, Kim D-H, Kim S-K, Lee K-J. The balneotherapy effect of hydrogen reduced water on UVB-mediated skin injury in hairless mice. *Mol. Cell Toxicol.*, **9**, 15–21 (2013).
- 22) Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn. Rev.*, **4**, 118–126 (2010).
- 23) Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann. Bot. (Lond.)*, **91** (Spec. No.), 179–194 (2003).
- 24) Qi XF, Teng YC, Yoon YS, Kim DH, Cai DQ, Lee KJ. Reactive oxygen species are involved in the IFN-gamma-stimulated production of Th2 chemokines in HaCaT keratinocytes. *J. Cell. Physiol.*, **226**, 58–65 (2011).
- 25) Grotto D, Maria LS, Valentini J, Paniz C, Schmitt G, Garcia SC, Pomblum VJ, Rocha JBT, Farina M. Importance of the lipid peroxidation biomarkers and methodological aspects for malondialdehyde quantification. *Quim. Nova*, **32**, 169–174 (2009).
- 26) Albanesi C. Keratinocytes in allergic skin diseases. *Curr. Opin. Allergy Clin. Immunol.*, **10**, 452–456 (2010).
- 27) Gros E, Busmann C, Bieber T, Förster I, Novak N. Expression of chemokines and chemokine receptors in lesional and nonlesional upper skin of patients with atopic dermatitis. *J. Allergy Clin. Immunol.*, **124**, 753–760, e751 (2009).
- 28) Cooper PJ, Chico ME, Sandoval C, Nutman TB. Atopic phenotype is an important determinant of immunoglobulin E-mediated inflammation and expression of T helper cell type 2 cytokines to ascaris antigens in children exposed to ascariasis. *J. Infect. Dis.*, **190**, 1338–1346 (2004).
- 29) Williams CM, Rahman S, Hubeau C, Ma HL. Cytokine pathways in allergic disease. *Toxicol. Pathol.*, **40**, 205–215 (2012).
- 30) Carmi-Levy I, Homey B, Soumelis V. A modular view of cytokine networks in atopic dermatitis. *Clin. Rev. Allergy Immunol.*, **41**, 245–253 (2011).
- 31) Ohta S. Molecular hydrogen is a novel antioxidant to efficiently reduce oxidative stress with potential for the improvement of mitochondrial diseases. *Biochim. Biophys. Acta*, **1820**, 586–594 (2012).