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Effects of H_2-Rich Water Consumption on Oxidative Stress, PBMC Profiles and Their Transcriptome: A Randomized, Double-blind, Controlled Study

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Abstract

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Oxidative stress indicates a state where excessive oxidants overwhelm the biological antioxidant system, leading to various pathological conditions such as chronic inflammation and cellular dysfunctions. Recently, molecular H_2 has been proposed as a novel antioxidant, and its therapeutic effects against various diseases were demonstrated with animal models and clinical trials. However, the antioxidant effect of the H_2 administration has not been examined in the healthy subjects, and its systemic effect has not been elucidated. Here, we aimed to investigate the effects of H_2-rich water (HW) consumption in healthy

adults through the extensive analysis of antioxidant capacity, immune cell profiles, and transcriptome of peripheral blood mononuclear cells (PBMCs), and to compare the effects of HW consumption with those of plain water (PW) consumption in resting state and exercise-induced oxidative stress state. A total of 38 participants (20-59 y) completed a double-blind, randomized, controlled intervention trial. They consumed either 1.5 L/d of PW (n = 18) or HW (n = 20) for 4 weeks. When the participants were at rest, we measured biological antioxidant potential (BAP), derivatives of reactive oxygen metabolites (d-ROMs), and 8-Oxo-2'-deoxyguanosine (8-OHdG) in serum, and also analyzed the apoptosis and subpopulations of PBMCs. At week 4, we conducted the treadmill test to induce acute oxidative stress, and analyzed the level of oxidative stress, peripheral immune cell subpopulations and PBMC transcriptome. In resting state, BAP increased to a greater extent in HW group (n = 10) than in PW group (n = 8) in those who aged ≥30 y (P = 0.028), with no difference between groups in <30 y (P = 0.534). Also, HW group (n = 18) showed a lower percentage of PBMC apoptosis than PW group (n = 18) (P = 0.042) and HW consumption decreased the frequency of CD14 positive PBMCs (P = 0.042). In the exercise-induced oxidative stress state, HW group (n = 18-20) did not significantly differ from PW group (n = 18) in the serum biomarkers of BAP, d-ROMs, and 8-OHdG and in the frequencies of PBMC subpopulations (All P > 0.05). Transcriptome profiling of PBMCs, however, revealed a clearly classified transcriptional response of HW group. Particularly, HW con-

sumption most influenced on the genes belonging to the functional category of inflammatory response and significantly down-regulated the NF-κB signaling pathway. Furthermore, the expression levels of NF-κB responsive genes including IL1B, IL8, IL6R, and TNFRSF10B were significantly lower in HW group. In conclusion, a 4-week HW consumption reduces oxidative stress by improving antioxidant capacity, which leads to the decreases in cellular damages of PBMCs and the frequency of circulating monocytes. In the condition of acute oxidative stress, 4-week HW consumption reduces the inflammatory response by down-regulating NF-κB-mediated signal transduction and NF-κB responsive genes. These findings suggest that the H_2 administration exhibits the antioxidant effect in healthy population and our study may help understanding the molecular mechanism by which H_2 exhibits the anti-inflammatory effect against intense oxidative stress.

Keywords **:** Antioxidant, oxidative stress, H_2-rich water, transcriptome profiling, peripheral blood mononuclear cell (PBMC), Inflammation

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ROS	reactive oxygen species
PBMC	peripheral blood mononuclear cell
BAP	biological antioxidant potential
d-ROMs	derivatives of reactive oxygen metabolites
8-OHdG	8-Oxo-2'-deoxyguanosine
RPKM	reads per kilobase million
IPA	ingenuity pathway analysis
DEG	differently expressed genes
NF-κB	nuclear factor-kappa B

I. Introduction

Oxidative stress indicates a state where excessive reactive oxygen species (ROS) overwhelm the biological antioxidant capacity, leading to disruption of ROS homeostasis and cellular damages (Sies, 2015a). ROS are natural by-products of normal cell metabolism such as the mitochondrial aerobic respiration, and they also can be generated by the environmental stimuli like pollutants, smoking, infection, and exposure to irradiation rays (Yoshikawa and Naito, 2002). Although ROS perform physiologically critical roles to maintain the redox signaling and the normal cellular functions, they are highly responsible for oxidative damages of vital biomolecules such as carbohydrates, proteins, lipids and nucleic acids because of their high reactivity (Schieber and Chandel, 2014). Also, excessive ROS and the oxidized molecules generated by ROS can stimulate pro-inflammatory immune cells and consequently induce the chronic inflammation (Reuter *et al*., 2010). As a result, oxidative stress can act as a trigger of various diseases including cardiovascular diseases, metabolic syndrome, neurodegenerative disorders, and cancer if they are not properly eliminated by antioxidant enzymes, endogenous or dietary antioxidants (Vaziri and Rodríguez-Iturbe, 2006, Furukawa *et al*., 2017, Emerit *et al*., 2004, Khansari *et al*., 2009).

Recently, molecular hydrogen (H_2) has been noticed as a novel antioxidant since the molecules have been reported to selectively scavenge the strong

oxidants such as hydroxyl radical in the cells (Ohsawa *et al*., 2007). Lots of animal studies have demonstrated the positive effect of H_2 on the various human diseases and the experimental condition of oxidative stress. In the models of ischemia/reperfusion (I/R) injury, H_2 effectively prevented the tissue damage and reduced the infarct size (Zheng *et al*., 2009, Hayashida *et al*., 2008, Fukuda *et al*., 2007). In the rat models of neurodegenerative disorders including Parkinson's disease and Alzheimer's disease, administration of H_2 improved the memory function of the rats and retarded the development and progression of the diseases (Fu *et al*., 2009, Li *et al*., 2010). In the apolipoprotein E knockout mouse model, H_2-dissolved water significantly reduced the atherosclerotic lesions (Ohsawa *et al*., 2008). Some clinical trials have also determined the positive effect of H_2 on various diseases including metabolic syndrome, rheumatoid arthritis, chronic hepatitis B and Parkinson's disease (Song *et al*., 2015, Song *et al*., 2013, Ishibashi *et al*., 2012, Xia *et al*., 2013). In the patients with diabetes mellitus type 2, drinking of H_2-rich water improved a lipid profile and glucose tolerance (Kajiyama *et al*., 2008). Also, H_2-rich water decreased DNA oxidation and disease activity in the patients with rheumatoid arthritis which is characterized by a chronic inflammatory disease (Ishibashi *et al*., 2012). In recent years, clinical examinations demonstrating the therapeutic effects of H_2 are continuously on the rise.

The capacity of antioxidants can be estimated with various *in vivo* models exposed to oxidative stress-causing factors such as obesity, cigarette smok-

ing, radiotherapy, I/R, and inflammation (Furukawa *et al*., 2017, Valavanidis *et al*., 2009b, Riley, 1994, Gonzalez-Flecha *et al*., 1993, Reuter *et al*., 2010). Exercise stress is one of the available *in vivo* conditions in that it instantly induces systemic oxidative stress inside the body by causing the production of free radicals in various tissues including skeletal muscles, heart, lungs and white blood cells (Powers and Jackson, 2008). Although the extent of stress is dependent on duration, intensity and modality of the physical activity, in general, intense aerobic exercise influences on the redox status of the endogenous antioxidants and increases the oxidation of biomolecules (Bouzid *et al*., 2014, Powers and Jackson, 2008). In this regard, we can evaluate the candidate expected to present the antioxidant activity by investigating how effectively it defends against the exercise-induced oxidative stress.

Despite the increasing evidence asserting the positive effects of H_2, to our knowledge, few studies have been conducted in healthy population. Furthermore, the systemic effect of H_2 administration has not been elucidated because most of the preceding studies have only focused on measuring the limited markers of oxidative stress. Here, we aimed to investigate the effects of H_2-rich water consumption in healthy adults through the extensive analysis of antioxidant capacity, immune cell profiles, and transcriptome of peripheral blood mononuclear cells (PBMCs). Also, we sought to determine the effects of H_2-rich water consumption by comparing with those of plain water consumption in the two different circumstances: when the participants were in

resting state and when they were exposed to acute oxidative stress by the treadmill exercise. These findings may provide the insights in the impact of H_2 administration on antioxidant state and immune responses in healthy population and contribute to understanding the underlying mechanism by which H_2 elicits the anti-inflammatory effect under the condition of acute oxidative stress.

II. Subjects and Methods

1. Subjects

158 individuals were recruited to the study which was advertised on the school portal site and the bulletin boards. They were assessed for eligibility according to the following inclusion criteria: men and women aged 20-59 y; no medical history of acute or chronic diseases; and consumption of plain water ≥500 and ≤2500 mL per a day. Exclusion criteria were as follows: consumption of beverages including coffee, tea, soft drinks and alcohol >500 mL per a day; consumption of alcohol containing beverages >2 days per a week; regular use of antioxidant supplements including vitamins and minerals within last 3 months; and habits of smoking and strenuous exercise.

2. Study design

This study was a parallel-designed, randomized, double-blind, placebo-controlled trial. Eligible participants were randomly assigned to either plain water group (PW group) or H_2-rich water group (HW group), and the random assignment was stratified by sex and age (<30 y and ≥30 y) with the use of online randomization service (Sealed Envelope, London, UK). We provided all participants with intervention water through the delivery service not to let them know what others had been provided. All participants perceived themselves as belonged to HW group, and lab technicians were also kept in blinded to the allocation until every analysis was completed. At the baseline and after the follow-up when the participants were at rest, we assessed serum biomarkers indicating oxidative stress level, and analyzed the apoptosis and subpopulations of PBMCs. Also, we determined whether HW consumption could ameliorate acute oxidative stress which exceeded the ordinary level and whether HW group would be distinguished from PW group regarding PBMC subpopulations and transcriptome profile. Accordingly, we adopted the treadmill exercise test lasting about 15-20 min on the day after the end of the intervention. This study was conducted at the Department of Food and Nutrition, and Department of Physical Education in Seoul National University between Jun and Sep 2016, and was approved from the Institutional Review Board of Seoul National University. All participants provided a written informed consent prior to the intervention.

3. Water intervention

Each participant was delivered either PW or HW which was individually packed in a capacity of 500 mL 1-2 times a week during the 4 weeks of intervention periods. Because the participants in PW group also perceived themselves as belonged to HW group, all of participants were instructed to drink the 500 mL of water within an hour after initial open for preventing the loss of dissolved H_2. Also, to achieve a regular consumption throughout the study, participants were asked to drink intervention water before and after every meal for three times a day (a total of 1500 mL per a day). They were not allowed to drink any other water except for provided one and the total consumption of extra beverages including coffee, tea, soft drinks and alcohol was limited to ≤500 mL per a day. Participants were instructed to record every day whether/when they had consumed intervention water. They also recorded the kind/amount of any other extra beverages which they had consumed for a day. The records were reviewed 1-2 times a week to enhance their compliance to the study. Participants in both PW and HW arms were advised to maintain their usual diet and physical activities and to avoid taking any antioxidant supplements throughout the experimental periods. Particularly, the day before each visit, they were refrained from strenuous exercise and alcohol consumption and required to comply with overnight fasting. On each visit, participants filled in a questionnaire containing the questions about daily dietary intake and physical activities. We used commercially

available H_2-rich water (Hi susosu, Seoul, Korea) and purified plain water (Coway Co., Ltd, Seoul, Korea). Before starting the intervention, the concentration of dissolved H_2 was measured with the use of dissolved hydrogen meter (ENH-1000; Trustlex, Tokyo, Japan) in both of PW (0 mg/L) and HW (1 mg/L).

4. Blood sampling and induction of exercise-induced oxidative stress

As shown in Figure 1, blood samples were collected total three times: T_0, T_1, and T_2. At the first visit on the day before starting the intervention, fasted blood samples were collected at rest (T_0) (Figure 1). The second visit took place on the day subsequent to the last day of the intervention. First, we collected fasted blood samples when the participants were at rest (T_1) (Figure 1). After 10-15 min from the end of the first blood collection, participants completed physical activity readiness questionnaires (PAR-Q) to determine any problem or hazard of partaking in an exercise test. Subsequently, they accomplished the submaximal graded exercise test which was employed to induce a standardized and consistent level of exertion across all participants (Evans and White, 2009). The test was performed according to modified Bruce protocol (Okin *et al*., 1986). Briefly, each participant started to walk slowly on the treadmill at 1.7 mph and 0% grade, and both of the speed and the grade gradually increased as time passed. Two supervisors monitored heart rate and checked rating of perceived exertion at each stage to continue the test until the participant was totally exhausted. The test lasted for an average of 15-20 min and was immediately followed by the second blood sampling (T_2) (Figure 1). Fasting venous blood samples from antecubital fossa were collected into 8-mL serum separator tubes (BD bioscience, Franklin Lakes, NJ, USA), 8-mL EDTA-containing tubes (BD bioscience,

Franklin Lakes, NJ, USA), and BD vacutainer mononuclear cell preparation tubes with sodium citrate (BD bioscience, Franklin Lakes, NJ, USA). Upon collection, plasma and serum samples were aliquoted in 1.5mL ep-tubes (Eppendorf, Hamburg, Germany) and were frozen at -80℃ for a later analysis.

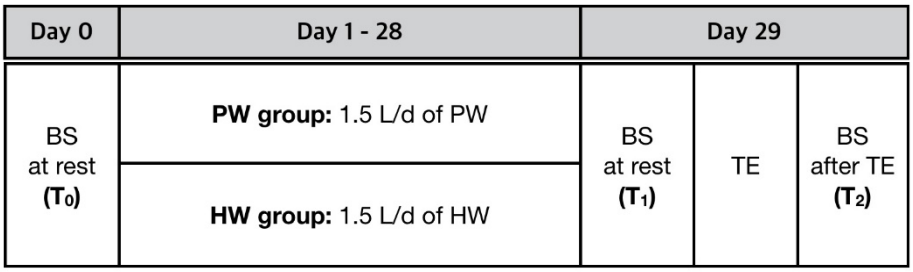

Figure 1. Schematic outline of the study protocol.

Blood samples of T_0 and T_1 were collected when participants were at rest, whereas those of T_2 were collected immediately after the treadmill exercise. BS, blood sampling; PW, plain water; HW, H_2-rich water; TE, treadmill exercise; T_0, day 0 at rest (baseline); T_1, day 29 at rest; T_2, immediately after the treadmill exercise on day 29.

5. Measurement of antioxidant capacity and oxi-dative damages

Antioxidant capacity was determined by measuring biological antioxidant potential (BAP) in serum samples. Oxidative damages were assessed by the concentration of derivatives of reactive oxygen metabolites (d-ROMs) and 8-Oxo-2'-deoxyguanosine (8-OHdG) in serum samples. BAP test (BAP Kit; Diacron Srl., Grosseto, Italy) and d-ROMs test (d-ROMs Kit; Diacron Srl., Grosseto, Italy) were performed with the use of colorimetric analyzer (Cobas 8000 c702; Roche, Mannheim, Germany) according to manufacturer's instructions. Briefly, addition of reagent solution containing ferric chloride ($FeCl_3$) to serum sample caused ferric iron ($Fe3^+$) to be reduced as ferrous ($Fe2^+$), which induced the decolorization of the solution. The amount of reduced ferric ions (μmol/L) was considered as proportional to antioxidant capacity of the serum sample. For d-ROMs test, the acidic buffer was added to serum sample, which caused the releasing of metal ions from the serum proteins. These metals catalyzed the cleavage of organic hydroperoxides (ROOH), generating free radicals. The radicals oxidized the aromatic amine, which induced the color formation. The intensity of the resulting color was expressed in relative units (1 CARR U = 0.08 mg/100ml H_2O_2) and was considered as positively correlated with a concentration of organic hydroperoxides in the serum sample. 8-OHdG, an indicator of DNA damage by oxidative stress, was measured with the use of enzyme-linked

immunosorbent assay (8-OHdG Check ELISA; Jaica, Fukuroi, Japan) in accordance to manufacturer's instructions. The concentration of serum 8-OHdG was expressed as ng/mL.

6. Analysis of PBMC subpopulation and apoptosis

PBMCs were isolated from the whole blood by density-gradient centrifugation using Ficoll-Paque PLUS density gradient media (GE healthcare, Songdo, Korea). PBMCs were stained with Alexa Fluor 488-conjugated anti-human CD4 (OKT4, eBioscience, San Diego, CA, USA), PE-conjugated anti-human CD8 (3B5, eBioscience), APC-Cy7-conjugated anti-human CD20 (B-Ly-1, eBioscience), APC-Cy7-conjugated anti-human CD11b (ICRF44, BD Biosciences, San Jose, CA, USA), APC-conjugated anti-human CD14 (61D3. eBioscience) antibodies in FACS buffer (0.1% bovine calf serum and 0.05% sodium azide in 1x PBS [phosphate buffered saline]) at 4°C for 30 min. Annexin V staining was performed using PE-conjugated anti-annexin V antibody (eBioscience) in annexin V binding buffer (10mM HEPES [pH7.4], 140mM NaCl. 2.5mM $CaCl_2$) at RT for 15 min and DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA) staining was used for excluding dead cells and apoptotic analysis with annexin V staining cells. The stained cells were analyzed using BD LSRFortessa (BD Biosciences) and data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

7. Profiling of PBMC transcriptome

Transcriptome profiling of PBMCs was performed according to the following protocol. PBMCs from the samples of T_2 were isolated immediately after the blood collection with the use of BD vacutainer mononuclear cell preparation tubes with sodium citrate (BD bioscience, Franklin Lakes, NJ, USA) and then total RNA was extracted from PBMCs (RNAqueous-4PCR Kit; Ambion, TX, USA). We assessed the quality and concentration of the extracted total RNA using Agilent 2100 BioAnalyzer (Agilent Technologies, CA, USA). Out of the samples with RNA integrity number (RIN) greater than seven, a total of 6 samples (3 samples per a group) were randomly selected to be sequenced. Subsequently, intact mRNA was captured from the total RNA with the use of Dynabeads mRNA DIRECT Micro Kit (Ambion, TX, USA). And then, total mRNA samples were depleted of 5S, 5.8S, 18S, and 28S ribosomal subunits up to 99.9% using RiboMinus Eukaryote System v2 (Life Technologies, Carlsbad, CA, USA). We verified the absence of ribosomal peaks using Bio-analyzer instrument (Agilent Technologies, CA, USA) and RNA 6000 Pico Kit (Agilent Technologies, CA, USA). Barcoded cDNA libraries were pre-pared from the ribo-depleted mRNA samples and constructed with the use of reagents in Ion Total-RNA Seq Kit v2 (Life Technologies, Carlsbad, CA, USA). First, the ribo-depleted mRNA was fragmented with RNase Ⅲ at 37℃ for 3 min. The fragmented RNA was purified on nucleic acid-binding beads

and hybridized with Ion Adaptor Mix v2. Subsequently, ligation was performed at 30℃ for 1 h. The adaptor-ligated libraries were pre-incubated with a reverse transcription primer at 70℃ for 10 min and then converted to cDNA by reverse transcription at 42℃ for 30 min. The cDNA libraries were purified on nucleic acid-binding beads and then amplified by PCR using barcoded primers (Ion Xpress RNA-Seq Barcode 01-16 Kit; Life Technologies, Carlsbad, CA, USA). After the bead-purification, molarity of the final library was determined using Bioanalyzer instrument (Agilent Technologies, CA, USA) and High Sensitivity DNA Kit (Agilent Technologies, CA, USA). Whole transcriptome libraries were diluted to 100 pM using BioAnalyzer (Agilent Technologies, CA, USA) and amplified on Ion Sphere Particles (ISPs) by emulsion PCR with the use of Ion One Touch 2 system (Life Technologies, Carlsbad, CA, USA) and Ion PI Hi-Q OT2 200 Kit (Life Technologies, Carlsbad, CA, USA). Enrichment of template-positive ISPs were performed using Ion OneTouch Enrichment System (ES) (Life Technologies, Carlsbad, CA, USA) where biotinylated adaptor sequences were selected by binding to streptavidin beads. Subsequently, the template-positive ISPs were sequenced with the use of Ion PI Hi-Q Sequencing 200 Kit (Life Technologies, Carlsbad, CA, USA). Sequencing primers were annealed to the template fragments attached to ISPs, and the template positive ISPs samples were loaded on a chip in Ion PI Chip Kit v3 (Life Technologies, Carlsbad, CA, USA) and incubated with polymerase. Finally, the chip was placed on Ion Proton System (Life Technologies,

Carlsbad, CA, USA) for sequencing which worked on the principal that hydrogen ion release was detected when new nucleotides were incorporated into the growing DNA template (Pareek *et al*., 2011). All procedures were performed according to the manufacturer's instructions.

8. Bioinformatics analysis of RNA sequencing data

Raw reads generated by the sequencer were uploaded as FASTQ files to the Torrent Suite software where low quality reads were trimmed and filtered. Trimming was performed to remove the adapter sequence and lower-quality 3' ends with low quality scores. Read filtering was carried out to remove adapter dimmers, reads lacking a sequencing key and polyclonal reads. High quality reads were mapped and aligned with the computational pipeline of Bowtie 2 and TopHat (Trapnell *et al*., 2010). After mapping and aligning, the resulting BAM files were imported into Partek Genomics Suite v6.6 (Partek Inc., Saint Louis, MI, USA) and converted into gene transcript levels as reads per kilobase of exon per million mapped reads (RPKM) with the use of a mixed-model approach. A one-way ANOVA model was applied and the database was filtered based on fold-change (greater than 5 or less than -5) and *P* value ($P < 0.01$). Genes that passed our statistical criteria were analyzed with the bioinformatics software Ingenuity Pathway Analysis (IPA; www.ingenuity.com). IPA used Fisher's exact test to analyze the gene set significantly modulated within the context of biological systems including cellular functions and gene regulatory network.

9. Statistical analysis

Statistical analysis was performed with the use of SPSS version 23 for Macintosh (IBM corp., Chicago, IL, USA). All data were tested for normality before selecting the appropriate statistical method. General characteristics and measures at baseline were analyzed on the basis of an unpaired *t* test or Mann-Whitney *U* test to identify whether there were statistical differences between groups. A one-way repeated measures ANOVA was used for within-group comparisons between day 0 at rest (T_0) and day 29 at rest (T_1), and between before (T_1) and immediately after the treadmill exercise on day 29 (T_2). Regarding the changes from baseline at rest (ΔT_1T_0), comparisons between PW and HW groups were performed on the basis of a general linear model with an adjustment for the value at T_0 as a covariate. Regarding the change induced by exercise stress on day 29 (ΔT_2T_1), we compared HW group with PW group with the use of an unpaired *t* test or Mann-Whitney *U* test. We conducted a two-way ANOVA with an adjustment for the value at T_0 as a covariate to determine the interaction between the effects of treatment (PW or HW) and age (<30 y or ≥30 y) regarding the changes in BAP, d-ROMs, 8-OHdG, and PBMC subpopulations and apoptosis from baseline to week 4. When a significant interaction was discovered, simple main effects analysis was performed. $P < 0.05$ was considered statistically significant.

III. Results

1. General characteristics of participants

The flow diagram of the participants throughout the study is presented in Figure 2. A total of 158 participants were assessed for eligibility according to the inclusion and exclusion criteria, and finally 41 participants were found to be eligible and were included in the study. They were randomly assigned to either PW group (n = 19) or HW group (n = 22). Out of 3 participants who were withdrawn from the study, 1 participant in PW group dropped out before starting the intervention, and 2 participants in HW group dropped out on the 4th day and the 10th day, respectively. As a result, a total of 38 participants successfully completed the 4 week of intervention and were included in the final analysis (n = 18 in PW group; n = 20 in HW group) (Figure 2).

As shown in Table 1, there were no statistical differences in age, height, weight, BMI and daily pain water intake at baseline between PW and HW groups (all $P > 0.05$).

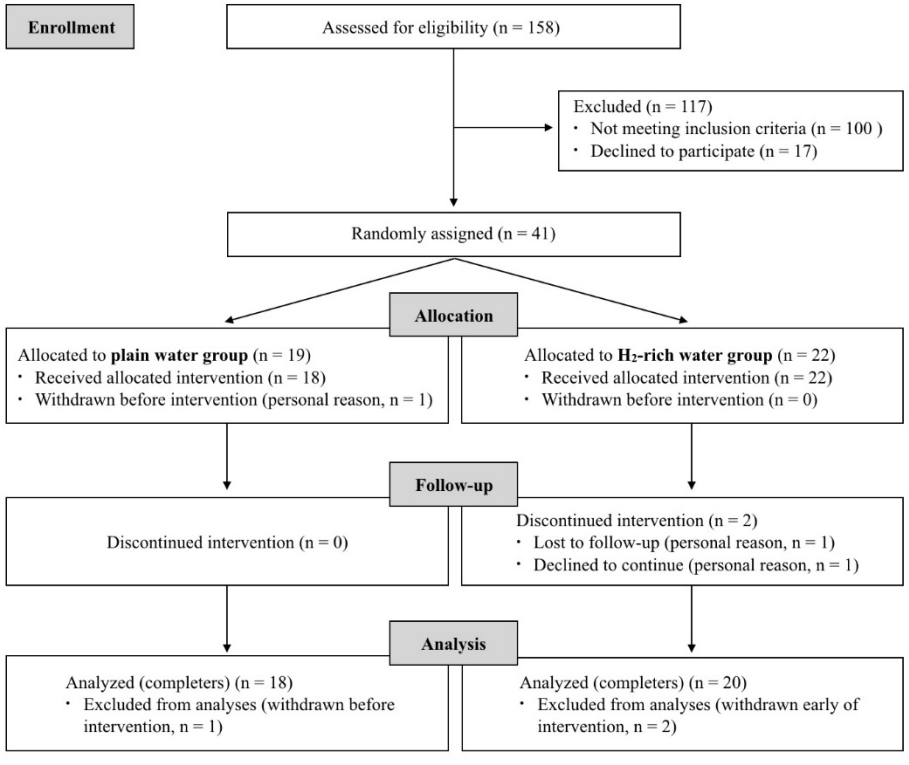

Figure 2. Flow diagram of the participants throughout the study

Table 1. General characteristics of participants at baseline[1]

Characteristics	PW group (n = 18)	HW group (n = 20)	P^2
Subjects, n	18	20	-
Sex, M/F, n	9/9	10/10	-
Age, y	32.9 ± 10.9	29.6 ± 8.1	0.393
Height, cm	167.6 ± 7.4	169.0 ± 9.1	0.602
Weight, kg	66.5 ± 12.6	68.8 ± 15.7	0.493
BMI, kg/m^2	23.1 ± 2.7	23.8 ± 3.9	0.530
Daily plain water intake[3], L/d	1.2 ± 0.5	1.2 ± 0.3	0.393

[1]All values are means ± SDs. PW, plain water; HW, H_2-rich water.

[2]There were no statistical differences between PW and HW groups on the basis of an unpaired *t* test or a Mann-Whitney *U* test.

[3]Obtained from self-reported questionnaires at baseline.

2. Effects of H_2−water in resting state

2-1. Antioxidant capacity and oxidative damages

A 4 week of intervention did not influence the concentrations of d-ROMs in PW and HW groups (both $P > 0.05$) (Table 2). In contrast, the concentration of 8-OHdG significantly decreased in both groups (for PW, Δ = -0.94 ± 1.44 ng/mL and $P < 0.05$; for HW, Δ = -1.32 ± 1.05 ng/mL and $P < 0.001$) (Table 2). For BAP, only HW group showed a significant increase (Δ = 297.8 ± 274.2 μmol/L; $P < 0.001$) (Table 2). In between-group comparisons regarding the change from baseline, however, there were no statistical differences in d-ROMs, 8-OHdG, and BAP (all $P > 0.05$) (Table 2).

Because age is intimately correlated with the extent of ordinary oxidative stress at rest (Kregel and Zhang, 2007), we hypothesized that the antioxidant effect of HW consumption would be variable depending on the age of the participants. Accordingly, after categorizing participants according the two age groups (<30 y or ≥30 y), we investigated the interaction between the effects of treatment (PW or HW) and age (<30 y or ≥30 y) regarding the changes (Δ T_1T_0) in BAP, d-ROMS, and 8-OHdG. Only for the change in BAP, the significant interaction was observed: in younger age group (<30 y), HW consumption did not show a statistical difference from PW consumption ($P = 0.534$) (Figure 3); in older age group (≥30 y), however, HW group showed a greater increase in BAP than PW group ($P = 0.028$) (Figure 3).

Table 2. Antioxidant capacity and oxidative damage markers in resting state[1]

	PW group (n = 18)			HW group (n = 20)			PW *vs.* HW
Measure	T_0[2]	T_1	ΔT_1T_0[3]	T_0[2]	T_1	ΔT_1T_0[3]	ΔT_1T_0 P[4]
BAP, μmol/L	2086.6 ± 236.6	2275.0 ± 394.5	194.4 ± 315.4	2109.5 ± 234.5	2407.3 ± 303.6	297.8 ± 274.2***	0.267
d-ROMs, CARR.U	354.9 ± 70.7	336.9 ± 54.8	–18.0 ± 31.9	375.3 ± 68.9	367.6 ± 63.1	–7.6 ± 39.9	0.142
8-OHdG, ng/mL	1.99 ± 1.27	1.04 ± 0.59	–0.94 ± 1.44*	2.05 ± 0.95	0.73 ± 0.60	–1.32 ± 1.05***	0.144

[1]All values are means ± SDs. T_0, day 0 at rest (baseline); T_1, day 29 at rest; PW, plain water; HW, H_2-rich water; BAP, biological antioxidant potential; d-ROMs, derivatives of reactive oxygen metabolites; 8-OHdG, 8-Oxo-2'-deoxyguanosine.

[2] There were no significant differences between PW and HW groups for all measures at T_0 on the basis of an unpaired t test.

[3]ΔT_1T_0 indicates the change from baseline at rest. Significant differences between T_0 and T_1 within each group were determined with the use of a one-way repeated measures ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001.

[4]P values were obtained with the use of a general linear model adjusting for the value at T_0 as a covariate.

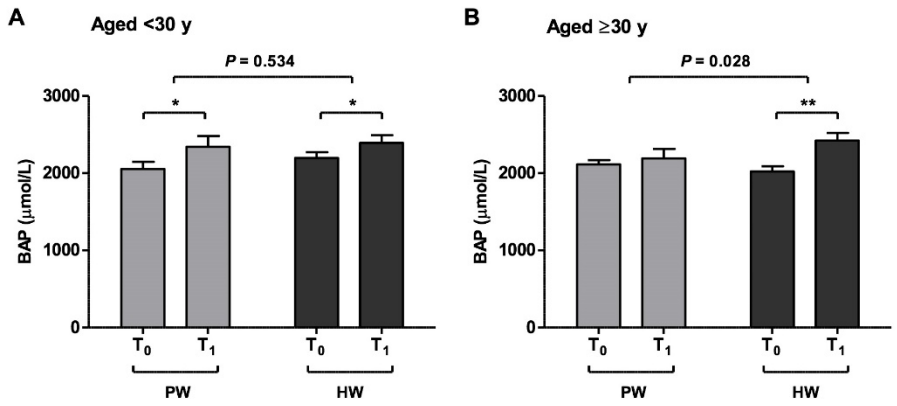

Figure 3. Antioxidant capacity by age (<30 y and ≥30 y) in resting state

Data are presented as means ± SEMs. Significant differences between T_0 and T_1 within each group were determined with the use of a paired *t* test. *P* values were obtained with the use of simple main effects analysis. $P < 0.05$ was considered statistically significant. **(A)** Within the participants aged <30 y, there was no significant difference between PW group ($n = 10$) and HW group ($n = 10$) for the change in BAP ($P = 0.534$). **(B)** HW group aged ≥30 y ($n = 10$) showed a greater increase in BAP compared with PW group aged ≥30 y ($n = 8$) ($P = 0.028$). PW, plain water; HW, H_2-rich water; BAP, biological antioxidant potential; T_0, day 0 at rest (baseline); T_1, day 29 at rest.

2-2. PBMC apoptosis

Next, we investigated if 4 week of HW consumption would affect the level of PBMC apoptosis. There was no significant difference between two groups in the baseline measure of PBMC apoptosis ($P = 0.739$) (Figure 4). After 4 week of water consumption, however, HW group showed a significantly lower percentage of PBMC apoptosis compared with PW group ($P = 0.042$) (Figure 4). No significant interaction [treatment (PW or HW) × age (<30 y or ≥30 y)] was observed in PBMC apoptosis.

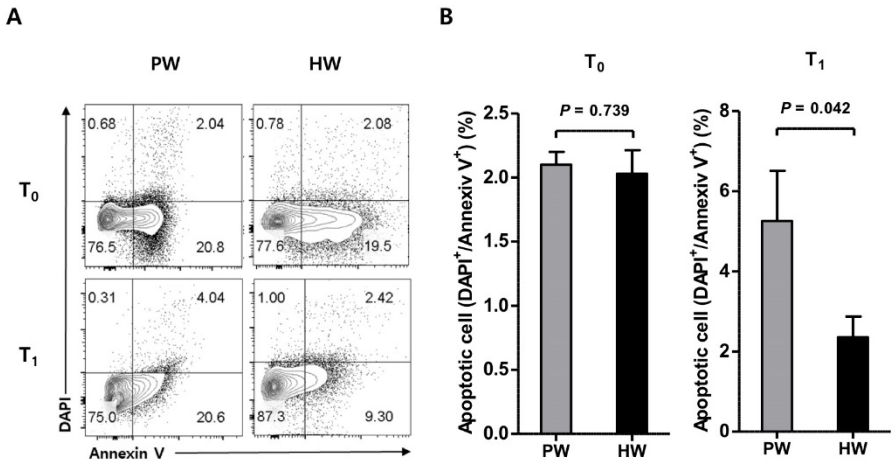

Figure 4. The percentage of PBMC apoptosis in resting state

(A) Representative flow cytometric data are displayed. **(B)** Data are presented as means ± SEMs. Significant differences between PW group (n = 18) and HW group (n = 18) were determined with the use of an unpaired t test. PW, plain water; HW, H_2-rich water; T_0, day 0 at rest (baseline); T_1, day 29 at rest.

2-3. Subpopulations of PBMCs

We analyzed the subpopulations of PBMCs with the antibodies of cell surface markers including CD4, CD8, CD20, CD14, and CD11b. After 4 weeks, PW and HW groups presented similar patterns of change in $CD4^+$ (for PW, Δ = -3.5 ± 4.8% and $P < 0.05$; for HW, Δ = -2.7 ± 3.5% and $P < 0.05$) and $CD8^+$ (for PW, Δ = -4.8 ± 2.1% and $P < 0.001$; for HW, Δ = -4.3 ± 2.7% and $P < 0.001$) cells, respectively (Table 3; Figure 5). In contrast, we observed a significant between-group difference in the frequency of $CD14^+$ cells ($P = 0.042$) (Table 3; Figure 5) as two groups showed opposite patterns of change: PW group showed the trend of increase (3.0 ± 11.9%), whereas HW group showed that of decrease (-0.7 ± 3.6%) (Table 3; Figure 5). No significant interaction [treatment (PW or HW) × age (<30 y or ≥30 y)] was observed in PBMC subpopulations including $CD4^+$, $CD8^+$, $CD20^+$, $CD14^+$, and $CD11b^+$ cells.

Table 3. Subpopulations of PBMCs in resting state[1]

	PW group (n = 18)			HW group (n = 18)			PW *vs.* HW
Cell type, %	T_0[2]	T_1	ΔT_1T_0[3]	T_0[2]	T_1	ΔT_1T_0[3]	ΔT_1T_0 P[4]
CD4	40.2 ± 9.0	36.7 ± 10.2	–3.5 ± 4.8*	37.6 ± 7.0	34.9 ± 8.0	–2.7 ± 3.5*	0.561
CD8	30.9 ± 6.6	26.1 ± 7.0	–4.8 ± 2.1***	32.9 ± 7.0	28.6 ± 6.5	–4.3 ± 2.7***	0.475
CD20	10.0 ± 3.2	11.0 ± 3.3	1.1 ± 3.0	9.0 ± 2.1	10.6 ± 2.9	1.5 ± 2.6	0.881
CD14	7.2 ± 3.6	10.2 ± 10.7	3.0 ± 11.9	5.6 ± 2.3	4.9 ± 3.0	–0.7 ± 3.6	0.042
CD11b	34.1 ± 6.6	36.8 ± 12.3	2.7 ± 13.6	31.6 ± 6.3	34.1 ± 10.6	2.5 ± 8.8	0.714

[1]All values are means ± SDs. Each percentage indicates the percent of live cells expressing the indicated cell surface marker. T_0, day 0 at rest (baseline); T_1, day 29 at rest; PBMC, peripheral blood mononuclear cell; PW, plain water; HW, H_2-rich water.

[2]There were no significant differences between PW and HW groups for all immune cell frequencies at T_0 on the basis of an unpaired *t* test.

[3]ΔT_1T_0 indicates the change from baseline at rest. Significant differences between T_0 and T_1 within each group were determined with the use of a one-way repeated measures ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001.

[4]P values were obtained with the use of a general linear model adjusting for the value at T_0 as a covariate.

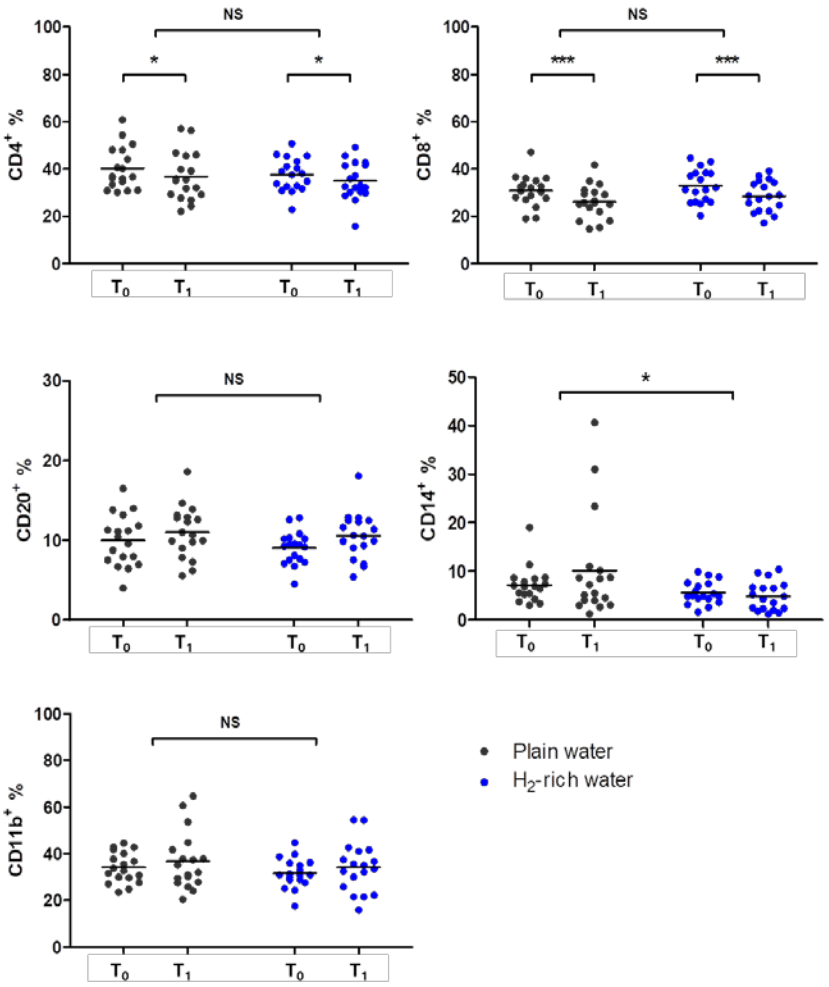

Figure 5. Subpopulations of PBMCs in resting state

Each line of a scattered dot plot indicates a mean value. Significant differences between T_0 and T_1 within each group were determined with the use of a one-way repeated measures ANOVA. Significant differences between two groups were determined with the use of a general linear model adjusting for the value at T_0 as a covariate. *P <0.05; ***P < 0.001; NS, not significant.

3. Effects of H_2-water in exercise-induced oxidative stress state

3-1. Antioxidant capacity and oxidative damages

We determined the effect of HW consumption on acute oxidative stress which was induced by the exercise protocol of treadmill test. There was no significant change in 8-OHdG in PW and HW groups even after the intense exercise (both $P > 0.05$) (Table 4). On the other hand, both groups showed significant increases in d-ROMs (for PW, Δ = 38.4 ± 14.5 CARR.U and $P < 0.001$; for HW, Δ = 35.5 ± 19.8 CARR.U and $P < 0.001$) and BAP (for PW, Δ = 161.8 ± 195.8 μmol/L and $P < 0.01$; for HW, Δ = 164.4 ± 239.0 μmol/L and $P < 0.01$) (Table 4). We observed no statistical between-group differences in the changes in 8-OHdG, d-ROMs, and BAP (all $P > 0.05$) (Table 4).

Table 4. Changes in antioxidant capacity and oxidative damage markers after acute oxidative stress [1]

	PW group (n = 18)			HW group (n = 20)			PW *vs*. HW
Measure	T_1	T_2	ΔT_2T_1[2]	T_1	T_2	ΔT_2T_1[2]	ΔT_2T_1 P[3]
BAP, μmol/L	2275.0 ± 394.5	2436.8 ± 382.9	161.8 ± 195.8**	2407.3 ± 303.6	2571.6 ± 230.5	164.4 ± 239.0**	0.972
d-ROMs, CARR.U	336.9 ± 54.8	375.3 ± 52.2	38.4 ± 14.5***	367.6 ± 63.1	403.1 ± 63.2	35.5 ± 19.8***	0.652
8-OHdG, ng/mL	1.04 ± 0.59	0.74 ± 0.56	–0.31 ± 0.78	0.73 ± 0.60	0.81 ± 0.90	0.07 ± 0.78	0.310

[1]All values are means ± SDs. T_1, day 29 at rest; T_2, immediately after the treadmill exercise on day 29; PW, plain water; HW, H_2-rich water; BAP, biological antioxidant potential; d-ROMs, derivatives of reactive oxygen metabolites; 8-OHdG, 8-Oxo-2-deoxyguanosine.

[2]ΔT_2T_1 indicates the change induced by the treadmill exercise on day 29. Significant differences between T_1 and T_2 within each group were determined with the use of a one-way repeated measures ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001.

[3]P values were obtained with the use of an unpaired t test or a Mann-Whitney U test depending on the distribution.

3-2. Subpopulations of PBMCs

Due to intense exercise stress, the frequencies of PBMC subpopulations were dramatically altered in PW group: the frequencies of $CD4^+$, $CD20^+$, and $CD14^+$ cells were significantly decreased (for $CD4^+$, Δ = -15.4 ± 4.8% and $P < 0.001$; for $CD20^+$, Δ = -4.0 ± 2.4% and $P < 0.001$; for $CD14^+$, Δ = -4.1 ± 6.3% and $P < 0.05$), whereas those of $CD8^+$ and $CD11b^+$ cells were significantly increased (for $CD8^+$, Δ = 3.8 ± 4.4% and $P < 0.01$; for $CD11b^+$, Δ = 18.4 ± 11.2% and $P < 0.001$) (Table 5; Figure 6). Although HW group had no statistical changes in the frequencies of $CD8^+$ and $CD14^+$ cells compared with those at T_1 (both $P > 0.05$), there were no statistical differences from PW group (all $P > 0.05$) (Table 5; Figure 6).

Table 5. Changes in PBMC subpopulations after acute oxidative stress[1]

	PW group (n = 18)			HW group (n = 18)			PW *vs.* HW
Cell type, %	T_1	T_2	ΔT_2T_1[2]	T_1	T_2	ΔT_2T_1[2]	ΔT_1T_0 P[3]
CD4	36.7 ± 10.2	21.3 ± 7.5	–15.4 ± 4.8***	34.9 ± 8.0	21.2 ± 5.6	–13.8 ± 4.8***	0.304
CD8	26.1 ± 7.0	29.9 ± 8.6	3.8 ± 4.4**	28.6 ± 6.5	30.0 ± 7.7	1.4 ± 3.3	0.072
CD20	11.0 ± 3.3	7.1 ± 2.1	–4.0 ± 2.4***	10.6 ± 2.9	6.6 ± 2.1	–4.0 ± 1.5***	0.993
CD14	10.2 ± 10.7	6.1 ± 8.2	–4.1 ± 6.3*	4.9 ± 3.0	3.1 ± 1.9	–1.8 ± 3.1	0.279
CD11b	36.8 ± 12.3	55.1 ± 10.8	18.4 ± 11.2***	34.1 ± 10.6	54.5 ± 9.8	20.4 ± 10.5***	0.586

[1]All values are means ± SDs. Each percentage indicates the percent of live cells expressing the indicated cell surface marker. T_1, day 29 at rest; T_2, immediately after the treadmill exercise on day 29; PBMC, peripheral blood mononuclear cell; PW, plain water; HW, H_2-rich water.

[2]ΔT_2T_1 indicates the change induced by the treadmill exercise on day 29. Significant differences between T_1 and T_2 within each group were determined with the use of a one-way repeated measures ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001.

[3]P values were obtained with the use of an unpaired t test or a Mann-Whitney U test depending on the distribution.

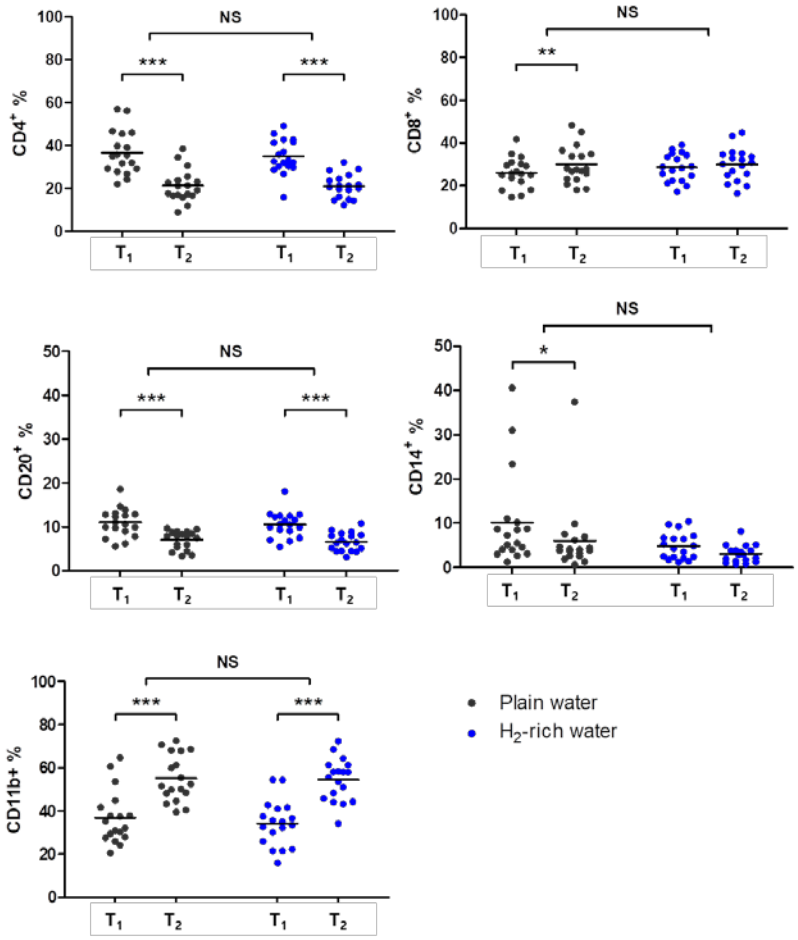

Figure 6. Changes in PBMC subpopulations after acute oxidative stress

Each line of a scattered dot plot indicates a mean value. Significant differences between T_1 and T_2 within each group were determined with the use of a one-way repeated measures ANOVA. Significant differences between two groups were determined with the use of an unpaired *t* test or a Mann-Whitney *U* test depending on the distribution. **P* <0.05; ***P* < 0.01; ****P* < 0.001; NS, not significant.

3-3. Transcriptome profiles of PBMCs

Next, we performed RNA sequencing analysis to determine the effect of HW consumption on global gene expression profiles under exercise-induced oxidative stress. Total 6 mRNA samples (3 samples per a group) were prepared and loaded on ISP beads. After amplifying the loaded samples, ISP beads were stacked to the Ion PI^{TM} chip. Loading density of the chip was 95% (Figure 7). The sequencing was successfully conducted and summary of the results of RNA sequencing is presented in Table 6.

As shown in Figure 8, principal component analysis (PCA) showed that HW group had a clearly classified transcriptome profile compared with PW group. We determined differentially expressed genes (DEGs) among the total transcripts with the statistical criteria (fold-change >5 or <-5; $P < 0.01$) and consequently, a total of 605 DEGs were discovered. As a result of hierarchical clustering analysis of DEGs, we readily observed that DEG profiles of two groups were obviously distinguishable as shown in Figure 9. Next, we conducted the biological functional category analysis with the use of IPA to gain further insight about these DEGs. HW consumption-altered biological functional categories which were ranked within top 5 were as follows; inflammatory response, immune cell trafficking, hematological system development and function, infectious diseases and immunological disease (Figure 10). Accordingly, we focused on the first-ranked category of inflammatory response and examined the expression levels of genes related to nuclear factor-kappa B (NF-κB)-mediated signal transduction: key genes including TLR1, TLR2,

TLR4, TLR6, TLR7, TLR8, TLR9, MYD88, NFKB1, NLRP12, MAP3K1, FOS, and RELB were significantly less expressed in HW group (Figure 11). Also, we compared the expression level of genes encoding pro-inflammatory cytokines and their receptors as well as those known to be responsive to NF-κB. Consequently, we observed that expression levels of IL1B, IL8, IL6R, and TNFRSF10B were significantly lower in HW group (Figure 12). As shown in Figure 13, furthermore, HW consumption influenced the expression of genes related with ROS secretion, synthesis, and production.

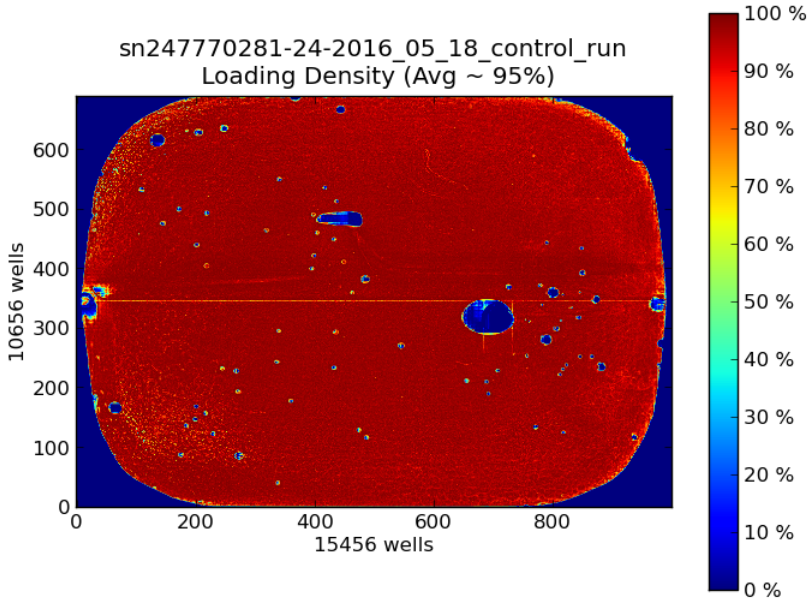

Figure 7. Loading density of ISP beads on the Ion PI™ chip

Colors indicate the degree of loading density of the chip.

Table 6. Summary of results from RNA sequencing

Group	Sample ID	Total reads	Aligned reads	Percent aligned	Mean read length (bp)	Genes Detected
PW	011	17,517,814	17,026,727	97.20%	98.4	16,207
	008	17,423,847	17,076,945	98.01%	126	16,720
	010	16,300,405	16,021,170	98.29%	128.5	16,046
HW	009	15,443,495	15,180,179	98.30%	103	15,759
	007	15,537,600	15,274,351	98.31%	136.5	16,539
	006	16,754,653	16,473,151	98.32%	112.7	16,076

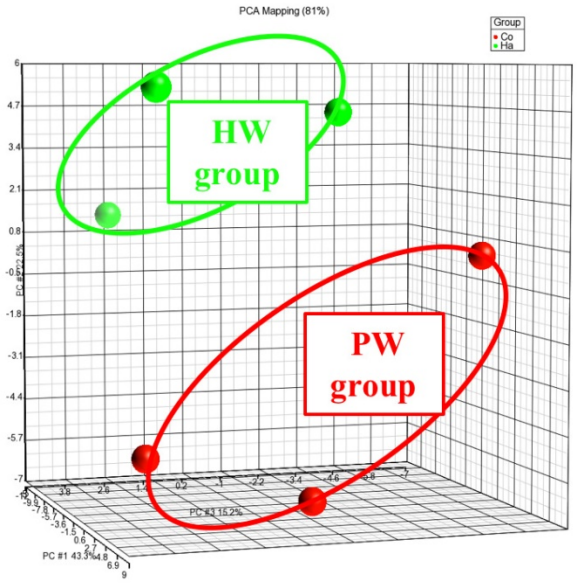

Figure 8. 3-D score plot of principal component analysis (PCA)

Each sphere on the plot represents an individual participant randomly selected.

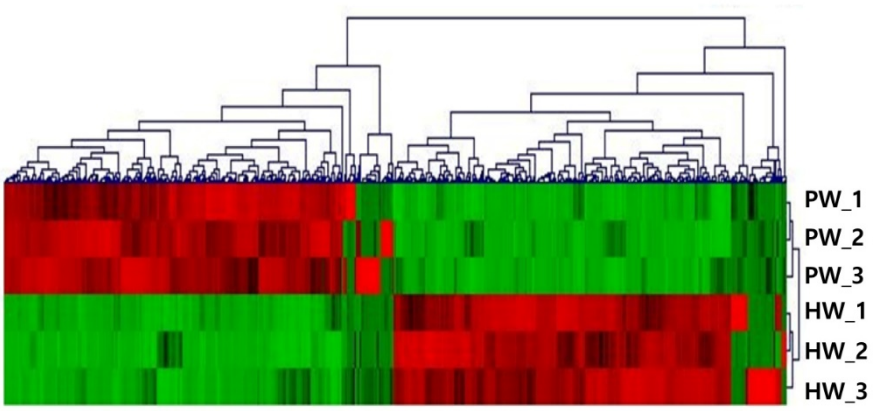

Figure 9. Hierarchical clustering analysis of PBMC transcriptome

The heatmap shows differentially expressed genes in PBMC transcriptome. Each column and row respectively represents an individual participant and a single gene. The red color indicates up-regulated genes, whereas the green color indicates down-regulated genes.

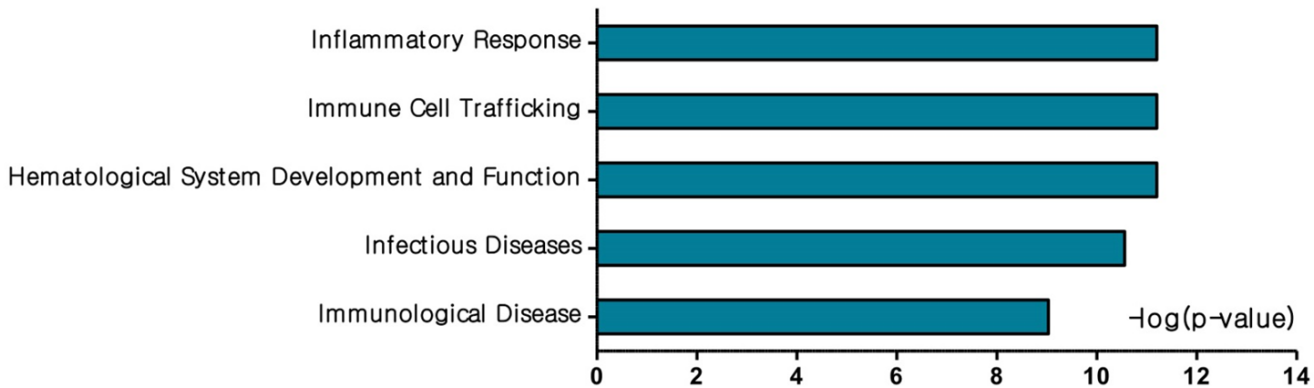

Figure 10. Biological functional categories by IPA

Top 5 biological functional categories were discovered with IPA of DEGs. Statistical significance was calculated by the Fisher's exact test and noted as a log(p-value).

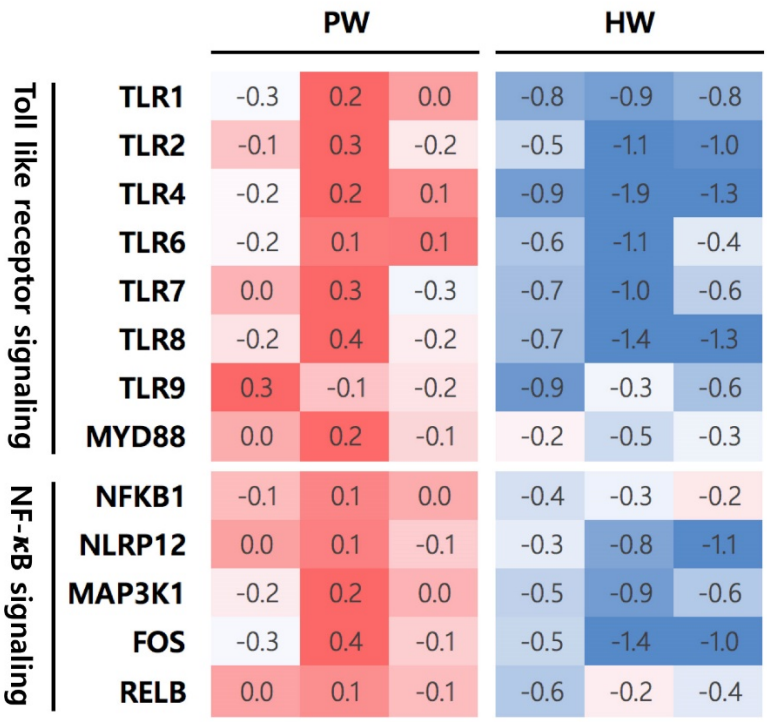

Figure 11. Heatmap of expression levels of key genes related with toll like receptor and NF-κB signaling

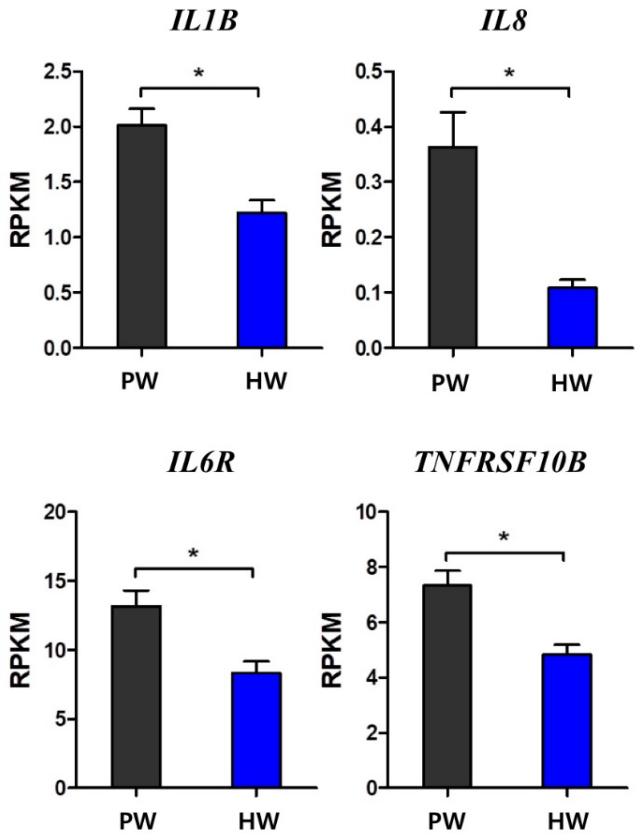

Figure 12. Expression levels of IL1B, IL8, IL6R and TNFRSF10B

HW group presented the lower expression levels in NF-κB responsive genes including IL1B, IL8, IL6R and TNFRSF10B, compared with PW group. PW, plain water; HW, H_2-rich water; RPKM, reads per kilobase million.

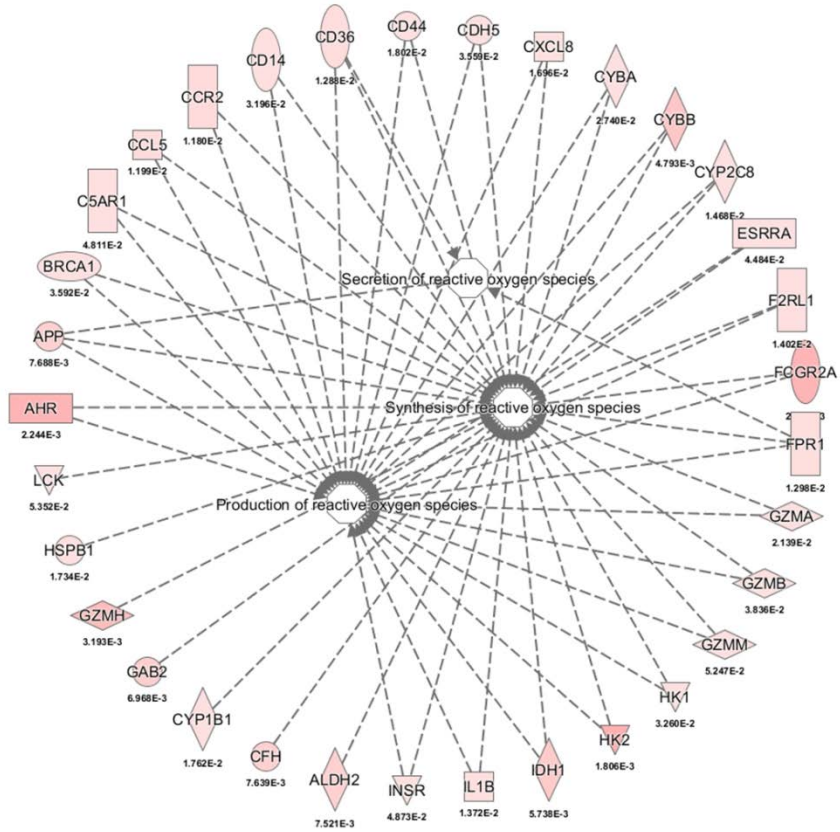

Figure 13. Differentially expressed genes involved in secretion, synthesis, and production of reactive oxygen species

Among differentially expressed genes, the genes related with reactive oxygen species secretion, synthesis, and production are presented. Each value under the gene symbol indicates a *p*-value.

IV. Discussion

The purposes of this study were to investigate the effects of HW consumption on antioxidant status and oxidative stress and on subsets and transcriptome of PBMCs in healthy adults and to determine the effects of HW consumption under the two different circumstances: in resting state and exercise-induced oxidative stress state. In the state of rest, HW group showed the increase in the serum BAP (aged ≥30 y) and the decreases in apoptosis and frequency of peripheral $CD14^+$ PBMCs compared with PW group. Under exercise-induced oxidative stress, the transcriptional response of HW group was clearly distinguished from PW group and HW consumption resulted in the significant down-regulation in the NF-κB-mediated signaling pathway.

It is important for cells to maintain a balance between oxidants and antioxidants for performing their functions normally. When the balance is collapsed because of the excessive amounts of oxidants or the decline of antioxidant capability, a state of oxidative stress results in the disruption of redox signaling and control (Sies, 2015b). ROS, strongly affecting this balance, are natural by-products of normal cell metabolism such as the mitochondrial aerobic respiration, and also can be produced by the environmental stimuli like pollutants, smoking, infections and ultraviolet radiation (Lobo *et al*., 2010). Although having vital roles including cell signaling (Schieber and Chandel, 2014), ROS can cause cellular injuries and aging when they are not eliminated

properly by enzymes or dietary antioxidants (Reuter *et al*., 2010, Finkel and Holbrook, 2000). To deal with ROS, several enzymes are involved in the biological antioxidant system. For example, superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anion (O_2^-) into oxygen and hydrogen peroxide (H_2O_2). Also, glutathione peroxidase (GPx) and catalase (CAT) react with the hydrogen peroxide to form water and oxygen harmless to body (Lobo *et al*., 2010, Noori, 2012). However, uncontrollably increased hydrogen peroxide can be converted rapidly to hydroxyl radical (OH•) via the Fenton reaction mediated by the transition metals (Reuter *et al*., 2010). Furthermore, some external stimulation such as the exposure to ionizing radiation can make intracellular water lose an electron and consequently generate the hydroxyl radical (Riley, 1994). The hydroxyl radical is known as the most reactive form among ROS and have the great responsibility for the oxidation of the biomolecules such as lipids and DNA (Filippin *et al*., 2008). Given the fact that there is no intrinsic enzymatic mechanism to eliminate the hydroxyl radical instantly, ingestion of the dietary antioxidants having the capacity to neutralize it is highly demanded.

In this regard, molecular H_2 has been noticed because its antioxidant effect is based on the ability to selectively scavenge the specific oxidant such as hydroxyl radical (Ohsawa *et al.*, 2007). Accordingly, we assessed the protective effect of H_2 from lipids and DNA oxidation by the radicals measuring d-ROMs and 8-OHdG, respectively, in the serum samples. Although the d-

ROMs test mainly detects the organic hydroperoxides primary products of lipid peroxidation (Costantini, 2016), consumption of HW for 4 week did not lower the concentration of serum d-ROMs. Inconsistent with this study, the uncontrolled clinical work revealed that the patients with potential metabolic syndrome showed a significant decrease in serum malondialdehyde, another marker of lipid peroxidation, after 10-week HW consumption (Song *et al*., 2013). In addition, the previous placebo-controlled trial showed that 6-week HW consumption mitigated the radiotherapy-induced elevation of d-ROMs (Kang *et al*., 2011). It was noteworthy that the concentration of 8-OHdG, an indicator of oxidative stress on DNA (Valavanidis *et al*., 2009a), decreased by half in HW group after the intervention, confirming the previous report that patients with rheumatoid arthritis showed a significant reduction in urinary 8-OHdG after the intake of 530 mL/d of HW for 4 week (Ishibashi *et al*., 2012). However, HW consumption did not show the extra antioxidant effect on DNA oxidation compared with PW consumption. In addition to the effect on the specific biomolecules, we sought to examine the impact of HW consumption on the total antioxidant capability by measuring serum BAP. The BAP test is based on measuring the ferric iron-reducing ability of the antioxidants in the sample (Kim *et al*., 2014). Although the main rationale for the antioxidant effect of H_2 is derived from the ability of the molecule to scavenge the certain radicals (Ohsawa *et al*., 2007), the previous study revealed that H_2-dissolved water exhibited the greater ability to reduce ferric iron when it was compared with pure water or tap water (Kato *et al*., 2015). Therefore, we had anticipated

that HW consumption would contribute to the increase in BAP. Indeed, we observed that the BAP significantly increased after HW consumption. However, HW group showed no statistical difference from PW group, which was inconsistent with the previous study that reported a maintained blood BAP in the radiotherapy treated-patients compared to those receiving placebo water (Kang *et al.*, 2011).

Aging is generally characterized by a state where systemic oxidative stress is elevated indicating the elevation in redox balance and the accumulation of oxidative damages (Kregel and Zhang, 2007). Therefore, we categorized all the participants into two age group based on age 30, and compared the older age group (≥30 y; n = 18) with the younger age group (<30 y; n = 20) regarding the oxidative stress-related indicators at baseline including BAP, d-ROMs and 8-OHdG. The measures of the younger age group in BAP, d-ROMs and 8-OHdG were 2125.8 ± 266.8 μmol/L, 355.2 ± 61.0 CARR.U, and 1.72 ± 0.87 ng/mL, respectively, and the older age group had those values as 2062.5 ± 189.9 μmol/L in BAP, 377.4 ± 78.1 CARR.U in d-ROMs, and 2.39 ± 1.30 ng/mL in 8-OHdG. Even though no statistical differences were observed between the two age groups, the older age group showed the trend of higher oxidative stress. Therefore, we hypothesized that the effect of HW consumption on antioxidant status and/or oxidative stress level might be different depending on the age group. We performed the subgroup analysis on all markers measured in resting state, and observed the significant interaction

between the effects of age and HW consumption on BAP showing the greater improvement of antioxidant capacity in the participants aged ≥30 y. As a result, the participants in the older age group may have been more susceptible to the antioxidant effect of HW consumption because they had a larger capacity for improvement.

Apoptosis is one of the consequences of excessive ROS generation (Kannan and Jain, 2000). As the mitochondrial respiratory chain is the major source of endogenous ROS, mitochondrial DNA, proteins and lipids are susceptible to attack by ROS (Ott *et al*., 2007). Oxidative stress in mitochondria is a major cause leading to the programmed cell death, and apoptosis is regulated by mitochondrial pro-apoptotic factors including cytochrome c, apoptotic protease-activating factor-1, and procaspase-9 (Kannan and Jain, 2000, Sinha *et al*., 2013). These factors are released from mitochondria into cytosol and ultimately activate downstream caspases which lead to apoptotic cell death (Sinha *et al*., 2013, Simon *et al*., 2000). Excessive destruction of normal cells mediated by these apoptotic pathways constitutes a major cause of aging (Kujoth *et al*., 2005), diabetes (Wali *et al*., 2013) and neurodegenerative diseases (Ozawa *et al.*, 1997). In this respect, we expected that ingestion of H_2 could contribute to protecting the peripheral cells from apoptosis by lowering cellular oxidative stress. Indeed, HW group showed the significantly lower percentage of PBMC apoptosis at week 4 compared with PW group, which apparently indicated that HW consumption was effective in ameliorating the

severe cellular damages. Even though the mechanisms by which H_2 decreased the PBMC apoptosis were not fully understood, it is assumed that the molecule may have directly affect the ROS balance by scavenging the radicals generated in the cell because it has small size and low molecular weight enough to diffuse across the cellular membrane and enter intracellular compartments (Ohta, 2015). In addition, it has been reported that H_2 is involved in regulation the gene expression related to the apoptotic pathway. Inhalation of H_2 gas significantly up-regulated the anti-apoptotic genes including B-cell lymphoma-2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xL) in the I/R-induced rats (Kawamura *et al*., 2010). In the rat model of hypoxia-ischemia brain injury, one of the major cause of neuronal cell death, H_2 therapy suppressed the expressions of caspase-3 and -12 which induce apoptosis (Cai *et al*., 2008). Uncontrolled clinical trial conducted in the patients with potential metabolic syndrome also indirectly demonstrated the anti-apoptotic effect of HW consumption showing the enhancement in HDL function of protecting endothelial cells from the apoptosis induced by tumor necrosis factor (TNF)-α (Song *et al*., 2013). The decrease in PBMC apoptosis in the current study may have linked with the result that HW consumption also decreased the frequency of CD14 positive PBMCs. CD14 is mainly expressed on the surface of human circulating monocytes (Yang *et al*., 2014) and the previous report demonstrated that CD14 positive monocytes were strongly migrated by damaged cells which were exposed to sublethal oxidative stress (Geiger-Maor *et al*., 2012). Considering the fact that monocytes are recruited to phagocytose

the dying cells (Mikołajczyk *et al*., 2009), the decrease in apoptotic cells by HW consumption consequently may have resulted in the reduction of the monocyte frequency. In summary, the effects of HW intake in resting state included the significant improvement in the antioxidant capacity compared with PW consumption, although it was evident only in those aged ≥30 y. Also, HW consumption decreased the PBMC apoptosis and consequently induced the decrease in the percentage of peripheral monocytes.

Exercise has a powerful influence on antioxidant defense system and oxidative stress level. Various tissues actively produce ROS during exercise, and it has been proposed that the moderate levels of cellular ROS are essential for force production in the skeletal muscle (Powers and Jackson, 2008) and muscle adaptation to exercise training (Powers *et al*., 2010). However, high levels of ROS induced by exercise and the consequence of oxidative stress are responsible for the oxidative damage and muscle fatigue (Powers and Jackson, 2008, Powers *et al*., 2011). Numerous studies have supported exercise-induced oxidative stress by showing an increase in lipid peroxidation, a redox change of endogenous antioxidant such as glutathione and accumulation of protein carbonyls (Fisher-Wellman and Bloomer, 2009, Powers *et al*., 2011). It is obvious that antioxidant enzymatic activities and antioxidant capacity are also influenced by the strenuous physical work, although the results of change patterns are conflicting (Fisher-Wellman and Bloomer, 2009). The results of our study indicated that oxidative stress was successfully induced as intended,

given that PW group showed a significant increase in d-ROMs and BAP confirming the previous studies reporting the exercise-induced increases in lipid peroxidation and total antioxidant status (Sacheck *et al*., 2003, Vider *et al*., 2001). In agreement with our result, the majority of previous studies have shown no alteration in 8-OHdG measured immediately after the short-duration exhaustive exercise (Fisher-Wellman and Bloomer, 2009). Unfortunately, 4-week HW consumption did not alleviate the increased lipid peroxidation and not elicit the further elevation in antioxidant capacity. The previous placebo-controlled study also failed to discover the superiority of HW consumption against exercise-induced oxidative stress when those markers were investigated (Aoki *et al*., 2012).

In addition to elevation of oxidative stress, exercise stress induces profound changes in peripheral cells. The changes are characterized by an immediate influx of lymphocytes to bloodstream because exercise induces an increase in the catecholamine level which makes lymphocytes detached from the vascular endothelium (Turner *et al*., 2011, Murray *et al*., 1992). As a result, phenotypic composition of peripheral cells is also affected by exercise (Pedersen and Hoffman-Goetz, 2000). These changes were also observed in our study: in response to treadmill exercise lasting for 15-20 min, the number of total PBMC was doubled, and the percentage of each PBMC subset was dramatically altered in PW group. Comparing with PW consumption, however, we observe no significant effect of 4-week HW consumption on the frequen-

cies of PBMC subsets in the condition of oxidative stress. In contrast to this, transcriptome profiles of PBMCs were obviously classified between PW and HW groups and included a total of 605 differentially expressed genes. To gain a further insight, we investigated which biological functional category was most associated with these differentially expressed genes. Interestingly, HW consumption had the greatest influence on the biological functional category of inflammatory response. Nuclear factor-kappa B (NF-κB), a family of transcription factors, mediates the expression of a large array of genes regulating inflammatory responses (Lawrence, 2009, Liu *et al*., 2017). Accordingly, we investigated if HW consumption had influenced on the expression levels in 84 key genes closely related to NF-κB-mediated signal transduction. Consequently, gene expression profile consisting of TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, MYD88, NFKB1, NLRP12, MAP3K1, FOS, and RELB was characterized by a significant down-regulation in HW group. Particularly, we observed that HW group had the lower expression levels in NF-κB responsive genes including IL1B, IL8, and TNFRSF10B compared with PW group (Hiscott *et al*., 1993, Kang *et al*., 2007, Kunsch and Rosen, 1993). Although there was no significant difference between groups in the expression level of IL6 coding a potent pro-inflammatory cytokine interleukin (IL)-6, HW group showed the significantly lower expression level in IL6R which encodes the receptor for IL-6 (Libermann and Baltimore, 1990, Schwantner *et al*., 2004). These findings suggest that down-regulation in NF-κB-mediated signal transduction may have contributed to amelioration of inflammatory response of

HW group under exercise-induced oxidative stress. Although we could not find the human study which have directly shown the positive effect of H_2 on inflammation, we found several studies with inflammation-induced animal models reporting that H_2-rich saline or H_2-rich water decreased the levels of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α (Wang *et al*., 2011, Zhang *et al*., 2011). Also, previous study has reported the H_2-induced suppression of NF-κB-regulated genes through the investigation of global gene expression in the healthy mouse liver and they also identified that H_2 attenuated the NF-κB activation (Sobue *et al*., 2015). ROS are thought to be involved in inflammation because high levels of ROS are generally observed in several human diseases characterized by chronic sterile inflammation such as neurodegenerative diseases, atherosclerosis, diabetes mellitus, and Crohn's disease (Reuter *et al*., 2010). Oxidative stress is implicated in triggering inflammatory responses in that excessive ROS promote the oxidation of lipids, proteins, and DNA which immune cells recognize as unsafe (Reuter *et al*., 2010, Hussain *et al*., 2016). Also, ROS are concerned with activating redox-sensitive proteins and transcription factors including mitogen-activated protein kinase (MAPK) and NF-κB which increase the expression of pro-inflammatory genes (Yoshikawa and Naito, 2002). Mitochondrial ROS have been reported to act as signaling molecules to trigger the production of pro-inflammatory cytokines such as IL-1 and IL-6 by influencing the transcriptional pathway (Naik and Dixit, 2011). Given the close association between ROS and inflammatory responses, the abilities of H_2 to improve the antioxidant capacity and reduce

the oxidative damage may have resulted in the significant change in the inflammation-related transcriptional responses. In respect to the anti-inflammatory effect of H_2, previous report has proposed the novel pathway by which molecular H_2 regulates the gene expression (Iuchi *et al*., 2016). Autoxidation of lipids by free radicals generates oxidized phospholipids which mediate Ca^{2+} signaling. They revealed that molecular H_2 contributed to generation of the modified phospholipid which appeared to act as an antagonist of oxidized phospholipids. Production of the antagonist by H_2 resulted in a decline in Ca^{2+} signaling and Ca^{2+}-dependent nuclear factor of activated T cells (NFAT) pathway which induces the production of pro-inflammatory cytokines (Iuchi *et al*., 2016). However, it is unclear if the proposed-pathway can be applied to understand the down-regulation in NF-κB-mediated signal transduction observed in our study. Our study may contribute to understanding the transcriptional regulation of H_2 attenuating inflammatory responses in the condition of intense oxidative stress, but further studies are demanded to discover the detailed and rational mechanisms by which H_2 regulates the gene expression in human.

To our knowledge, this is the first randomized, double-blind, placebo-controlled trial to demonstrate the extensive effects of HW consumption on oxidative stress, immune cell population, and transcriptional responses. To present the reliable and comprehensive findings in respect to the effects of HW consumption on acute oxidative stress, we used the standardized protocol

of the treadmill test and adopted the high-throughput RNA sequencing analysis which is a powerful tool for exploring global gene expression profile. On the other hand, this study had some limitations as well. If the negative control group (those who consumed very little water for the intervention periods) had been included in the study, the effect of H_2 would have been assessed more evidently. Additionally, the intervention period of the current study was relatively short in comparison with the previous placebo-controlled trials (Ito *et al*., 2011, Kajiyama *et al*., 2008, Kang *et al*., 2011). Nevertheless, the current findings cannot be overlooked as they suggest that HW consumption has a potential of exhibiting the antioxidant and immune modulating effects in healthy population.

In conclusion, HW consumption reduces oxidative stress by improving antioxidant capacity, which leads to the decreases in the cellular damage and the frequency of circulating monocytes. In the condition of acute oxidative stress, 4-week HW consumption reduces the inflammatory response by down-regulating NF-κB-mediated signal transduction and NF-κB responsive genes. These findings suggest that H_2 administration exhibits the antioxidant effect in healthy population, and our study may contribute to understanding the transcriptional regulation by which H_2 exhibits the anti-inflammatory effect against intense oxidative stress.

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국문초록

수소수 섭취가 인체 내 산화 스트레스와 말초혈액단핵구 분포 및 전사체에 미치는 영향 : 무작위 배정 이중 맹검 대조 연구

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심 민 주

산화 스트레스는 과도한 활성 산소종의 생성 혹은 체내 항산화 능력의 저하로 인해 oxidant와 antioxidant 사이의 균형이 깨진 상태를 가리키며, 다양한 질병의 발생 원인으로 작용할 수 있다. 최근에 수소 분자는 새로운 항산화제로서 주목 받고 있으며, 다양한 질병에 대한 수소의 긍정적인 효과가 동물 실험 및 임상 연구를 통해 보고되었다. 하지만 건강한 사람에 대한 수소의 효과 및 수소가 미치는 체내 전반적인 영향을 조사한 연구는 매우 미비한 실정이다. 따라서 본 연구는 건강한 사람을 대상으로 한 중재 연구를 통해, 수소수의 섭취가 항산화 능력과 면역 세포의 프로파일 및 말초혈액단핵구의 전사체에 미치는 영향을 종합적으로

규명하고자 하였다. 또한, 연구 참여자가 안정 상태일 때와 운동을 통한 고강도의 산화 스트레스에 노출된 상태일 때의 두 가지 조건에서 수소수의 효과를 조사하였다. 38명의 건강한 성인 남녀가 무작위 배정, 이중 맹검, 플라시보-대조 디자인의 중재 연구에 참여하여 하루 1.5 리터의 정수 혹은 수소수를 총 4주간 섭취하였다. 연구 참여자가 안정 상태일 때, 산화 스트레스 지표로서 biological antioxidant potential (BAP), derivatives of reactive oxygen metabolites (d-ROMs), 8-Oxo-2'deoxyguanosine (8-OHdG)를 측정하였고, FACS 분석을 통해 말초혈액단핵구의 분포 및 아폽토시스 비율을 조사하였다. 중재가 끝난 4주차에는 약 20분간 진행되는 트레드밀 운동을 실시함으로써 연구 참여자에게 고강도의 산화 스트레스를 유도한 후, 산화 스트레스 정도와 말초혈액단핵구의 분포를 조사하고, RNA 시퀀싱을 통해 전사체 프로파일링을 수행하였다. 본 연구 결과, 수소수군에서 4주 후 BAP의 유의한 증가가 관찰되었으며(30세 이상의 연구 참여자), 말초혈액단핵구의 아폽토시스 비율 및 $CD14^+$ 세포의 비율이 대조군과 비교하여 유의하게 낮은 것을 확인하였다. 운동을 통해 급격한 산화 스트레스가 유도된 조건에서, 수소수군은 산화 스트레스 지표 및 말초혈액단핵구의 분포에서 대조군과 유의한 차이를 나타내지 않았다. 그러나 말초혈액단핵구의 전사체를 프로파일링한 결과, 수소수군은 염증반응을 유도하는 NF-κB signal-

ing pathway가 대조군과 비교하여 유의하게 down-regulation 되었음을 관찰하였다. 또한, NF-κB에 의해 발현이 유도되는 IL1B, IL8, IL6R 및 TNFRSF10B의 발현량이 대조군보다 유의적으로 낮게 나타났다. 결론적으로, 건강한 성인의 4주간의 수소수 섭취는 항산화 능력의 향상과 함께 세포의 아폽토시스 감소와 혈중 단핵구의 감소를 유도함을 확인하였다. 또한, 수소수의 섭취는 고강도의 산화 스트레스 상황에서 NF-κB signaling pathway의 발현에 영향을 미침으로써 염증 반응을 완화하였다. 본 연구는 수소수의 섭취가 건강한 사람에서도 항산화 능력을 증진시킬 수 있다는 가능성을 제시하였으며, 더 나아가 고강도의 산화 스트레스 상황에서 수소가 나타내는 항 염증 효과의 분자 생물학적 메커니즘을 규명하였다는 점에서 그 의의가 있다.

주요어 : 항산화제, 산화 스트레스, 수소수, 전사체 프로파일링, 말초혈액단핵구, 염증 반응

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