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ORIGINAL PAPER

The balneotherapy effect of hydrogen reduced water on UVB-mediated skin injury in hairless mice

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Abstract Exposure to UVB radiation induced skin damage that results to increase risk of skin cancer. Despite the clinical importance of skin-induced damage, antioxidants imposed limited therapeutic success. Hydrogen molecule (H_2) has been known as a safe antioxidant in the prevention and therapeutic approach towards several diseases. Drinking hydrogen reduced water (HRW), inhalation of hydrogen gas, and injecting H_2-dissolved saline are widely accepted to incorporate H_2 in the body. However, there is no document about the beneficial effect of hydrogen water bath. Here, we investigated the effect of hydrogen bathing on the UVB-induced skin damage in hairless mice. For this, mice of the bathing group are allowed to freely swim on HRW, and let the HRW penetrate for 60 mins. Scoring of skin injury, reactive oxygen species (ROS) enzyme activity quantification, cytokine analysis, and ultrastructural change of corneocytes were measured after exposure to UVB radiation of 360-540 mJ/cm^2. In summary, the bathing with HRW significantly reduced the levels of skin damage, as well as increased activity of glutathione peroxidase. Further, the effect of HRW on cytokine network in the skin after UVB exposure revealed that HRW significantly decreased the level of inflammatory cytokines such as IL-1β, IL-6, TNF-α and IFN-γ. Finally, scanning electron microscopy data revealed low number of defected corneocytes and ultrastructural changes, suggesting that HRW bathing would protect UV-induced cell damage.

Keywords Antioxidant, Hydrogen molecule, Hydrogen reduced water, Skin damage, Bathing

Introduction

Skin is easily damaged by UV light as it is directly exposed. Among the types of solar radiation, UVB light corresponds to the most inducing damage to skin that is detrimental[1]. UVB exposure of the skin accounts to a number of biological effects such as damaged to skin cells and proteins, induced inflammation due to production of cytokines by keratinocytes and damage to DNA that leads to skin cancer[2].

Administration of antioxidant and topical application for scavenging ROS has been studied to lessen the skin damage mediated by UV exposure[2]. Hydrogen molecule (H_2) has been promoted to have a number of advantages as antioxidant[3]. Unlike other antioxidants which are unable to target organelles, H_2 has overcome this limitation through favorable distribution inside the cell. H_2 can penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus[4]. Moreover, H_2 has been reported to have OH-scavenging activity in culture medium[4]. Hydrogen reduced water (HRW) has high content of hydrogen that might be usable to treat UVB-mediated skin damage in hairless mice owing to its antioxidant and anti-inflammatory effect[5,6]. Drinking hydrogen reduced water, inhalation of hydrogen gas, and injecting H_2-dissolved saline are widely accepted to incorporate H_2 in the

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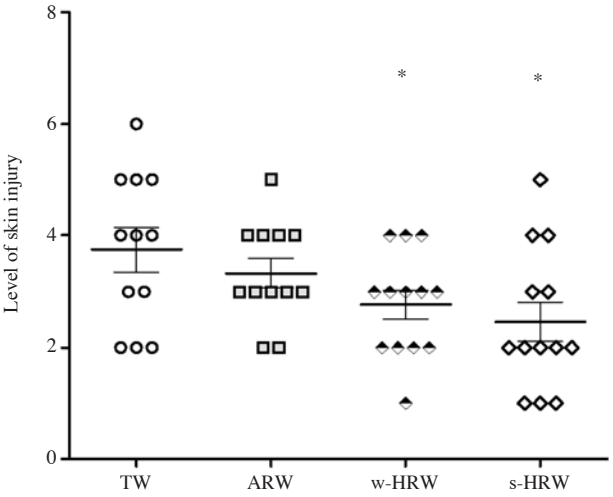

Figure 1. Levels of Skin Injury. Scoring of skin injury after UVB exposure and bathing. The grades were divided into 6 steps according to the degree of injury through observation by naked eyes, 6 being the highest (most damage) and 1 being the lowest (lesser extent of UVB mediated damage). Data were presented as mean ± S.D. *$P < 0.05$ vs TW bathing group.

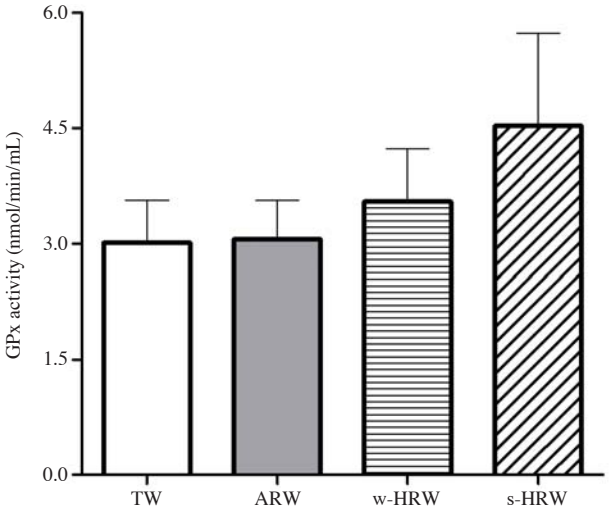

Figure 2. Effect of HRW-bathing on endogenous skin enzyme (Glutathione Peroxidase). Mice were simultaneously irradiated with UVB and bathed with tapwater, TW; alkaline reduced water, ARW; weak hydrogen reduced water, w-HRW and strong hydrogen reduced water, s-HRW. Data are mean ± S.D, n=12. Tukey's test was used for *post hoc* tests.

body, however hydrogen water bath is a new approach of incorporating hydrogen in the body. In this study, we investigated the effect of HRW-bathing against UVB-induced skin damage in hairless mice through scoring of skin injury, white blood cells counting, glutathione peroxidase activity, cytokine profiling, and histological examination of corneocytes. We found that HRW-bathing ameliorates the UVB-induced skin damage through enhancing endogenous enzyme activity, and influencing cytokine production.

Scoring of skin injury

Skin of hairless mice were assessed for the degree of injury. Skin injury scores of group bathed with HRW showed significantly lower ($P < 0.05$) compared to the tap water and ARW bathing groups (Figure 1). The degree of damage revealed this following pattern from least damaged to severe injured skin: s-HRW-bathing group < w-HRW-bathing group < ARW bathing group < TW-bathing group. Collectively, UVB-mediated skin injury of HRW bathing groups revealed significantly reduced damage, which means protecting effect of HRW against the adverse effect caused by UVB exposure.

WBC and differential cell counting

The number of lymphocyte of s-HRW bathing group significantly increased ($P < 0.05$), while that of basophil of HRW-bathing groups were significantly decreased [s-HRW ($P < 0.01$), w-HRW ($P < 0.001$)] compared to the TW and ARW bathing groups. The total WBC, neutrophils and monocytes had no significance among the groups (Table 2).

Glutathione peroxidase (GPx) activity

H_2O_2 scavenging capacity in plasma was measured by the principles of glutathione peroxidase (GPx) activity decomposing H_2O_2 induced by UVB exposure. GPx activity of both HRW-bathing groups namely s-HRW and w-HRW showed higher than that of TW and ARW-bathing groups although the result showed statistically insignificant (Figure 2).

Bio-plex cytokine assay

Comparing cytokine levels among bathing groups, HRW-bathing groups (s-HRW and w-HRW) showed significantly low level of proinflammatory cytokines (IL-1β, IL-6, IL-12p70, and TNF-α) compared to the TW and ARW bathing groups (Figure 3). In addition, the IFN-γ concentration of weak HRW-bathing group was significantly decreased ($P < 0.05$) compared to the other three bathing groups. However, the level of the anti-inflammatory cytokine such as IL-10 of the weak HRW-bathing group showed the same concentration with the TW-bathing group (control). Consistent with our previous studies, ARW-bathing group showed significantly increased anti-inflammatory cytokines ($P < 0.05$) compared to the TW and HRW-bathing groups.

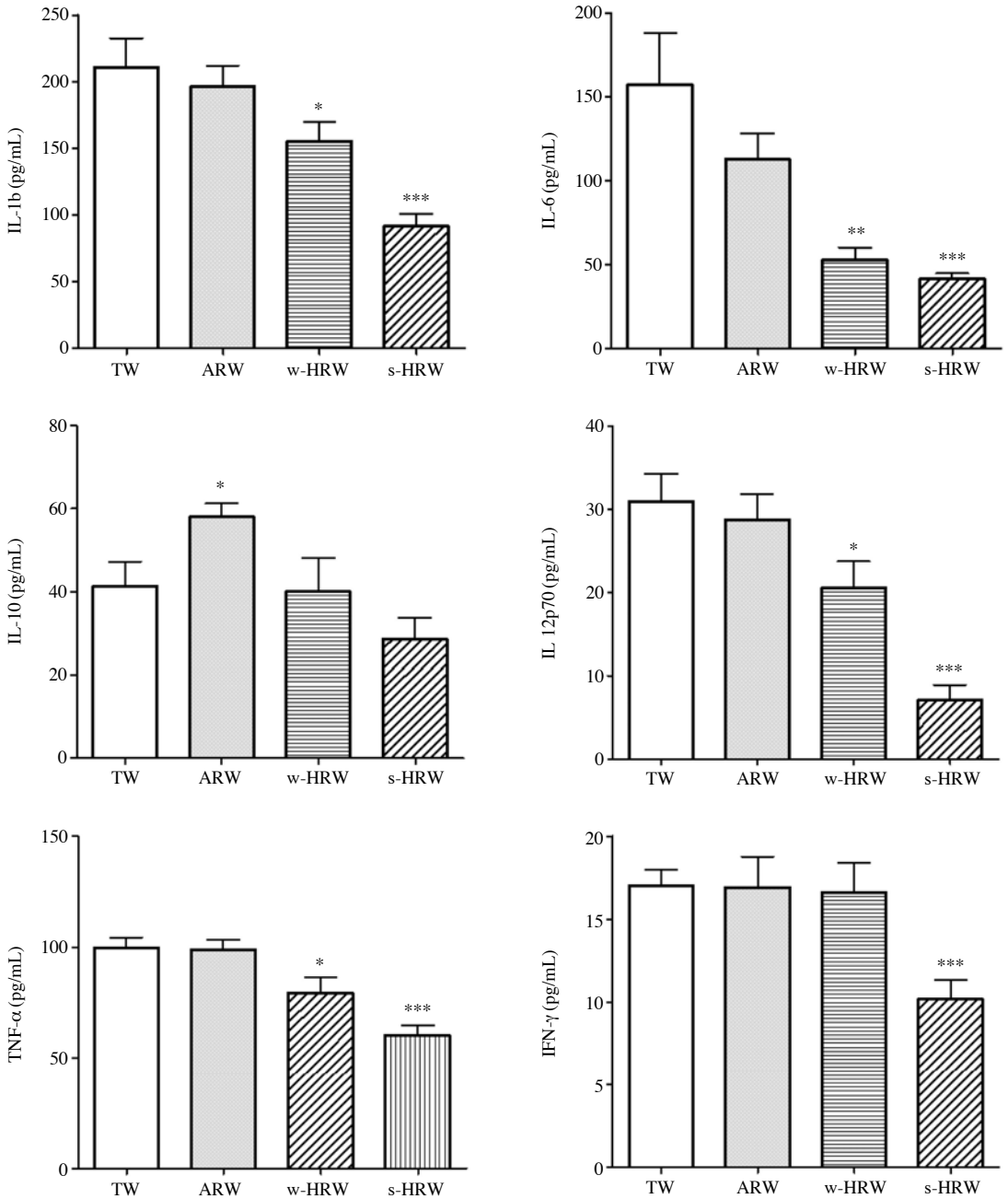

Figure 3. Cytokine profiling using Bio-Plex analysis. Hydrogen reduced water both weak and strong showed reduced IL-1β, IL-6, IL-12p70, TNF-α compared to tapwater (TW) and alkaline reduced water (ARW) bathing groups. Data are mean ± S.D., n=12. $*P<0.05$, $**P<0.01$, and $***P<0.001$ indicate significant differences with ANOVA. Tukey's test was used for *post hoc* tests.

Ultrastructural changes of dorsal skin surface by SEM

Scanning electron microscopy (SEM) images of the dorsal skin surface revealed higher number of defective corneocytes on TW-bathing group compared to the HRW bathing groups (Figure 4).

Discussion

It has been demonstrated previously that UVB exposure can lead to increased secretion of cytokines[7], ROS generation[8] and cell death[9]. In this paper, we showed that HRW bathing significantly decreased the level of pro-inflammatory cytokines, reduced skin damage as

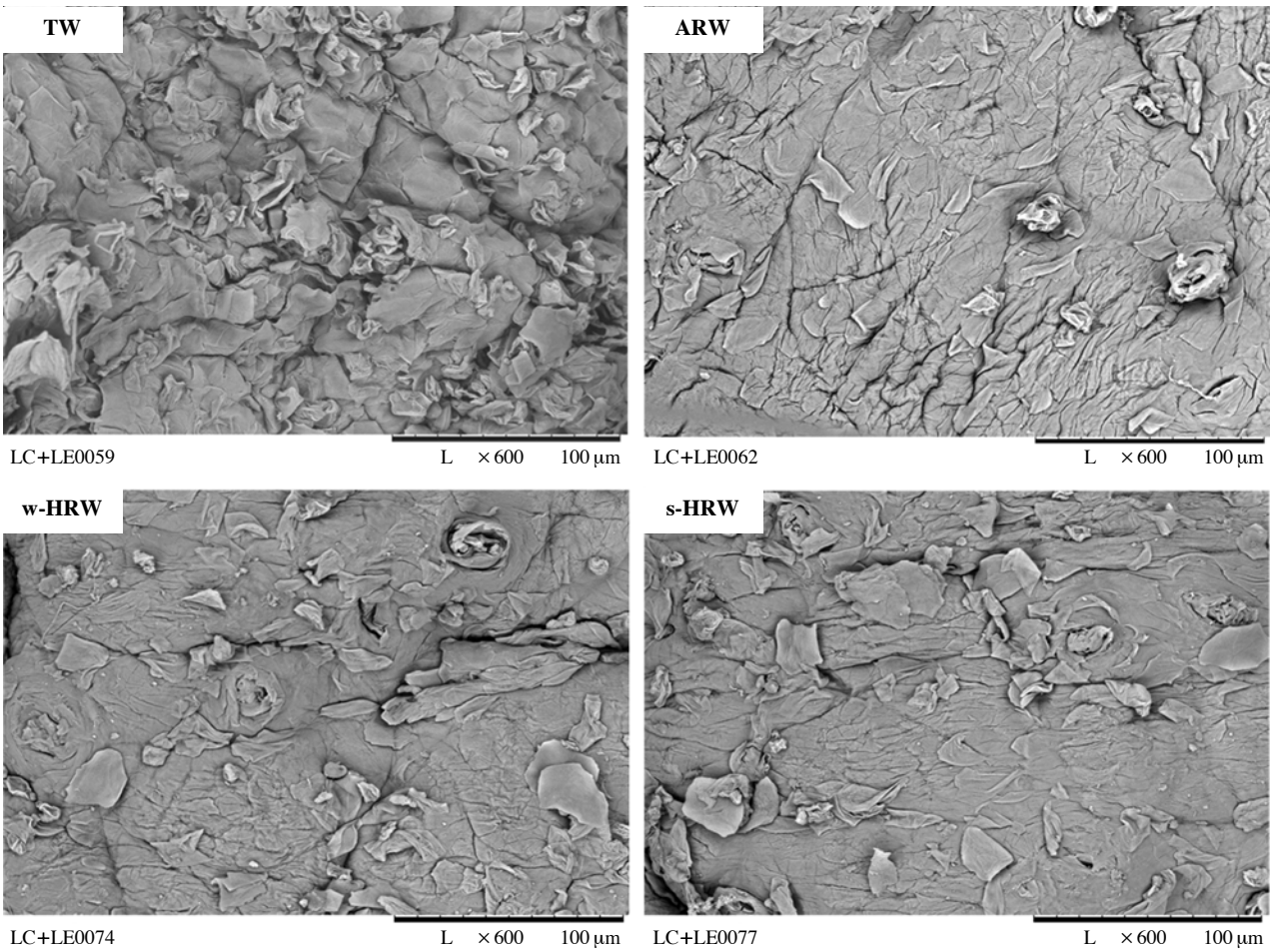

Figure 4. Representative scanning electron microscope image of dorsal skin surface, ×600 magnification. Alkaline reduced water (ARW), weak hydrogen reduced water (w-HRW), and strong hydrogen reduced water (s-HRW) bathing groups showed less defective corneocytes than tapwater (TW) bathing group. The scale bar represents 100 μm.

evidenced by quantitative scoring of skin injury, lower damage of corneocytes as revealed through SEM observation and enhanced glutathione peroxidase activity. Our findings demonstrated for the first time the antioxidant capacity of HRW through bathing which suggests favorable penetration of H_2 in the skin.

We first examined skin injury by scoring the degree of damage, 6 being the highest and 1 as the lowest. We showed that after bathing with HRW, the level of skin damage was significantly lowered compared to the TW-bathing group.

Next, we examined ROS scavenging ability of HRW via influencing enzymes that helps in the removal of destructive ROS in the body. Previous studies elucidated that UV irradiation results in the rapid depletion of several endogenous skin enzymes and antioxidants such as glutathione peroxidase, reductase and catalase[8]. Our data showed enhanced activity of glutathione peroxidase (GPx) compared to the TW-bathing group. GPx plays important roles in the protection of organisms from oxidative damage. This enzyme is responsible in reducing lipid hydroperoxides to their corresponding free hydrogen peroxides to water counterpart.

To further support our findings about ROS scavenging ability of HRW-bathing, we performed ultrastructure observation of corneocytes by SEM. HRW-bathing showed lower number of corneocytes damaged compared to TW-bathing group. This finding suggests that bathing with HRW is another method for the penetration of H_2 into the cell membrane and to its target organelles.

Finally, to delineate the inflammatory changes after exposure to UVB and bathing with HRW, we did cytokine profiling. It has been demonstrated previously that UVB irradiation caused abnormal production of

inflammatory cytokines that produces inflammation[7]. HRW-bathing groups showed lower level of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IFN-γ. This evidenced demonstrated that the dissolve H_2 in HRW can diffuse rapidly into the cell and restore the evoked inflammation of UVB exposure. This cytokine profiling is compatible with the previous studies on molecular hydrogen, which provides anti-inflammatory effect[10,11].

Our study uses both weak and strong hydrogen concentration, however the results are comparable. This suggests that hydrogen molecule was proven of no toxicity even at high concentration. Further, our study shows that bathing with HRW is alternative, convenient, and effective method to ingest molecular hydrogen in the body.

Collectively, this study shows that HRW bathing significantly decreases the level of pro-inflammatory cytokines, reduces skin damage as evidenced by quantitative scoring of skin injury, lower damage of corneocytes as revealed through SEM observation, and enhanced glutathione peroxidase activity. Further, these results importantly imply the potential antioxidant effect of HRW against UV-induced skin injury.

Materials & Methods

Preparation of HRW and ARW

Hydrogen reduced water (HRW) was generated from electrolyzing apparatus. pH and oxidative reduction potential (ORP) values of HRW were adjusted to obtain weak HRW (w-HRW) and strong HRW (s-HRW) as seen in Table 1. Alkaline reduced water (ARW) was produced by the continuously electrolyzing apparatus (HDr Co., Ltd., Korea). Main source of water was a tap water. ARW was prepared by physical filtering followed by electrolysis and collected in a cell equipped with a cathode platinum-coated titanium electrode (0.9±0.1 A, 1-2 L/min at 2 kgf/min). We used tapwater (TW) for positive control group. The characteristics of test waters were adjusted in terms of pH, oxidation reduction potential (ORP) and temperature (Table 1).

Animals

Six-week old male SKH-1 hairless mice weighing 25 ±2 g were purchased from Orient Bio Inc. (Korea). Mice were maintained in stainless steel cages in a controlled environment with temperature of 22±2°C and 40-60% humidity under a 12 : 12 hour light dark cycle. After 1 week of acclimation, the hairless mice were randomly assigned to four groups: TW-bathing group bathed with tap water after UV treatment (control, n=12), ARW-bathing group bathed in ARW after UV treatment (n=12), w-HRW-bathing group bathed with weak HRW after UV treatment and s-HRW-bathing group bathed in strong HRW after UV treatment. The animal use and care protocol for this animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) at Wonju Campus, Yonsei University, Wonju, Gangwon, Korea.

UVB irradiation and bathing

UVB fluorescent lamp (TL 40W/12RS Philips, Netherland Holland) emitting a continuous spectrum between 290-320 nm was used to induce skin damage. The intensity of UVB lamp was 0.10-0.15 mW/cm^2 and the total cumulative dose energy of UVB irradiated was 360-540 mJ/cm^2 (UV light meter YK-34 UV, Lutron Electronics Inc., Taiwan). The lamp was mounted 30 cm above surface of the bathing cage. TW-bath-

Table 1. Preparation of test water.

Test water	pH	ORP	Temperature
w-HRW	7.33±0.53	−83.3±44.8	30.0±0.3
s-HRW	7.32±0.34	−481±41.4	30.0±0.4
ARW	9.88±0.28	−225±117	30.5±0.4
TW	6.90±0.35	410±166	29.9±0.1

Abbreviations: w-HRW, weak-hydrogen reduced water; s-HRW, strong-hydrogen reduced water; ARW, alkaline reduced water; TW, tap water; ORP, oxidation reduction potential

Table 2. WBC count after UVB irradiation and bathing.

Groups	TW	ARW	w-HRW	s-HRW
WBC (K/μL)	7.41±3.21	7.13±2.82	7.22±1.80	7.77±1.10
Neutrophil (K/μL)	4.20±2.44	3.89±2.18	3.60±0.880	3.79±0.98
Lymphocyte (K/μL)	2.67±0.65	2.80±0.66	3.26±1.00	3.26±0.60*
Monocyte (K/μL)	0.397±0.12	0.392±0.12	0.314±0.09	0.378±0.09
Eosinophil (K/μL)	0.0133±0.00650	0.00917±0.00515	0.00539±0.00660	0.00308±0.00480
Basophil (K/μL)	0.0433±0.03	0.04±0.02	0.04±0.02**	0.04±0.04***

Data are means±S.D., n=12. *$P<0.05$, **$P<0.01$, ***$P<0.001$ indicate significant differences with ANOVA. Tukey's test was used for *post-hoc* tests. Abbreviation: TW, tapwater; ARW, alkaline reduced water; w-HRW, weak hydrogen reduced water; s-HRW, strong hydrogen reduced water.

ing, ARW-bathing and weak/strong HRW-bathing groups were irradiated with UVB lamp for 15 minutes prior to and after 30 minutes freely swimming for 4 times. The mice of bathing groups were then bathed individually for 50 minutes, two times for one day without UVB irradiation. All mice were sacrificed at the end of experimental period, and damage of the dorsal skin surface was examined using scanning electron microscope (SEM). Activity of ROS related enzyme and cytokine analysis were also measured. Moreover, scoring of the degree of skin injury was also recorded.

Scoring of skin injury

The next day after UVB exposure, mice were observed for skin injury and the degree of dorsal skin damage was measured. The scored grade of damage was divided into 6 steps according to the severity of skin injury, 6 being the most damage. Score were presented as mean ± S.D.

Hematological examination

Mice were anesthetized with CO_2 and blood samples were collected from the retro-orbital plexus for the analysis of total WBC and its differential count (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) measured by an automatic blood analyzer (HEMAVET HV950 FS, Scientific Inc., USA).

Glutathione peroxidase (GPx) activity assay

GPx activity in plasma was measured for H_2O_2 scavenging capacity by modified Cayman's GPx assay kit (Cayman Chemical Company, Ann Arbor MI USA) according to the manufacturer's instruction. Oxidized glutathione, produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm.

Bio-plex cytokine assay

Cytokines secreted in the blood were investigated. Serum concentrations of IL-1β, IL-6, IL-10, IL-12 (p70), TNF-α and IFN-γ were measured using Multiplex kit (Bio-Rad, San Diego, USA) and run on Luminex technology (Bio-Plex Multiplex Bead Array System™, Bio-Rad Hercules, CA, USA) according to the manufacturer's instruction. Raw fluorescence data were analyzed by the software using a 5-parameter logistic method.

Scanning electron microscopy (SEM)

Specimens were fixed in 2.5% glutaraldehyde for 2 h at 4°C, washed in 0.1 M phosphate buffer (pH 7.4) and post fixed in 1% osmium tetroxide for 90 mins. The specimens were dehydrated in graded ethanol, exchanged through isoamylacetate, and critical point dried. The specimens were coated with gold-palladium and examined in scanning electron microscope (TM-1000, Hitachi, Japan).

Statistical analysis

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

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