

Effect of Molecular Hydrogen Saturated Alkaline Electrolyzed Water on Disuse Muscle Atrophy in Gastrocnemius Muscle

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Abstract The objectives of this paper were to determine the level of oxidative stress in atrophied gastrocnemius, and to verify the effect of molecular hydrogen (H₂) saturated alkaline electrolyzed water (HSW) on gastrocnemius atrophy by modifying the redox status, indicated by 8-hydroxy-2'-deoxyguanosine (8-OHdG), malondialdehyde (MDA), and superoxide dismutase (SOD)-like activity. Female Wistar rats were divided into four groups: (1) the control (CONT); (2) the Hindlimb unloading (HU, for 3 weeks) given purified normal water (HU-NW); (3) the HU given alkaline electrolyzed reduced water (HU-AEW); and (4) the HU given HSW (HU-HSW). We showed that 8-OHdG, but not MDA, significantly increased by 149% and 145% in HU-NW and HU-AEW, respectively, when compared with CONT. In contrast, there was a trend toward suppression in 8-OHdG levels (increased by 95% compared with CONT) by treatment of HSW, though this effect was not prominent. Additionally, SOD-like activity significantly increased in both HU-NW (184%) and HU-AEW (199%) when compared with CONT. This result suggests the elevation of O₂⁻ in the atrophied gastrocnemius. However, upregulation of SOD-like activity in the HU-HSW was increased by only 169% compared with CONT, though this difference is too small to detect statistical significance. HU led to 13% and 15% reduction of gastrocnemius wet weights in HU-NW and HU-AEW, respectively, compared with CONT. And the reduction of gastrocnemius wet weights in HU-HSW was attenuated by 7% compared with CONT. The gastrocnemius wet weights in the HU-HSW group were significantly greater than those in the HU-AEW, but not statistically significant with HU-NW. These results indicate that HU causes an increase in oxidative stress, but, in this experimental protocol, continuous consumption of HSW during HU does not demonstrate successful attenuation of oxidative stress and HU-mediated gastrocnemius atrophy. *J Physiol Anthropol* 30(5): 195–201, 2011 <http://www.jstage.jst.go.jp/browse/jpa2>

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Introduction

The loss of muscle mass and function due to chronic bed rest, physical inactivity, and aging is associated with not only a decline in quality of life but also increased mortality (Metter et al., 2002). Knowledge of disuse muscle atrophy and the development of anti-catabolic strategies that retard protein loss are important and challenging for not only the care of the geriatric population, but all of humanity.

Oxidative stress, in which antioxidant proteins and scavenger protection are overwhelmed by reactive oxygen species (ROS) production, is recognized as one of central causes of disuse muscle atrophy (Kondo, 2000; Powers et al., 2005, 2007). Furthermore, recent evidence suggests that overproduction of ROS can result in DNA fragmentation, lipid peroxidation and protein oxidation, which can lead to apoptosis, depending on the severity of oxidative stress (Cai et al., 2008; Leeuwenburgh et al., 2004; Nagano et al., 2008). Therefore, ROS may serve as an important trigger for signaling pathways leading to muscle atrophy during prolonged periods of disuse, and countermeasures designed to suppress the ROS during disuse are a promising strategy to prevent muscle atrophy.

Vitamin E is most commonly recognized as a beneficial antioxidant. Several early studies reported that the level of thiobarbituric acid-reactive substance (TBARS) in atrophied muscle and the degree of atrophy were significantly lower in vitamin-E-supplemented animals compared to those in placebo

animals (Appell et al., 1997; Servais et al., 2007). As these reports suggest, the intake of an antioxidant substance such as vitamin E, is regarded as one of the countermeasures against muscle atrophy during prolonged periods of disuse. In addition to conventional antioxidants, recent studies demonstrated that oxidative stress damage induced in the brain by focal ischemia-reperfusion was markedly suppressed by the inhalation of molecular hydrogen (H₂) (Cai et al., 2008; Ohsawa et al., 2007; Wood and Gladwin, 2007). Thus H₂ could be used as an effective antioxidant therapy, owing to its ability to rapidly diffuse across membranes, providing powerful protection against oxidative stress through its ability to react with the hydroxyl radicals ($\cdot\text{OH}$), the most highly reactive oxygen species (Hayashida et al., 2008; Ohsawa et al., 2007). Koyama et al. (2008) have shown that H₂-saturated alkaline electrolyzed water, produced by an alkaline ionized water apparatus, suppresses urinary excretion of exercise-induced 8-hydroxy-2'-deoxyguanosine (8-OHdG) in humans compared with a placebo group. Furthermore, Sato et al. (2008) have found that consumption of H₂-saturated water prevents the formation of superoxide anion radicals (O₂⁻) in the brain *in vivo*. These findings also strongly indicate that H₂ rich water is effective in suppressing oxidative stress.

To date, information supporting a causal relationship between H₂ and disuse muscle atrophy does not exist. Given that hydrogen either suppresses or scavenges ROS such as O₂⁻ and $\cdot\text{OH}$ generated during prolonged periods of disuse, it provides protection against oxidative stress and preserves muscle mass after disuse. Further, from the point of view of practical application for humans, the study of hydrogen-saturated water on disuse muscle atrophy using an animal model is an indispensable step.

Moreover, there is research showing that muscle atrophy during prolonged periods of disuse is induced in mixed-fiber type muscles, such as in the gastrocnemius, which contains both slow and fast myosin-heavy chains (Hofer et al., 2008; Siu et al., 2005). These findings indicate that disuse muscle atrophy may not be just confined to the soleus muscle (Thomason and Booth, 1990). Furthermore, since the gastrocnemius contains a variety of muscle fiber types, the results may be applied to other muscles. But to our knowledge, not many studies about mixed-fiber type muscle atrophy have been done.

Hence, the goal of this study was to investigate the levels of oxidative stress in the atrophied gastrocnemius after hindlimb unloading (HU), and to determine the effect of H₂-saturated alkaline electrolyzed water (HSW) on oxidative stress and muscle mass after HU. We hypothesized that ingestion of HSW during HU would decrease oxidative stress and attenuate HU-mediated muscle atrophy compared with rats exposed to HU given purified normal water.

Methods

Animals

This investigation was carried out in accordance with the guidelines of the ethical committee of animal experiments at the University of Yamanashi.

Twenty-six female Wistar rats (15 weeks old) were housed in individual cages under 12: 12 h light-dark cycles. For the first two weeks, rats were housed and provided tap water in individual cages without any procedure to prevent a decrease in food intake associated with the stress of a new environment. After 2 weeks, rats were randomly assigned to hindlimb unloading (HU) groups ($n=18$) or a control group (CONT, $n=8$).

Experimental protocols

After rats had been anesthetized with diethyl ether, pentobarbital sodium was injected intraperitoneally (50 mg/kg body weight). While the rats were anesthetized, the center part of the 14th caudal tail vertebra was penetrated with Kirschner's wire using a pin vise. An equilateral triangle was made to connect to a hook, which was connected to the center of a steel bar running across the cage. The hooks were placed between two nuts on the center of the steel bar so that the rats could not move left or right. However, the rats were allowed to perform free 360° rotation. The hindlimbs were elevated to the spinal orientation of 45° above horizontal. This design meant that the rat's hindlimbs had no contact with any sides of the cage for 3 weeks. The forelimbs were maintained in contact with the ground allowing the rats to move back and forth and access food and water ad libitum throughout the experiment.

HU rats were further randomly assigned to one of three groups: (1) HU given purified normal water (HU-NW, $n=6$); (2) HU given alkaline electrolyzed water (HU-AEW, $n=6$); (3) HU given H₂-saturated alkaline electrolyzed water (HU-HSW, $n=6$).

Purified normal water, alkaline electrolyzed water and dihydrogen saturated alkaline electrolyzed water

Purified normal water (NW) and alkaline electrolyzed water (AEW) were generated with an alkaline ionized water apparatus (National TK 7505-S, Panasonic Electric Works Co., Ltd., Osaka, Japan). To generate H₂-saturated alkaline electrolyzed water (HSW), H₂ gas produced by a hand-manufactured H₂ gas generator was dissolved in AEW until saturated. Rats assigned to CONT were provided with NW.

The voltage and current of the H₂ gas generator was 4.0 V or below and 3.5 A respectively. The water flow of the alkaline ionized water apparatus was set at 2.5 L/min. H₂ concentrations (pH) of NW, AEW, and HSW were 0 (7.76), 0.19 (9.15), and 1.5 (9.15) mg/L, respectively. After one day, hydrogen concentrations (pH) of AEW and HSW dropped to 0.01 (9.20) and 0.73 (9.22) mg/L respectively. H₂ concentration was measured with a dissolved hydrogen meter (DH-meter, DH-35A, DKK-TOA Corporation). In addition, to reduce the loss

of the H₂, glass bottles were used to supply water and changed at 10 a.m. every day. Bottles were kept pointing downward to prevent ingress of air.

Muscle preparation

After 3 weeks of HU, the rats were anesthetized with diethyl ether and the gastrocnemii were quickly dissected, weighed, and frozen in liquid nitrogen. The muscles were stored at -80°C until analysis. The muscle weights referred to below are the averages of the two legs. Gastrocnemii contain a variety of muscle fiber types. To minimize the histochemical differences of each sample for all the analyses, medial gastrocnemii were used for all analyses.

8-hydroxy-2'-deoxyguanosine (8-OHdG) content

DNA was isolated by the NaI extraction technique with the DNA Extractor WB Kit (NIPPON GENE, Tokyo, Japan) described by Hamilton et al. (2001). Gastrocnemii (up to 120 mg) were homogenized in lysis solution on crushed ice. The homogenates were centrifuged ($10,000\times g$, 20 s, 4°C) and the pellets were resuspended in enzyme reaction solution (200 μl) with 20 g/ml RNase at 50°C for 10 min. Protease (10 μl) was added and the resulting solution was incubated at 50°C for a further 1 h. Samples were then centrifuged at $10,000\times g$ for 5 min at room temperature. The supernatants were collected and mixed with NaI solution. DNA was precipitated by ice-cold isopropyl alcohol and resuspended in TE buffer. The DNA concentrations were determined by measuring the absorbance at 260 nm. Fifteen μl of 200 mM sodium acetate buffer (pH 4.8) and 12 μl of 1 mg/ml nuclease P1 were also added to 135 μl DNA samples. The samples were incubated at 37°C for 1 h to digest the DNA. Fifteen μl of 1 M Tris-HCl (pH 7.4) and 7 μl of 286 units/ml alkaline phosphatase were then added to the samples, and the samples were incubated at 37°C for 1 h to hydrolyze the nucleotides to nucleosides, followed by centrifugation (14,000 rpm, 10 min) using Microcon YM-10 (Millipore, Japan) to remove proteins from the samples. The nucleoside samples were used for the determination of 8-OHdG by competitive ELISA using a monoclonal antibody to 8-OHdG (Highly Sensitive 8-OHdG Check, Institute for the Control of Aging, Nikken SEIL Co., Ltd., Shizuoka, Japan). Briefly, 50 μl of nucleoside samples or standard 8-OHdG solution were added to the wells of a 96-well plate pre-coated with 8-OHdG, then 50 μl of primary antibody was added and the plate was incubated at 4°C overnight. After incubation, the wells were washed with 250 μl /well of 0.05% Tween 20/phosphate-buffered saline and 100 μl /well of secondary antibody were added and the plate was incubated for 1 h at room temperature. Following incubation, 100 μl of peroxidase substrate (3,3',5,5'-tetramethylbenzidine/hydrogen peroxide/citric acid phosphate buffered saline) was added. After incubation at room temperature for 15 min in the dark, 100 μl of phosphoric acid (1 M) was added and the absorbance was read at 450 nm on the microplate reader.

Malondialdehyde (MDA) content

TBARS levels, as an index of lipid peroxidation, were measured with the NWK-MDA01 assay kit (Northwest Life Science Specialties, LLC, WA, USA). This kit is based on the reaction of MDA with thiobarbituric acid (TBA) forming an MDA-TBA2 adduct that is absorbed at 532 nm. Gastrocnemii (up to 140 mg) were homogenized in 10% (w/v) cold phosphate buffer (pH 7.0) on crushed ice. The homogenates were centrifuged ($10,000\times g$, 10 min, 4°C) to remove nuclei and debris. The supernatants were mixed with butylated hydroxytoluene, phosphoric acid, and TBA reagent, and then the samples were incubated at 60°C for 1 h. After cooling the samples to room temperature, 650 μl butanol (BuOH) was added to each and immediately vortexed. Following centrifugation ($10,000\times g$, 3 min, room temperature), 520 μl of the BuOH fraction was transferred to a new tube and 650 μl of 1 N sodium hydroxide (NaOH) was added to each sample. After the samples were vortexed, 520 μl of the NaOH fraction was transferred into a new tube and 130 μl phosphoric acid (3.7 M) was added to each sample. The absorbance was recorded at approximately 532 nm. Using tetramethoxypropane, a standard curve was prepared and the values of the samples were determined from the curve. MDA content was normalized to the gastrocnemius wet weights. Results are expressed as nano mol MDA per gram of gastrocnemius wet weights.

Superoxide dismutase (SOD)-like activity

SOD-like activity was determined with a SOD assay kit-WST (Dojindo Molecular Technology, Inc., Kumamoto, Japan). This kit uses Dojindo's highly water-soluble tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon reduction with a superoxide anion. Portions of gastrocnemii (up to 200 mg) were briefly washed with saline to remove as much blood as possible then dried with paper towels. The muscle samples were homogenized in 9% (w/v) sucrose buffer (0.25 M sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA) on crushed ice. The homogenates were centrifuged ($10,000\times g$, 1 h, 4°C), and supernatants were collected and diluted with dilution buffer to prepare the sample solution. The sample solution (20 μl) was added to each sample well and blank 2 well and ddH₂O (20 μl) was added to each blank 1 and blank 3 well. The WST working solution (200 μl) was added to all wells, and dilution buffer (20 μl) was added to each blank 2 well and blank 3 well. Then enzyme working solution (20 μl) was added to each sample well and blank 1 well. After incubation at 37°C for 20 min, the absorbance was then read at 450 nm on the microplate reader. IC 50 (50% inhibition activity of SOD) was calculated using the following equation:

$$\begin{aligned} \text{SOD-like activity (inhibition rate \%)} \\ = \{[(\text{blank 1} - \text{blank 3}) - (\text{sample} - \text{blank 2})] \\ / (\text{blank 1} - \text{blank 3})\} \times 100 \end{aligned}$$

Results are expressed as unit per gram of gastrocnemius wet weights.

Statistical analysis

All data are expressed as means±SE. Statistical analyses were performed using SPSS ver. 10.0 (Japan Inc., Tokyo, Japan). The results obtained from the experiments were analyzed by one-way ANOVA followed by a Tukey honestly significant difference post hoc multiple-comparison test. Statistical significance was evaluated for all statistics at $p<0.05$.

Results

Body weights and gastrocnemius wet weights

There were no differences in body weights between the groups at the beginning of the study (CONT=180±7 g, HU-NW=179±3 g, HU-AEW=181±12 g, HU-HSW=180±7 g). After 3 weeks of HU, body weights of HU groups were significantly less than those for CONT (CONT=200±8 g, HU-NW=174±6 g, HU-AEW=171±2 g, HU-HSW=167±7 g). But the differences among the HU groups were not statistically significant.

HU groups showed significantly ($p<0.05$) lower gastrocnemius wet weights (HU-NW=725.2±33.5 mg, HU-AEW=697.2±10.6 mg, HU-HSW=741.2±27.1 mg) compared with CONT (952.1±15.0 mg). But differences among HU groups were not statistically significant. Furthermore, when expressed as muscle wet weights to body weights ratio, HU-AEW resulted in a significant 15% ($p<0.05$) reduction in gastrocnemius wet weights compared with CONT (Fig. 1). On the other hand, reduction in gastrocnemius wet weights in HU-HSW was attenuated by 7% compared with CONT.

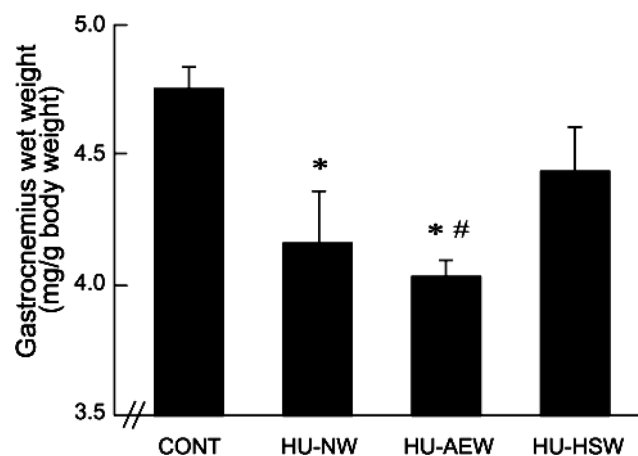


Fig. 1 Effect of hindlimb unloading on gastrocnemius wet weights normalized to body weights in CONT, HU-NW, HU-AEW, and HU-HSW. HU resulted in significant reduction in gastrocnemius wet weights. The gastrocnemius wet weights in the HU-HSW group were significantly greater than those in the HU-AEW, but not statistically significant with HU-NW.

* Significantly different from CONT and # significantly different from HU-HSW ($p<0.05$).

Intriguingly, there was a significant ($p<0.05$) difference between HU-AEW and HU-HSW (Fig. 1). The difference for the statistical result between absolute and relative value of gastrocnemius wet weights in HU-AEW and HU-HSW is interpreted as the result of a slightly smaller body weight in HU-HSW than in HU-AEW after HU. Also, HU-NW was associated with 13% significant ($p<0.05$) reduction in gastrocnemius wet weights compared with CONT. Although gastrocnemius wet weights in HU-HSW were heavier than those in HU-NW, the difference between the two groups was not statistically significant.

8-OHdG content

HU caused a significant ($p<0.05$) increase in 8-OHdG contents in the HU-NW (149%) and the HU-AEW (145%) groups compared with CONT (Fig. 2). On the other hand, in the HU-HSW group, there was a trend toward a depression in 8-OHdG elevation (95%) by treatment with HSW, but this was not statistically significant when compared with either HU-NW or HU-AEW.

MDA content

No significant ($p<0.05$) change in MDA contents after HU was detected (Fig. 3). HSW did not affect MDA contents either.

SOD-like activity

HU caused a significant ($p<0.05$) upregulation of SOD-like activity (HU-NW=184%, HU-AEW=199%) compared with CONT (Fig. 4). On the other hand, there was no significant difference between CONT and HU-HSW because of small upregulation of SOD-like activity in HU-HSW (169%). But the slight differences between HU groups were not statistically significant.

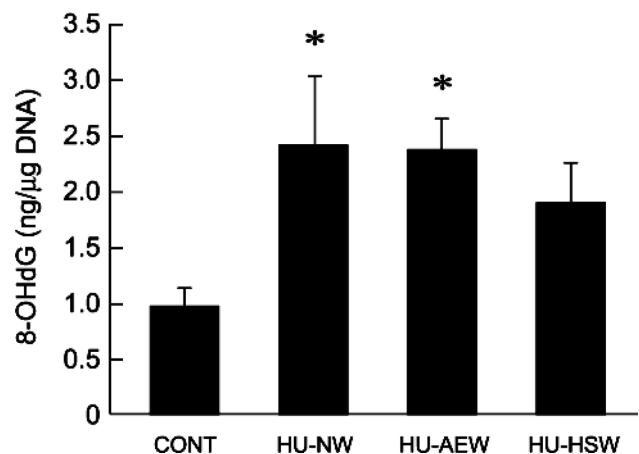


Fig. 2 Effect of hindlimb unloading on 8-hydroxy-2'-deoxyguanosine (8-OHdG) contents in the gastrocnemius of CONT, HU-NW, HU-AEW and HU-HSW. HU caused a significant increase in 8-OHdG contents compared with CONT. There was a trend toward a decrease in 8-OHdG with HSW, but this reduction was not statistically significant when compared with either HU-NW or HU-AEW. * Significantly different from CONT ($p<0.05$).

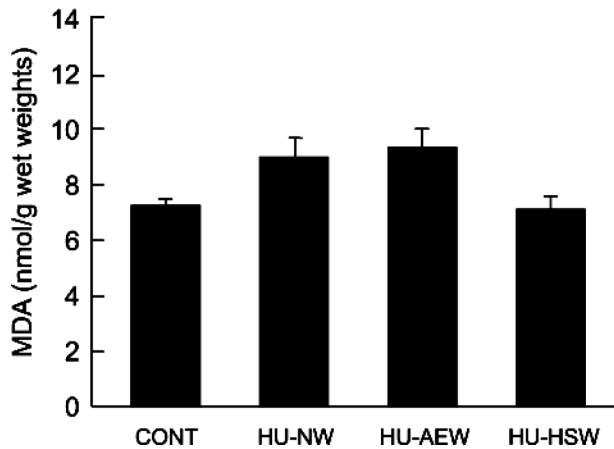


Fig. 3 Effect of hindlimb unloading on malondialdehyde (MDA) contents in the gastrocnemius of CONT, HU-NW, HU-AEW, and HU-HSW. There was no significant difference among the groups.

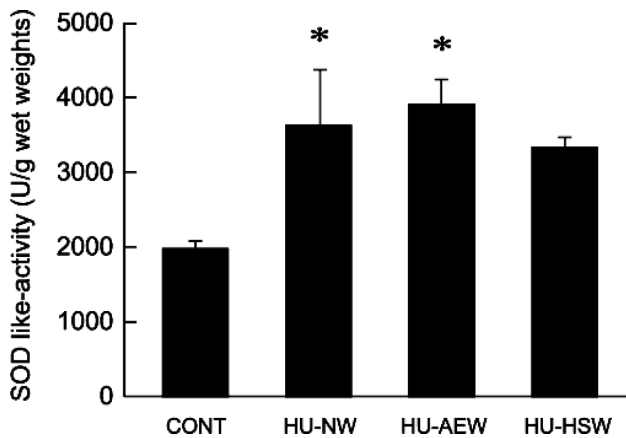


Fig. 4 Effect of hindlimb unloading on superoxide dismutase (SOD)-like activity in the gastrocnemius of CONT, HU-NW, HU-AEW, and HU-HSW. SOD-like activity in HU-NW and HU-AEW was significantly elevated compared with CONT. Upregulation of SOD-like activity after HU tended to be suppressed in HU-HSW, but this suppression was not significant. * Significantly different from CONT ($p < 0.05$).

Discussion

The aim of this study was to investigate the effects of HSW on oxidative stress associated with HU. Body weight losses were observed in HU groups, but were within the range shown in previous studies (Koesterer et al., 2002; Nagano et al., 2008). Notably, gastrocnemius wet weights normalized to body weights were significantly greater in the HU-HSW compared with those in the HU-AEW, but not in HU-NW. However, H_2 saturated water does not suppress the elevation of oxidative stress, as indicated by 8-OHdG levels and SOD-like activity in the gastrocnemius after HU. Thus, this data indicates that HSW might not be an effective countermeasure to prevent the disuse muscle atrophy associated with HU in our experiment.

As shown by this study, HU causes an increase in 8-OHdG levels, a finding that is in line with our hypothesis. The

elevation of 8-OHdG levels could be the result of an increased ROS production, a decreased antioxidant status, or a combination of both. On the other hand, MDA contents, a measure of lipid peroxidation, tend to be higher for HU-NW than for CONT, but this increase is not statistically significant. The work by Koesterer et al. (2002) has reported no change in TBARS level in the gastrocnemius after HU, which is in harmony with our finding. On the other hand, in the previous work of Kondo (2000), an increased TBARS level in atrophied soleus muscle after 12-day immobilization was observed. The disparity may be due to different experimental models (immobilization vs. unweighting) and period, as well as differences in muscle fiber susceptibility (soleus vs. gastrocnemius) to oxidative stress (Hollander et al., 1998; Kondo et al., 1992; Kondo, 2000). But the reasons for this disparity are still unclear and warrant further studies.

We found that SOD-like activity, which acts as a converter of O_2^- to hydrogen peroxide (H_2O_2), was significantly elevated in the HU groups. This result is in accord with the findings of Lawler et al. (2003) showing that 28 days of HU causes a significant elevation of total SOD activity, suggesting increased production of O_2^- in the skeletal muscle.

To our knowledge, this is the first study to demonstrate a trend toward an attenuation of increase in SOD-like activity after HU as a result of ingestion of H_2 -saturated water. This is deemed to be because H_2 not only has an ability to scavenge $\cdot OH$ but it also has the ability to prevent formation of O_2^- . Support for this hypothesis comes from a study by Sato et al. (2008) using H_2 -saturated water to prevent the formation of O_2^- in brains exposed to hypoxia-reoxygenation treatment. However, because no statistical significant for SOD-like activity in our study was observed among HU, the result should be interpreted with caution. Moreover, in view of the imbalance among the antioxidant enzymes that was followed by oxidative stress (Meneghini, 1997; Peled-Kamar et al., 1997; Pinho et al., 2006), further studies are needed to clarify the interaction between SOD and other antioxidants such as catalase and glutathione peroxidase associated with disuse and HSW treatment.

One point of our experiment was the degree to which HSW provided protection against oxidative stress and muscle atrophy after HU. We hypothesized that consumption of HSW attenuates oxidative stress and oxidative stress-induced muscle atrophy during HU. Because previous studies showed that H_2 selectively neutralizes $\cdot OH$ in living cells and acts as an effective antioxidant (Kajiyama et al., 2008; Ohsawa et al., 2007; Wood and Gladwin, 2007). Another possibility is that H_2 prevents O_2^- formation leading to lower reduced forms of transition metal ion, suggesting a decreased production of $\cdot OH$, also cannot be ruled out.

However, there is a likelihood that consumption of HSW ad libitum during HU suppressed the increase in the 8-OHdG contents, a product of oxidative damaged DNA formed by $\cdot OH$, though there is just a trend toward a reduction of 8-OHdG contents in the present study. In addition,

gastrocnemius wet weights in the HU-HSW group were greater than those in the HU-NW and HU-AEW groups, but not statistically significant with HU-NW. These findings are not inconsistent with previous studies that have demonstrated successful antioxidant challenges such as vitamin E and heat treatment could be a therapeutic candidate for the prevention of muscular atrophy (Selsby and Dodd, 2005; Servais et al., 2007). Therefore, we would have to consider that ingestion of HSW in this HU model is not a likely promising agent for a therapeutic antioxidant to suppress oxidative stress during HU and progression of disuse muscle atrophy. However, considering previous evidence in animal studies that drinking H₂-saturated water *ad libitum* reduces oxidative stress (Nagata et al., 2008; Ohsawa et al., 2008; Sato et al., 2008), our results, such as the trend toward suppression of 8-OHdG and SOD-like activity in HSW, suggest something that we cannot ignore. We observed that H₂ concentrations of both AEW and HSW had plunged to nearly one-half after one day. Taking these facts into consideration, we believe that the results in the present study underestimate the effects of AEW and HSW on oxidative stress and disuse muscle atrophy. Therefore, we are now developing a new tool for maintaining hydrogen concentration in water. A further redesigned study is necessary to confirm the effect of hydrogen-saturated water on disuse muscle atrophy.

A potential weakness of our study is that we did not check how much water each rat drank, although most antioxidants such as vitamin E are given at set dosages. An ongoing study in our group demonstrated that hindlimb unloading had an effect on loss of amount of drinking, but significant differences among the hindlimb unloading groups treated either with hydrogen-saturated water or purified normal water, were not found (unpublished data). Taken together, dose-dependent effects between the groups may not be biased toward the results observed in our study.

In conclusion, we examined the effects of HSW on oxidative stress associated with disuse muscle atrophy after HU. Although an increase in 8-OHdG contents was induced by HU, drinking HSW failed to suppress the increase in 8-OHdG contents, SOD-like activity, and the reduction of muscle mass after HU. Our initial hypotheses were refuted in this experiment; however, the effectiveness and application of hydrogen-saturated water were not completely denied. Further studies are required to determine more precisely the effects of H₂ on disuse muscle atrophy induced by oxidative stress that will ultimately provide an insight into therapeutic approaches to prevent or retard loss of muscle mass associated with inactivity or aging in humans.

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