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Hydrogen Treatment Protects against Cell Death and Senescence Induced by Oxidative Damage

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Hydrogen has potential for preventive and therapeutic applications as an antioxidant. However, micro- and macroparticles of hydrogen in water disappear easily over time. In order to eliminate reactive oxygen species (ROS) related with the aging process, we used functional water containing nanoparticle hydrogen. Nanoparticle hydrogen does not disappear easily and collapse under water after long periods of time. We used murine embryonic fibroblasts that were isolated from 12.5-day embryos of C57BL/6 mice. We investigated the ability of nanoparticle hydrogen in water to suppress hydroxyurea-induced ROS production, cytotoxicity, and the accumulation of β-galactosidase (an indicator of aging), and promote cell proliferation. The accumulation of β-galactosidase in the cytoplasm and the appearance of abnormal nuclei were inhibited by daily treatment of cells with hydrogen water. When the aging process was accelerated by hydroxyurea-induced oxidative stress, the effect of hydrogen water was even more remarkable. Thus, this study showed the antioxidant and anti-senescence effects of hydrogen water. Nanoparticle hydrogen water is potentially a potent anti-aging agent.

Keywords: Hydrogen-rich water, hydrogen nanoparticles, anti-aging, antioxidant

Introduction

Reactive oxygen species (ROS) include oxygen-derived free radicals such as hydroxyl radicals (·OH), superoxide anions ($O_2^{\bullet -}$), alkoxyl radicals (RO·), and peroxyl radicals ($RO_2^{\bullet}$), as well as oxygen-derived non-radical species such as hydrogen peroxide (H_2O_2) [1]. As major ROS generators, mitochondria are constantly exposed to high levels of ROS, with detrimental consequences, including oxidative damage to mitochondrial DNA [2]. Thus, increased $O_2^{\bullet -}$ and ·OH associated with mitochondrial DNA damage have been implicated in cellular apoptosis [3].

The deleterious role of free radicals in aging and senescence was first suggested by Dr. Denham Harman in 1956 [4]. Sixty years after his publication, numerous studies have provided evidence supporting the central hypothesis, and various reviews on free radicals and aging have been published [5–8].

Hydrogen, the most abundant and lightest chemical element in nature, has anti-apoptotic, antioxidant, anti-inflammatory, and anti-allergy effects, and thus can be used to treat various diseases [9]. Molecular hydrogen diffuses rapidly into cells and tissues, and selectively reduces ·OH and peroxynitrite anions, which are the most cytotoxic ROS species. The effects of hydrogen occur without affecting normal ROS signaling or disrupting normal metabolic redox reactions that influence cell signaling [10]. Hydrogen is combustible in air at concentrations between 4% and 75%. However, in water, hydrogen is a stable gas that can only react with oxide radical ions ($\cdot O^-$) and ·OH at low reaction rates [11]. The reaction rate constants of $\cdot O^-$ and ·OH with other biomolecules are generally in the order of 10^9–10^{10} $M^{-1}{\cdot}s^{-1}$, whereas those with hydrogen are in the order of 10^7 $M^{-1}{\cdot}s^{-1}$. Nevertheless, hydrogen, being a small molecule that can easily distribute throughout cells and the body, is expected to have high collision rates with other

molecules that overcome its low reaction rates [12].

Hydrogen particles in water that are more than a micrometer in size (*i.e.*, macroparticles) disappear over time. Microparticles of hydrogen that have a diameter <50 μm have important technical applications due to their tendency to decrease in size and collapse in water [13–15]. Because micro- and macroparticles of hydrogen disappear over time, it is difficult to quantify the exact amount of hydrogen in water; thus, in previous studies, hydrogen water was prepared every week for study. In the present study, we tested various previously published methods to produce hydrogen nanoparticles that are less than 1 μm in diameter and do not evaporate or collapse in water. Because water rich in hydrogen nanoparticles can provide a stable supply of known amounts of hydrogen to cells and tissues, we used it to determine whether hydrogen had an effect on cellular phenotypes associated with aging.

Materials and Methods

Preparation of Water Enriched in Hydrogen Nanoparticles

Various sizes of hydrogen particles were produced with a bubbler made from bamboo (NanoSight; NNB, Korea). Water was pressurized quickly to induce magnetic pressurization, and the particles that were generated first were eliminated to form hydrogen particles of stable sizes. Hydrogen microparticles were then selectively filtered to form hydrogen nanoparticles. In the present study, we prepared water enriched in hydrogen nanoparticles using the method described above in which nearly 70% of total hydrogen nanoparticles had diameters <100 nm. Briefly, the nanobubble generator used consisted of a gas tank and a gas-liquid dispersion system as described in our previous study [16]. The hydrogen gas was fed into the membrane module from the gas tank, by using a gas regulator; the gas pressure was 0.5 MPa. The gas-liquid dispersion system has a membrane module (75 mm long and 20 mm in diameter) with a tubular membrane. The bubble size distribution in nano-scale was measured by the nanoparticle tracking analysis method (NanoSight LM1O-HSBFT14 with 405 nm blue laser; Quantum Design Korea, Korea). Brownian motion of the nanoparticles was analyzed in real-time by a high-sensitivity electron-multiplying CCD camera, based on a laser-illuminated microscopical technique.

Cell Isolation and Culture

Murine embryonic fibroblasts (MEFs), isolated from embryonic day 12.5 C57BL/6 mouse embryos, were provided from Cefobio (Korea). Cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, USA) containing 10% fetal bovine serum (FBS; GE Healthcare, USA) and 2% penicillin/streptomycin (Sigma-Aldrich, USA). Unless noted otherwise, cells that were passaged twice were used.

Quantification of Cell Proliferation

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega, USA) following the manufacturer's protocol. Briefly, cells were seeded in 96-well plates at a density of 5×10^3 cells per well. In the presence or absence of hydroxyurea (10 μmol/ml), cells were incubated with 5 mg/ml MTT for 4 h. The medium was then removed and 150 μl of solubilization solution and stop solution were added to the cells and incubated at 37°C for 4 h. The absorbance of the solution at 570 nm was then measured using a microplate reader. The percentage inhibition of cell growth was calculated as (1 − absorbance of experimental group/absorbance of control group) × 100.

Quantification of Cytotoxicity

MEFs were seeded in 24-well plates at a density of 5×10^4 cells per well and then incubated in the presence or absence of hydrogen nanoparticle water for 1 to 7 days. Media prepared with water enriched in hydrogen nanoparticles were prepared separately. To quantify cytotoxicity, a lactate dehydrogenase (LDH) Cytotoxicity Assay kit (Cayman Chemical Company, USA) was used, following the manufacturer's protocol. Briefly, after culturing in 24-well plates to reach passage 2, MEFs were seeded in 96-well plates at a density of 2×10^4 cells per well and grown at 37°C and in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. After 48 h, 100 μl of the extant medium was transferred to corresponding wells in a new 96-well plate, and 100 μl of reaction solution was added to each well. Plates were incubated at room temperature for 30 min with gentle shaking on an orbital shaker. Absorbance of the solution at 490 nm was measured using a microplate reader.

Characterization of Nuclear Morphology

Nuclei were observed by fixing MEFs with 4% paraformaldehyde for 20 min at room temperature and post-fixing with 70% ethanol. 4′,6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) was diluted in cell culture medium and added at a final concentration of 10 ng/ml for 30 min at room temperature. DAPI-stained nuclei were imaged with a fluorescence microscope (Nikon, Japan).

Quantification of β-Galactosidase Staining

Senescence-associated β-galactosidase staining was performed using the Senescence β-Galactosidase Staining Kit following the manufacturer's instructions (Biovision, USA). Briefly, after washing with phosphate-buffered saline (PBS), cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde (Sigma-Aldrich) in PBS for 15 min at room temperature. After washing with PBS, cells were incubated with X-gal staining solution at 37°C for 24 h. Cells were visualized with a light microscope (Nikon) using a 200× objective. The numbers of positive (blue) cells in four random fields of view were counted.

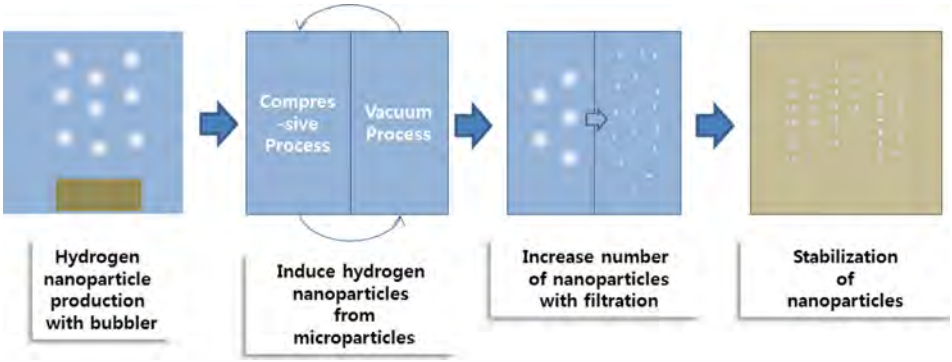

Fig. 1. Production of water rich in hydrogen nanoparticles.
Hydrogen nanoparticles were produced with a bamboo bubbler, further induced from microparticles, and then increased in number by filtration and stabilized.

ROS Production

MEF cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated in the absence or presence of hydroxyurea (10 μmol/ml) and/or hydrogen for 4 h. The culture medium was then replaced with fresh medium containing 2′,7′-dichlorofluorescin diacetate, and ROS production was assessed after 1 h by measuring fluorescence with a microplate reader using excitation and emission filters at 488 and 543 nm, respectively.

Statistical Analyses

Data are expressed as the mean ± SD. Data were analyzed by ANOVA followed by Dunnett's multiple comparison test, or Student's *t*-test when only two groups were compared. Differences between groups were considered significant at *p*-values less than 0.05. Statistical analyses were performed using SPSS 11.0 software (IBM, USA).

Results

Stable Hydrogen Nanoparticles in Water

Hydrogen nanoparticles, 10–100 nm in diameter, do not evaporate or contract/dissolve in water [17]. In the present study, we obtained water with a hydrogen concentration of 400–1,800 ppb and an oxidation-reduction potential of −550 mV. The concentration of nanoparticles was 200 million/ml of water, as determined by a nanobubble test analysis, and the diameter of the nanoparticles was <100 nm. Bubble size distribution was subsequently measured by using each particle, which was visualized and tracked simultaneously but separately by a dedicated particle tracking image analysis program (Fig. 1).

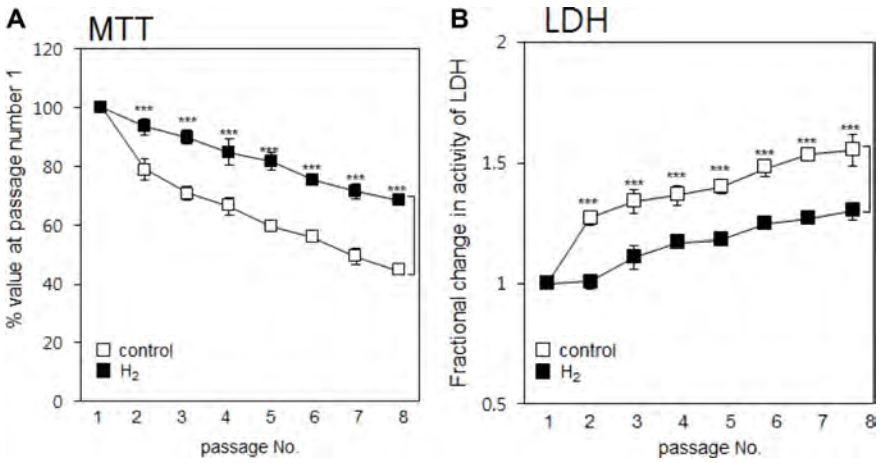

Fig. 2. Effect of hydrogen on hydroxyurea-induced cytotoxicity.
(**A**) Murine embryonic fibroblasts were subcultured until passage number 8. The enzymatic activity of NAD(P)H-dependent cellular oxidoreductase enzymes in live cells was assessed using an MTT assay in the presence or absence of hydrogen. (**B**) The activity of lactate dehydrogenase enzymes present in the culture media was assessed in the presence or absence of hydrogen. In both **A** and **B**, data were normalized to the absorbance value at passage number 1. Data are expressed as the mean ± SD from three independent measurements (***$p < 0.001$).

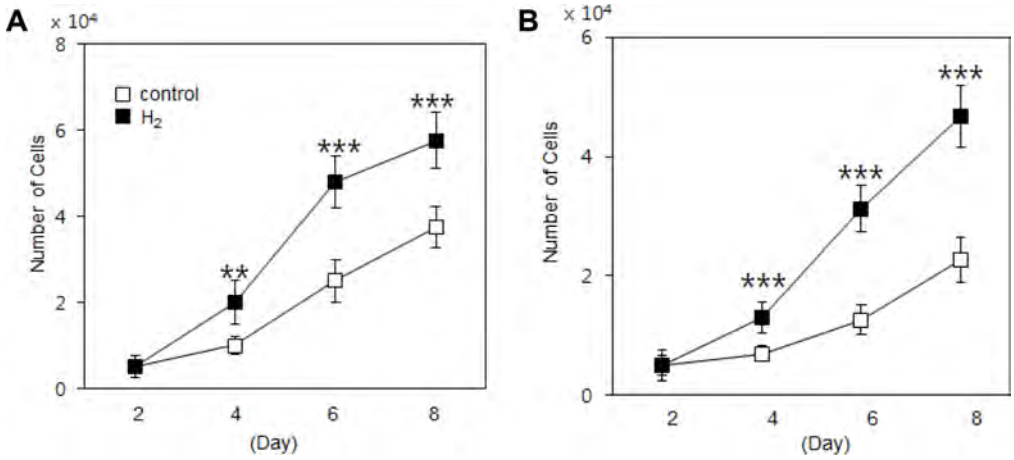

Fig. 3. Effect of hydrogen on cell proliferation.
(**A**) The number of cells was quantified in cultures grown in the presence or absence of hydrogen nanoparticle water via the MTT assay. (**B**) The number of cells was quantified in cultures pre-treated with hydroxyurea (10 μmol/ml) and grown in the presence or absence of hydrogen nanoparticle water. Data are expressed as the mean ± SD from three independent measurements (**$p < 0.01$, ***$p < 0.001$).

Hydrogen Enhances Cell Viability In Vitro

To determine whether hydrogen suppressed cytotoxicity, we treated primary MEFs with hydroxyurea to induce oxidative damage associated with the aging process. Cell proliferation was monitored using the MTT assay, which measures the number of viable cells present. A percentage decrease in normalized MTT values reflects the loss of viable cells or reduced proliferation. The proliferation of control MEFs decreased with the culture passage number (Fig. 2A). Although the proliferation of MEFs treated with hydrogen also declined with passage number, the decline was significantly less than that of control cells (Fig. 2A).

The effect of hydrogen on cytotoxicity was also assessed using the LDH assay. The amount of LDH present in the culture media of both control and hydrogen-treated cells increased over time. However, the increase was significantly lower with hydrogen treatment, suggesting that hydrogen inhibited hydroxyurea-induced cytotoxicity

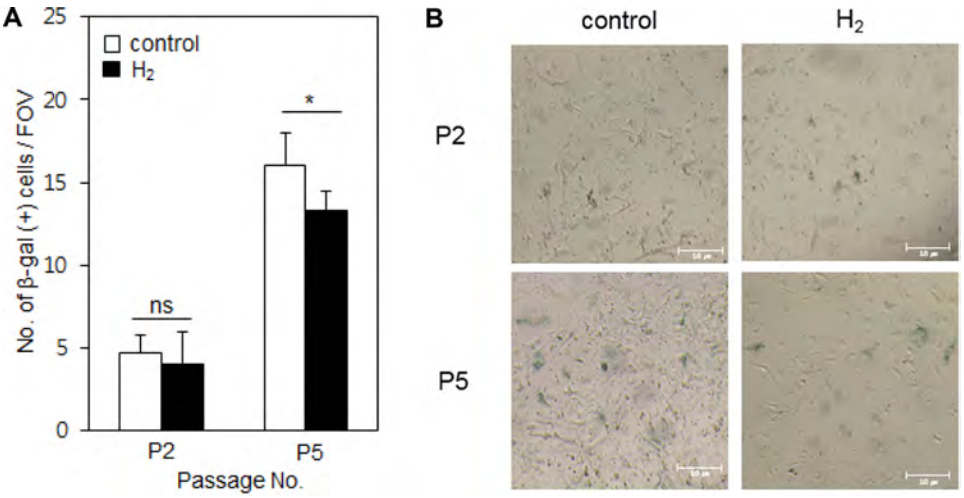

Fig. 4. Effect of hydrogen on β-galactosidase staining.
(**A**) The average number of β-galactosidase-positive cells per field of view was determined at passage number 2 and 5. The number of β-galactosidase-positive cells in cultures treated with 300 μM H_2O_2 only (control, white bars) was compared with that in cultures treated with H_2O_2 and hydrogen water (hydrogen, black bars). Data are shown as the mean ± SD from four independent experiments (*$p < 0.05$; ns, not significant). (**B**) Representative micrographs showing β-galactosidase-positive cells in randomly selected fields of view at passage number 2 and 5 under both control and hydrogen conditions. Scale bars: 10 μm.

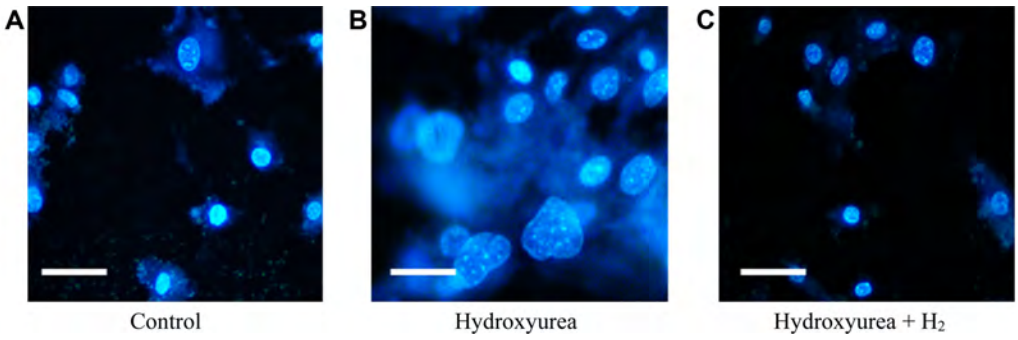

Fig. 5. Effect of hydrogen on hydroxyurea-induced nuclear morphological changes.
Representative images of DAPI-stained nuclei after treatment with 10 μmol/ml hydroxyurea or co-treatment with hydroxyurea and hydrogen. Hydroxyurea induced aberrant changes in nuclear morphology that were suppressed by hydrogen treatment. Scale bar = 1 μm.

(Fig. 2B). Taken together, these results suggest that hydrogen (nanoparticles in water) suppresses the cytotoxicity associated with oxidative damage and aging.

Hydrogen Promotes Cell Proliferation

We also examined the effect of hydrogen on the number of cells over 8 days in culture. The numbers of cells detected in cultures treated with hydrogen were significantly greater than control cultures at days 4, 6, and 8 (Fig. 3A). Similarly, the number of cells was significantly higher in cultures treated with both hydroxyurea and hydrogen than in cultures treated only with hydroxyurea at days 4, 6, and 8, particularly on day 8 (Fig. 3B).

Hydrogen Inhibits Cellular Senescence

We examined the effect of hydrogen on cellular senescence using β-galactosidase staining. The number of β-galactosidase-positive cells increased significantly from passage number 2 to 5 in cultures treated with 300 μM H_2O_2, indicating the accumulation of senescent cells (Fig. 4A). Co-treatment with hydrogen water reduced the number of β-galactosidase-positive cells compared with that in cultures treated only with H_2O_2 (Fig. 4B, $p < 0.05$).

Effect of Hydrogen on Hydroxyurea-Induced Morphological Changes of Cell Nuclei

The effect of hydrogen on the morphology of cell nuclei was examined by fluorescence imaging of DAPI-stained nuclei. Hydroxyurea (10 μmol/ml) treatment induced aberrant changes in nuclear morphology, including dramatic increases in nuclear volume and the formation of multiple lobes. Treatment with hydrogen suppressed the abnormal changes in nuclear morphology induced by hydroxyurea (Fig. 5).

Hydrogen Inhibits ROS Formation

Hydroxyurea increased ROS formation in both control and hydrogen-treated cells. The amount of ROS produced by control cells at passage 0 treated with hydroxyurea was set to 100%. This decreased to 62% at passage 2. Hydrogen treatment significantly reduced the rate of ROS formation in both control and hydroxyurea-treated cells at passage 2 (Fig. 6).

Discussion

Animal and clinical studies have demonstrated that

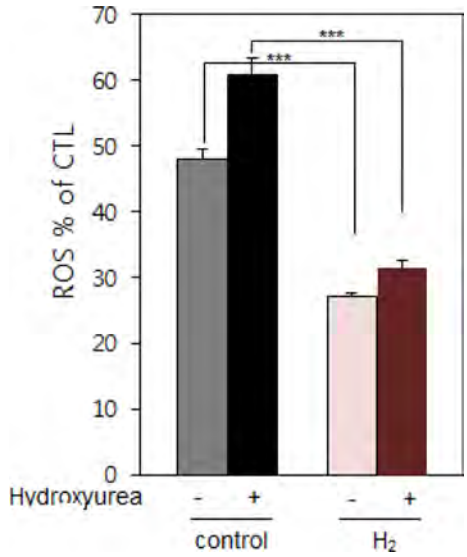

Fig. 6. Effect of hydrogen on ROS formation.
Hydroxyurea (10 μmol/ml) induced ROS formation in the culture media at passage 2, which was described as the ROS % of cytotoxic T lymphocyte (CTL). Hydrogen reduced the extent of ROS formation in both control and hydroxyurea-treated cells (***$p < 0.001$).

drinking hydrogen-rich water provides numerous health benefits, including improving serum lipid profiles of those at risk for the metabolic syndrome [18], improving quality of life after radiation exposure [19], and suppressing non-alcoholic steatohepatitis-related hepatocarcinogenesis [20]. Hydrogen-rich water also prevents ROS-induced aging effects by buffering against oxidative stress and superoxide formation [21].

Through the sonochemical effect, gases inside bubbles decompose to yield radicals such as H•, •OH, and HOO•. These radicals interact with the bulk liquid when bubbles fragment during transient collapse, and react through various chemical reactions [13]. The instability of hydrogen in water makes it difficult to quantify its exact concentration, which is important to know for understanding its potential therapeutic effects [19, 20, 22, 23]. Previous studies that used hydrogen-rich water did not determine the exact concentration of hydrogen that is maintained over time, and often only report the initial hydrogen concentration. In the present study, we generated water rich in hydrogen nanoparticles using previously published methods that relied on a bubbler made from bamboo (rather than an electrolytic method). The stability of hydrogen nanoparticles in water is better than hydrogen microparticles because nanoparticles do not readily evaporate or collapse in water.

Hydrogen-rich water prepared in this study contained nanoparticles, 70% of which had a diameter <100 nm. This water had a hydrogen concentration between 400 and 1,800 ppb, and an oxidation-reduction potential of −550 mV (compared with +100−200 mV for normal water). The concentration of hydrogen nanoparticles was over 200 million/ml of water, which was maintained for up to six months. Therefore, water rich in hydrogen nanoparticles can provide sufficient amounts of hydrogen to various tissues of the body [18, 24].

In the present study, we tested the effects of hydrogen nanoparticle-rich water on oxidative damage. Treatment with hydrogen suppressed cell death associated with the oxidative damage induced by hydroxyurea pre-treatment. In addition, hydrogen improved cell proliferation and significantly reduced cytotoxicity. The positive effects of hydrogen nanoparticle-rich water on cell survival and proliferation are likely due to the ability of hydrogen to reduce cytotoxic ROS such as $ONOO^-$ and •OH, which was demonstrated previously by Ohsawa *et al*. [21]. Another study suggested that hydrogen-rich water can act as an antioxidant in brain slices and prevent superoxide formation [24]. Compared with control water, administration of hydrogen-rich water reduced the formation of superoxide by 27.2% in ischemia-reperfusion animal models [21]. In addition, hydrogen reduced •OH and peroxynitrite levels in a dose-dependent manner [25]. Consistent with these previous findings, we showed that hydrogen inhibited hydroxyurea-induced ROS production.

Nanotechnologies are used mainly in fields such as agriculture and aquaculture [26, 27]. Nanoparticles that are 0.5−10 μm in size are generated from the contraction of microparticles. Compared with conventional alkaline electrolyzed reduced water, the abundance of hydrogen and the neutral pH of hydrogen-rich electrolyzed water are hypothesized to mediate its antioxidant potential by protecting other antioxidants such as ascorbic acid [28]. In the present study, we show that neutral pH hydrogen-rich water protects cells from the harmful effects of hydroxyurea in part by suppressing ROS production and cellular death, which are associated with the aging process.

Aging is a risk factor in the pathogenesis of various diseases. To decrease the incidence of these diseases, efforts to slow or prevent aging are being considered, including the use of antioxidants such as hydrogen. However, the therapeutic efficacy of hydrogen depends on its half-life in water, which determines how well it can distribute throughout the body. Thus, novel technologies to dissolve and maintain hydrogen in water will be important to consider. In the present study, we demonstrated the antioxidant properties of hydrogen-rich water. Although further studies are warranted to determine the optimal concentration of hydrogen for therapeutic efficacy, hydrogen nanoparticle-rich water represents a promising platform for anti-aging therapies or as an adjuvant to treatments for various other diseases.

Acknowledgments

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