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ORIGINAL ARTICLE

Treatment of hydrogen molecule abates oxidative stress and alleviates bone loss induced by modeled microgravity in rats

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Abstract
Summary Treatment with molecular hydrogen alleviates microgravity-induced bone loss through abating oxidative stress, restoring osteoblastic differentiation, and suppressing osteoclast differentiation and osteoclastogenesis.
Introduction Recently, it has been suggested that hydrogen gas exerts a therapeutic antioxidant activity by selectively reducing cytotoxic reactive oxygen species (ROS). The aim of the present study was to elucidate whether treatment with molecular hydrogen alleviated bone loss induced by modeled microgravity in rats.
Methods Hindlimb suspension (HLS) and rotary wall vessel bioreactor were used to model microgravity in vivo and in vitro, respectively. Sprague–Dawley rats were exposed to HLS for 6 weeks to induced bone loss and simultaneously administrated with hydrogen water (HW). Then, we investigated the effects of incubation with hydrogen-rich medium (HRM) on MC3T3-E1 and RAW264.7 cells exposed to modeled microgravity.
Results Treatment with HW alleviated HLS-induced reduction of bone mineral density, ultimate load, stiffness, and energy in femur and lumbar vertebra. Treatment with HW alleviated HLS-induced augmentation of malondialdehyde content and peroxynitrite content and reduction of total sulfhydryl content in femur and lumbar vertebra. In cultured MC3T3-E1 cells, incubation with HRM inhibited modeled microgravity-induced ROS formation, reduction of osteoblastic differentiation, increase of ratio of receptor activator of nuclear factor kappa B ligand to osteoprotegerin, inducible nitric oxide synthetase upregulation, and Erk1/2 phosphorylation. In cultured RAW264.7, incubation with HRM aggravated modeled microgravity-induced ROS formation, osteoclastic differentiation, and osteoclastogenesis.
Conclusion Treatment with molecular hydrogen alleviates microgravity-induced bone loss in rats. Molecular hydrogen could thus be envisaged as a nutritional countermeasure for spaceflight but remains to be tested in humans.

Keywords Bone loss · Hydrogen molecule · Microgravity · Osteoblastic differentiation · Osteoclast differentiation · Oxidative stress

Introduction

Microgravity (MG) is the condition of weightlessness experienced by astronauts during spaceflight or patients during long-term bed rest that causes severe physiological alterations in the human body, one of the most prominent of which is bone loss, which leads to an increase in fracture risk [1–3]. In the most severe forms of MG-induced bone loss, there is an approximately 2 % decrease in bone mineral density in only 1 month, equal to that of a postmenopausal woman in 1 year [4]. Long-term exposure to a MG environment leads to enhanced bone resorption in the early phase and reduced bone formation over the period of weightlessness [5, 6]. Space medicine tries to understand the underlying mechanism of MG-induced bone loss and to conceive potent countermeasures.

Several reports demonstrated that spaceflight or modeled microgravity resulted in oxidative stress not only in human and animals [7–9] but also in plants, fungi, and in vitro

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cultured cells [10–12]. Oxidative stress, resulting from excessive formation of reactive oxygen species (ROS) or dysfunction of antioxidant defense system, represents an important cause of age-associated pathological conditions including aging [13] and postmenopausal bone loss [14]. Though Kondo et al. found that short-term exposure to modeled microgravity hindlimb suspension (HLS) could not induced oxidative stress within mineralized tissue [15], we found long-term exposure to HLS in rats induced oxidative stress not only in the circulating system but also in bone tissue in the current study. In addition, it was reported that chronic resveratrol supplementation reversed the decrease of the glutathione versus glutathione disulfide ratio and maintained the bone mineral density and strength of the femur in suspended rats [9], indicating that oxidative stress might contribute to the HLS-induced bone loss.

To determine the role of oxidative stress in the progress of bone loss induced by modeled microgravity, we tried to use an antioxidant to treat it. Though different normal antioxidants such as vitamin C and vitamin E failed to show an improved antiosteoporotic outcome in clinical trials [16, 17], molecular hydrogen has a number of unique characteristics: hydrogen rapidly diffuses into tissues and cells, it is mild enough neither to disturb metabolic redox reactions nor to affect ROS that function in cell signaling, and it selectively reduces the hydroxyl radical and peroxynitrite anion (the most cytotoxic chemicals of ROS) [18].

In the present work, we explored whether long-term drinking of hydrogen water (HW) had antiosteoporotic property in rats exposed to HLS in vivo. In addition, we explored the effect of incubation with hydrogen-rich medium (HRM) on osteoblastic and osteoclastic function in vitro.

Material and methods

Material and animals

Chemicals, reagents, and drugs were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise stated. Cell culture supplies were obtained from Invitrogen except where indicated.

All the animals were entrained to controlled temperature (21–25°C), 12-h light and 12-h dark cycles (light, 08:00–20:00 hours; darkness, 20:00–08:00 hours), and free access to food and tap water. All the animals used in this study received humane care in compliance with institutional animal care guidelines. All the surgical and experimental procedures were in accordance with institutional animal care guidelines and were approved by the local institutional committee. Male Sprague–Dawley (SD) rats were purchased from the Vital-Aiver Animal Ltd (Beijing, China).

Hydrogen water preparation

For the saturated hydrogen water preparation, H_2 was dissolved into pure water for 2 h under 0.4 MPa. The saturated hydrogen water was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume. Hydrogen water was freshly prepared every week, which maintained a continuous concentration. Each day, hydrogen water from the aluminum bag was placed in a closed glass vessel, which ensured that the hydrogen concentration was greater than 0.4 mM after 1 day.

Hydrogen-rich medium preparation

Over a 2-h period, we dissolved H_2 into DMEM under 0.4 MPa pressure based on the method described by Ohsawa et al. [18]. We dissolved O_2 into a second medium by bubbling O_2 gas at the saturated level, and CO_2 into a third medium by bubbling CO_2 gas. All three media were maintained at atmospheric pressure. Then, we combined the three media (H_2 medium/O_2 medium/CO_2 medium) in the proportion 75:20:5 % (vol/vol/vol) and added fetal bovine serum to achieve a final concentration of 1 %. For culture, we put the combined medium into a culture flask. Then, we filled the culture flask with mixed gas consisting of 75 % H_2, 20 % O_2, and 5 % CO_2 (vol/vol/vol) and cultured cells in the closed culture flask.

Hindlimb suspension

Briefly, a strip of elastic tape was applied to the surface of the tail. A clip at the end of the tape was fastened to an overhead bar and adjusted to maintain the rats at a 30° head-down tilt with the hindlimbs elevated above the floor of the cage. The rats were subjected to HLS for 6 weeks. Control rats were housed individually in similar conditions, but not suspended. Parallel soleus muscle-to-body mass ratio was used to test the efficacy of simulated microgravity.

In vitro simulated microgravity

The rotary wall vessel bioreactor (RCCS; Synthecon, Houston, TX, USA), developed by the National Aeronautics and Space Administration, was used to model microgravity in vitro as previously described [19]. Cells were either rotated about a horizontal axis perpendicular to the gravitational vector to randomize gravitational vectors across the surface of the platelets and generate microgravity of 10^{-2} G or rotated about a vertical axis parallel to the gravitational vector to experience normal gravitational forces and serve as a control (1 G) environment.

Cell culture

The murine osteoblastic cell line MC3T3-E1 and a murine macrophage cell line RAW264.7 were cultured at 37°C in 5 % CO_2 and 90 % humidity in α-MEM medium, supplemented with 10 % fetal bovine serum and 100 μg/ml gentamicin before treatment.

Bone mineral density measurement

The rats were subjected to HLS for 6 weeks. Then, bone mineral density (BMD) of femur and lumbar vertebra was measured with a by dual energy X-ray absorptiometry NORLAND XR-46 (Norland Co., Fort Atkinson, WI, USA) using the small animal program.

Biochemical analysis

Serum concentration of Ca and inorganic phosphorus (P) was measured using an autoanalyzer (Hitachi 7170; Hitachi, Tokyo, Japan). Creatinine (CRE) was quantified with QuantiChrom Creatinine Assay Kits (BioAssay Systems, Hayward, CA, USA). Urinary deoxypyridinoline (DPD) excretion was measured using an OSTEOLINKS-DPD EIA kit (Sumitomo Seiyaku, Osaka, Japan), and the data were corrected for the urinary CRE concentration. Plasma pH was determined by using blood gas analysis in an automatic blood gas analyzer (ABL system, Radiometer, Copenhagen, Denmark). The levels of NOx, the stable end products of nitric oxide (NO), in serum were measured using a Total Nitrite/Nitrate Assay kit (Dojindo, Kumamoto, Japan) which employs the Griess method.

Measurement of mechanical properties

Using a mechanical strength analyzer (TK-252CC; Muromachi Kikai Co., Ltd., Tokyo, Japan), the mechanical strength including ultimate compressive load (newton), the stiffness (newton per millimeter), and the energy (megajoule) of the lumbar vertebra (L5) and left femur was measured using a compression test [20] and a three-point bending test [21], respectively.

Assay of malondialdehyde levels

Bone homogenates and plasma were used for the determination of malondialdehyde (MDA) using a kit (Cayman, Ann Arbor, MI, USA). MDA content of bone homogenates was calculated as micromole per gram of protein.

Measurement of serum total antioxidant capacity

Serum total antioxidant capacity (TAC) was measured with an Antioxidant Assay Kit (Cayman). The final results were expressed as micromole trolox equivalents per milliliter.

Assay of total sulfhydryl levels

Bone homogenates were used for the determination of total sulfhydryl (t-SH) using a Glutathione Assay Kit (Cayman). t-SH content of bone homogenates was calculated as micromole per gram of protein.

Assay of peroxynitrite levels in bone

The peroxynitrite ($OONO^-$) production in bone homogenate was detected based on the method described by Elks et al. [22]. Briefly, samples were incubated at 37°C with CMH (1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine, 200 μM) for 30 min, and then with CPH (1-hydroxy-3-carboxypyrrolidine, 500 μM) for 30 min for $OONO^-$ measurement.

Quantitative real-time PCR analysis

Total RNA was extracted using Trizol (Life Technologies Inc., Gaithersburg, USA) according to the manufacturer's protocol. RT-PCR analysis was performed with a QuantiTectTM SYBR® Green PCR (Tiangen, Shanghai, China) according to the manufacturer's instructions. The sequences of primers are listed in Table 1S (supplementary data). The highly specific measurement of mRNA was carried out for ALP, cathepsin K (CathK), integrin β_3 (Intβ3), tartrate-resistant acid phosphatase (TRAP), runt-related transcription factor 2 (Runx2), and 18 S using the LightCycler system (Bio-Rad, Carlsbad, USA). Each sample was run and analyzed in duplicate. Target mRNA levels were adjusted as the values relative to 18 S, which was used as the endogenous control to ensure equal starting amounts of cDNA. When comparison between two groups was performed, the control group was used as the calibrator with a given value of 1, and the other groups were compared with this calibrator.

Western blotting analysis

The protein concentration was determined with bovine serum albumin as a standard by a Bradford assay. Equal amount of protein preparations (10 μg in 10 μl buffer) was run on SDS-polyacrylamide gels, electrotransferred to polyvinylidine difluoride membranes, and blotted with a primary antibody against inducible nitric oxide synthetase (iNOS) (1:500, Santa Cruz), p-Erk1/2 (1:1,000, Santa Cruz), and Erk1/2 (1:400, Santa Cruz) overnight at 4°C using slow rocking. Then, they were blotted with HRP-conjugated secondary antibody (1:5,000) and HRP-conjugated monoclonal antibody against GAPDH (1:10,000). Immunoreactive bands were detected by a chemiluminescent reaction (ECL kit, Amersham Pharmacia).

Reactive oxygen species production

The culture medium was replaced with phenol-red-free DMEM containing 2′,7′-dichlorodihydrofluorescein diacetate (10 μM) before exposure to modeled microgravity. Then, the ROS production was measured with a fluorescence reader.

Alkaline phosphatase activity assay

The induction of alkaline phosphatase (ALP) is an unequivocal marker for bone cell differentiation. After exposure to modeled microgravity and incubation with HRM for 7 days, the medium was removed and the cell monolayer was gently washed twice with PBS. Cells were then lysed with cell lysis buffer (0.5 ml for a 35-mm dish) and centrifuged at 12,000×*g* for 10 min. The resulting supernatant was used for the measurement of ALP activity and protein concentration with a commercially available ALP activity assay kit (Cell Biolabs, San Diego, CA, USA) and a BCA-protein assay kit (Bio-Rad, Hercules, CA, USA), respectively. ALP activity was expressed as nanomole per minute milligram protein.

Measurement of osteoprotegerin and receptor activator of nuclear factor kappa B ligand

After exposure to modeled microgravity and incubation with HRM for 96 h, the concentrations of osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL) proteins in the supernatant of cultured MC3T3-E1 were measured using the kits according to the manufacturer's recommendations (R&D Systems).

Determination of intracellular nitric oxide

After exposure to modeled microgravity and treated with HRM for 96 h, intracellular NO content of cultured MC3T3-E1 was measured using a membrane-permeable indicator dye, 4-amino-5-methylamino-2′,7′-difluororescein diacetate (DAF-FM), which reacts with NO to form a green fluorescent product. Fluorescence intensity was recorded at the excitation wavelength of 488 nm. The single cell intracellular NO concentration was analyzed as the average intensity of DAF-FM fluorescence.

Measurement of osteoclastogenesis

RAW264.7 cells (2.5×10^5) were exposed to modeled microgravity and stimulated with RANKL (20 ng/ml) and incubated with HRM for 4 days. Then, the cells were TRAP-stained based on a previously described method [23]. For further quantification, multinucleated TRAP-positive cells with different numbers of nuclei were counted in two different categories: 3 to 30 nuclei and those with greater than 30 nuclei.

Study design

Experiment 1

SD rats were divided into four groups of 12 animals each and treated for 6 weeks as follows: (1) control rats drinking normal water (Con); (2) hindlimb suspended rats drinking normal water (HLS); (3) control rats drinking HW (Con+HW); and (4) hindlimb suspended rats drinking HW (HLS+HW). Six weeks later, the extent of bone loss and oxidative stress-related markers were measured.

Experiment 2

The MC3T3-E1 cells were exposed to modeled microgravity and treated with HRM for 96 h. The intracellular ROS and NO formation, ALP activity, Runx2 mRNA expression, OPG and RANKL content in supernatant, iNOS, and p-Erk1/2 and Erk1/2 protein expression were determined. The MC3T3-E1 cells were exposed to modeled microgravity and treated with HRM for 7 days. The ALP activity was determined.

Experiment 3

The RAW264.7 cells were exposed to modeled microgravity and treated with HRM for 96 h. RANKL was used to stimulate osteoclastogenesis. ROS formation, CathK and Intβ3 and TRAP mRNA expression, and osteoclastogenesis were determined. The in vitro experiments were performed at least three times and each experiment was performed with replicates.

Statistical analysis

All data are expressed as mean±SD. Comparison between two groups was testified by two-way ANOVA. The Dunnett's post hoc test was performed subsequent to analysis of variance. $P<0.05$ was considered statistically significant. Statistical analysis was performed using SPSS 11.0.0 software (SPSS Inc., Chicago, IL, USA).

Results

Effects of treatment with HW in rats exposed to HLS

SD rats were exposed to HLS for 6 weeks and treated with or without HW. The soleus muscle-to-body mass ratios were significantly reduced in HLS and HLS+HW rats, which confirmed the efficacy of simulated microgravity in this set of experiments. Neither exposure to HLS nor treatment with HW had a significant effect on body weight, food intake, plasma pH, and serum Ca and P (Table 1).

Table 1 Effects of HW on general data in rats exposed to HLS (n=12 in each group)

	Con	Con+HW	HLS	HLS+HW
Body mass (g)	393±35	387±28	382±33	385±29
Soleus/body mass (mg/g)	0.39±0.06	0.41±0.07	0.20±0.05*	0.21±0.06*
Food intake (g/day)	20.3±2.8	19.7±3.2	20.4±2.2	21.5±3.5
Plasma pH	7.44±0.14	7.52±0.15	7.48±0.13	7.51±0.14
Serum Ca (mmol/l)	2.39±0.18	2.43±0.18	2.47±0.24	2.37±0.23
Serum P (mmol/l)	1.55±0.19	1.57±0.17	1.62±0.20	1.55±0.22

HW hydrogen water, *HLS* hindlimb suspension, *Ca* calcium, *P* inorganic phosphorus

*P<0.05 versus the control group

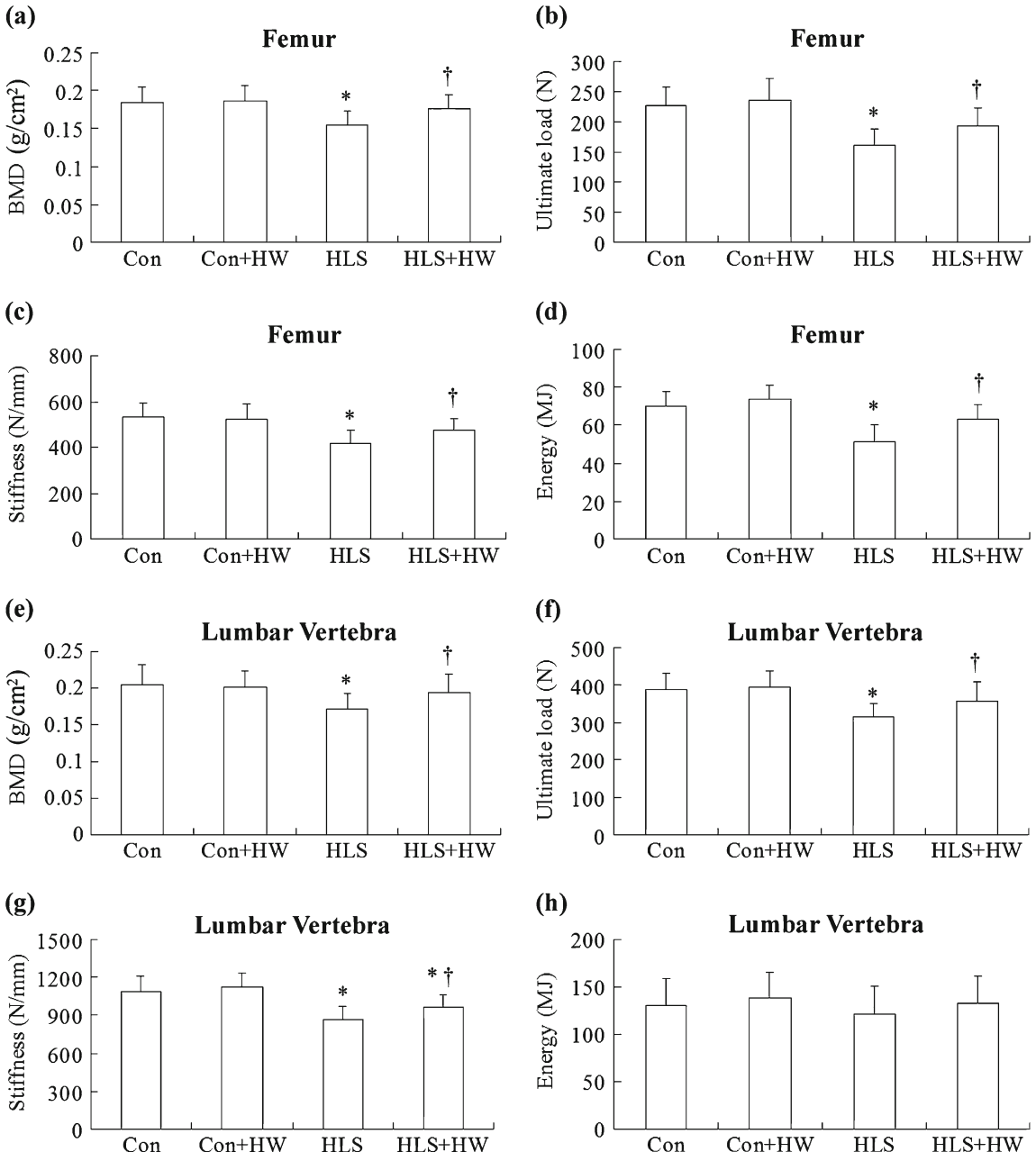

Fig. 1 Effects of HW on BMD and mechanical properties in femur and lumbar vertebra of rats exposed to HLS. SD rats were exposed to HLS and treated with or without HW for 6 weeks. Femur and lumbar vertebra were removed for the determination of BMD (**a**, **e**) and mechanical properties including ultimate load (**b**, **f**), stiffness (**c**, **g**), and energy (**d**, **h**). n=12 in each group. *HW* hydrogen water, *HLS* hindlimb suspension, *BMD* bone mineral density. *P<0.05 versus the control group; †P<0.05 versus the HLS group

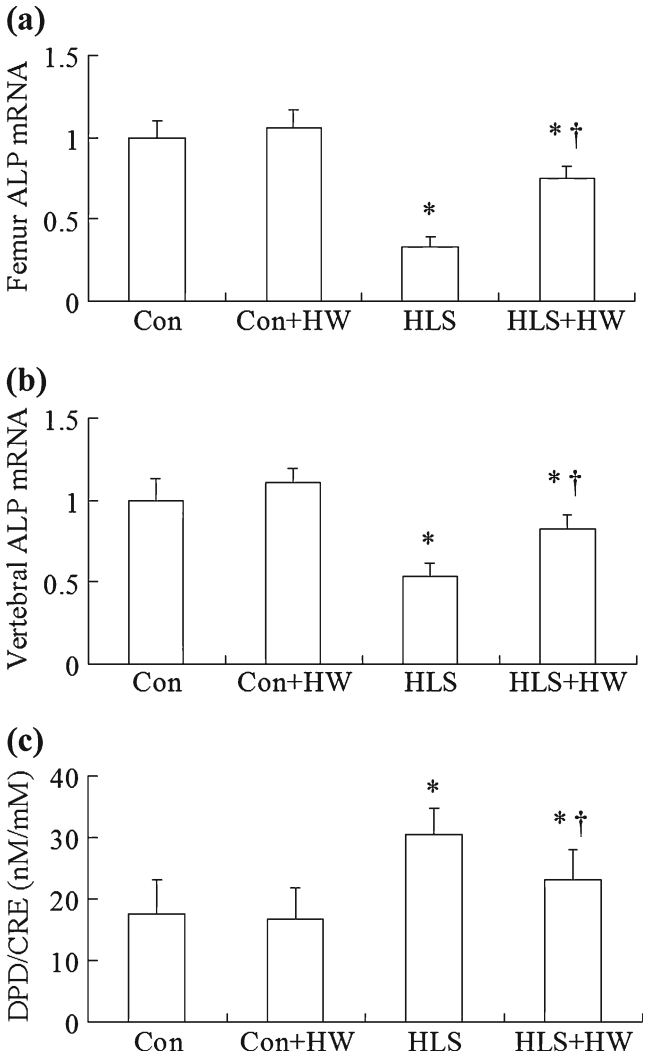

Fig. 2 Effects of HW on bone formation and bone resorption in rats exposed to HLS. SD rats were exposed to HLS and treated with or without HW for 6 weeks. Femur and lumbar vertebra were removed for the determination of ALP mRNA expression (biomarker of bone formation, **a**, **b**). Urine was collected for determination of DPD excretion (biomarker of bone resorption, **c**). $n=12$ in each group. *HW* hydrogen water, *HLS* hindlimb suspension, *ALP* alkaline phosphatase, *CRE* creatinine, *DPD* deoxypyridinoline. *$P<0.05$ versus the control group; †$P<0.05$ versus the HLS group

Exposure to HLS decreased femur BMD, ultimate load, stiffness, and energy significantly. Treatment with HW restored femur BMD, ultimate load, stiffness, and energy (Fig. 1a–d). Exposure to HLS decreased vertebral BMD, ultimate load, and stiffness significantly and decreased vertebral energy but not significantly. Treatment with HW restored vertebral BMD and ultimate load and restored stiffness partly (Fig. 1e–h). In addition, structural analysis revealed that exposure to HLS decreased BV/TV, Tb.N, and Tb.Th in lumbar vertebra and BV/TV and Tb.N in femur, which were also restored by treatment with HW (Table 2S). These results indicated that treatment with HW effectively reversed HLS-induced bone loss in rats.

Exposure to HLS decreased femur and vertebral ALP mRNA expression (Fig. 2a, b) and increased urinary DPD excretion (Fig. 2c). Treatment with HW restored femur and vertebral ALP mRNA expression and inhibited urinary DPD excretion (Fig. 2a–c), which indicated that treatment with HW improved bone formation and inhibited bone resorption in rats exposed to HLS.

Exposure to HLS increased plasma MDA content (Fig. 3a) and decreased serum TAC (Fig. 3b), but had no significant effect on serum NOx (Fig. 3c). Treatment with HW decreased plasma MDA content and increased serum TAC (Fig. 3a, b). Exposure to HLS increased MDA content (Fig. 3d, g) and $OONO^-$ content (Fig. 3f, i) and decreased t-SH content (Fig. 3e, h) in femur and lumbar vertebra. Treatment with HW decreased MDA content (Fig. 3d, g) and $OONO^-$ content (Fig. 3f, i) and increased t-SH content (Fig. 3e, h). These results demonstrated that exposure to HLS in rats resulted in oxidative stress and treatment with HW effectively abated oxidative stress both in the circulating system and in bone tissue.

Effects of incubation with hydrogen on the osteoblastic cell line MC3T3-E1

Exposure to modeled microgravity in MC3T3-E1 led to increased ROS formation (Fig. 4a) and reduced osteoblastic differentiation marked by decreased ALP activity (Fig. 4b) and Runx2 mRNA expression (Fig. 4c). Incubation with HRM suppressed ROS formation and restored osteoblastic differentiation (Fig. 4a–c).

Exposure to modeled microgravity in MC3T3-E1 resulted in a reduction of OPG (Fig. 4e) and RANKL content (Fig. 4d) in the supernatant, but led to an augmentation of the ratio of RANKL/OPG (Fig. 4f). Incubation with HRM increased OPG and RANKL content and decreased the ratio of RANKL/OPG (Fig. 4d–f).

Exposure to modeled microgravity in MC3T3-E1 increased intracellular NO content (Fig. 4g) and upregulated iNOS expression (Fig. 4h), which was reversed by treatment with HRM. Exposure to modeled microgravity in MC3T3-E1 increased Erk1/2 phosphorylation (Fig. 4i), which was also reversed by treatment with HRM.

Effects of incubation with hydrogen in RAW264.7

Exposure to modeled microgravity in RAW254.7 led to increased ROS formation (Fig. 5a) and enhanced osteoclastic differentiation marked by upregulated CathK (Fig. 5b) and Intβ3 mRNA expression (Fig. 5c) and increased osteoclastogenesis marked by increased TRAP mRNA expression (Fig. 5d) and TRAP-positive multinucleated osteoclasts (Fig. 5e). Treatment with HRM decreased ROS content,

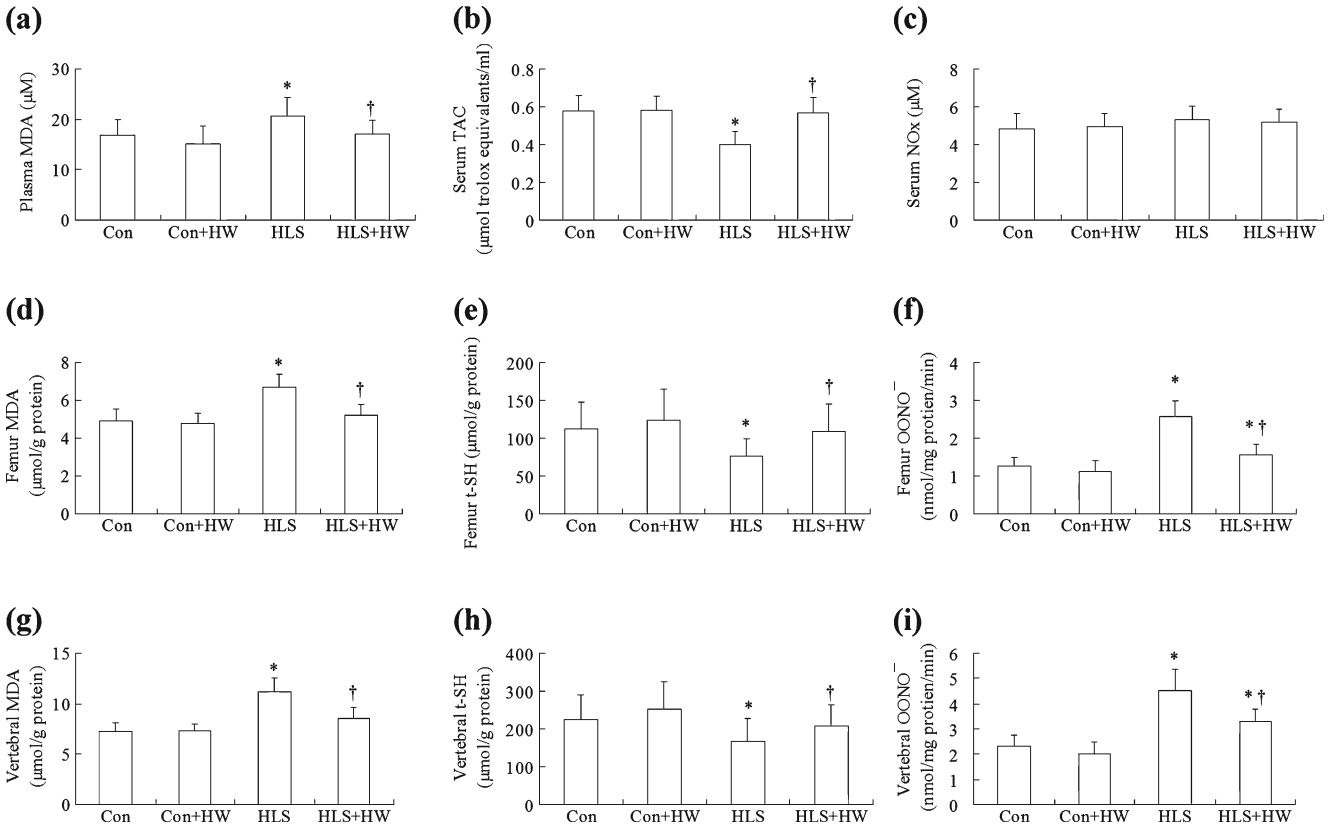

Fig. 3 Effects of HW on oxidative stress in rats exposed to HLS. SD rats were exposed to HLS and treated with or without HW for 6 weeks. Plasma was collected for determination of MDA content (**a**); serum was collected for determination of TAC (**b**) and NOx content (**c**); femur and lumbar vertebra were removed for the determination of MDA (**d**, **g**), t-SH (**e**, **h**), and $OONO^-$ (**f**, **i**). $n=12$ in each group. *HW* hydrogen water, *HLS* hindlimb suspension, *MDA* malondialdehyde, *OONO*$^-$ peroxynitrite, *TAC* total antioxidant capacity, *t-SH* total sulfhydryl, *NOx* nitrite and nitrate. *$P<0.05$ versus the control group; †$P<0.05$ versus the HLS group

osteoclast differentiation, and osteoclastogenesis induced by exposure to modeled microgravity.

Discussion

Exposure to microgravity resulted in aggravated oxidative stress not only in the circulating system [7], but also in the brain [9], liver [24], and muscle [25] in animals or humans. In the present study, increased lipid peroxidation and $OONO^-$ content and decreased t-SH in femur and lumbar vertebra in hindlimb unloaded rats for the first time revealed that modeled microgravity induced notable oxidative stress in bone tissue of rats. In vitro studies revealed that modeled microgravity induced significant ROS formation in both cultured osteoblast and osteoclast, which further demonstrated oxidative stress. Consumption of HW alleviated HLS-induced bone loss in femur and lumbar vertebra and incubation with HRM improved osteoblastic function and inhibited osteoclastogenesis, indicating that oxidative stress played an important role in the pathological process of bone loss and osteoblastic and osteoclastic function when exposed to modeled microgravity.

Administration of HW enhanced ALP expression in bone and decreased urinary DPD excretion, indicating that hydrogen had a beneficial effect on both bone formation and bone resorption when exposed to microgravity. Then, the protective effect of incubation with HRM on osteoblast cell line MC3T3-E1 and preosteoclast cell line RAW264.7 was investigated.

Thus, to investigate the pathophysiology during spaceflight, several ground-based systems, including the two-dimensional (2D) and three-dimensional (3D) clinostats, the rotating wall vessel, and diamagnetic levitation, have been developed to simulate microgravity using cultured cells and tissues [26–29]. In the present study, the rotating wall vessel developed by NASA was used to simulate microgravity in vitro.

Modeled microgravity induced oxidative stress in cultured MC3T3-E1, which further indirectly demonstrated that microgravity was able to induce oxidative stress in bone tissue. Incubation with HRM effectively eliminated excessive ROS induced by exposure to microgravity.

Previous reports about the effect of modeled microgravity on osteoblastic differentiation were contradictory. Rucci et al. found that microgravity modeled with the rotating wall vessel bioreactor failed to affect osteoblast differentiation and

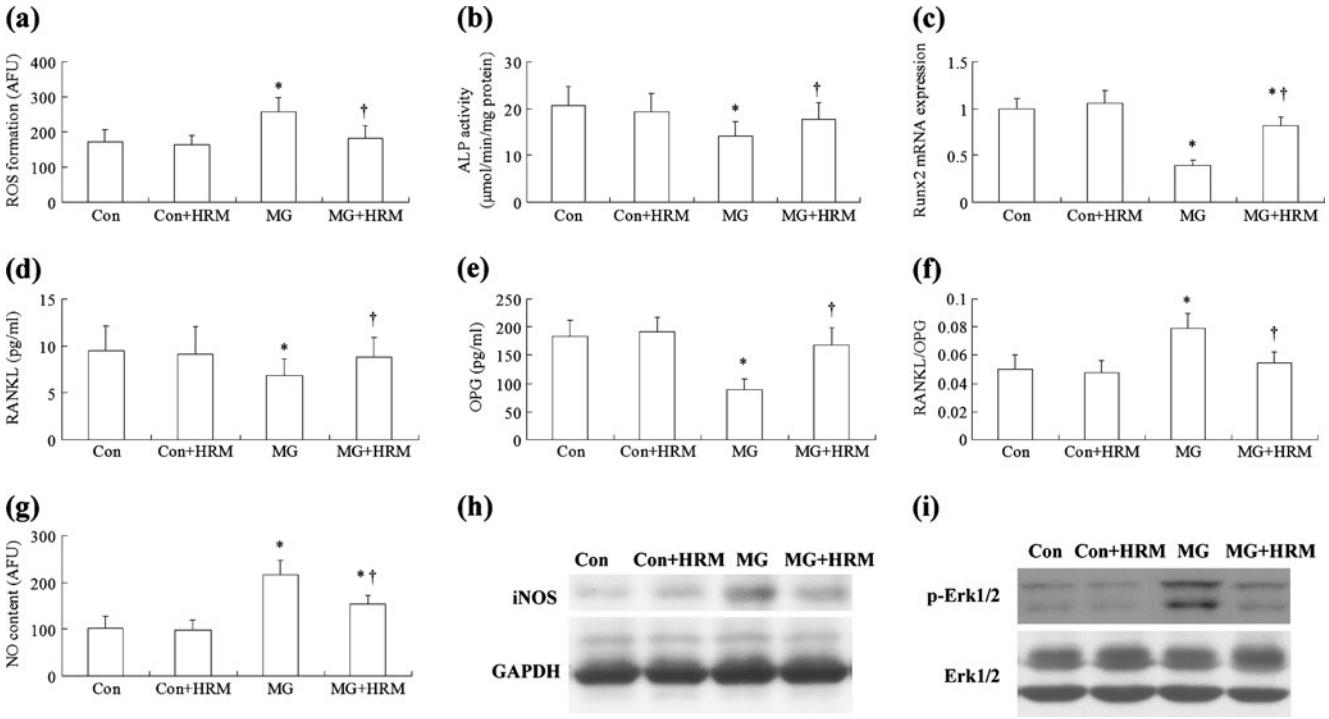

Fig. 4 Effects of HRM on osteoblast cell line MC3T3-E1 exposed to modeled microgravity. MC3T3-E1 cells were exposed to modeled microgravity and incubated with HRM. The intracellular ROS content (**a**), ALP activity (**b**), Runx2 mRNA expression (**c**), OPG and RANKL content in supernatant (**d**, **e**), ratio of RANKL/OPG (**f**), NO formation (**g**), iNOS protein expression (**h**), and Erk1/2 phosphorylation (**i**) were determined. *HRM* hydrogen-rich medium, *OPG* osteoprotegerin, *RANKL* receptor activator of nuclear factor kappa B ligand, *NO* nitric oxide, *iNOS* inducible nitric oxide synthase, *ROS* reactive oxygen species, *ALP* alkaline phosphatase, *Runx2* runt-related transcription factor 2, *MG* microgravity, *AFU* arbitrary fluorescence units. *$P<0.05$ versus the control group; †$P<0.05$ versus the MG group

function [30]. Boehrs et al. found that microgravity simulated by 3D clinostats enhanced osteoblast differentiation [31]. But most reports demonstrated that microgravity modeled by the rotating wall vessel bioreactor, 3D clinostats, and diamagnetic levitation inhibited osteoblastic differentiation in cultured calvaria, human mesenchymal stem cells, 2 T3 preostoblasts, and

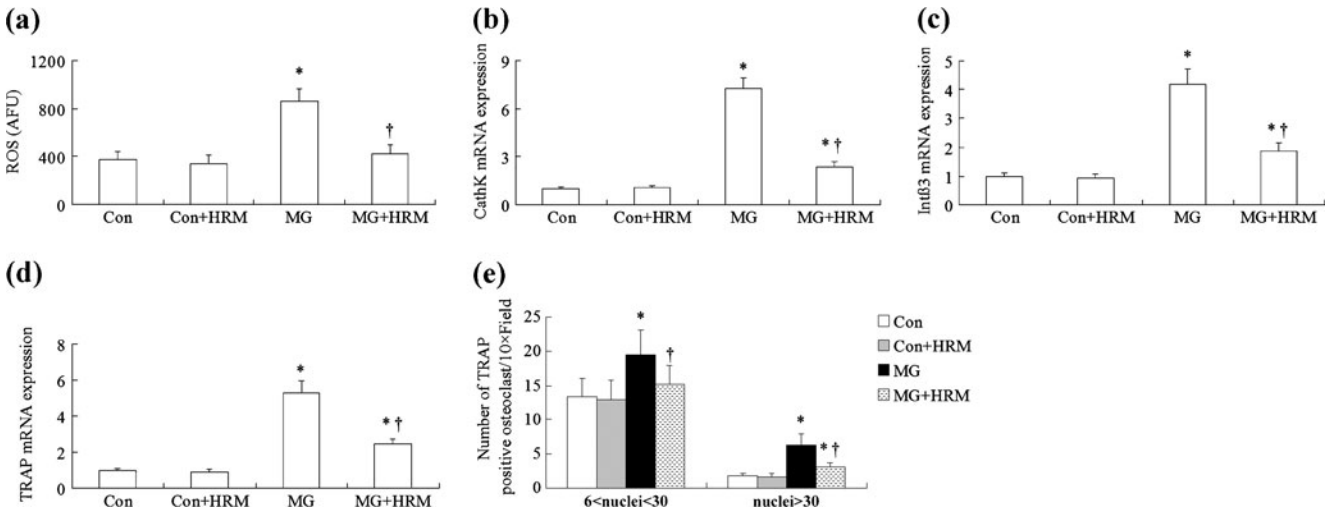

Fig. 5 Effects of HRM on preosteoclast cell line RAW264.7 exposed to modeled microgravity. RAW264.7 cells were exposed to modeled microgravity and incubated with HRM. Osteoclast differentiation and osteoclastogenesis were induced by incubation with RANKL (20 ng/ml). The intracellular ROS content (**a**), CathK (**b**) and Intβ3 (**c**) and TRAP mRNA expression (**d**), and multinucleated TRAP-positive cells (**e**) were determined. *HRM* hydrogen-rich medium, *RANKL* receptor activator of nuclear factor kappa B ligand, *MG* microgravity, *CathK*, cathepsin K, *Intβ3* integrin β_3, *TRAP* tartrate-resistant acid phosphatase, *AFU* arbitrary fluorescence units. *$P<0.05$ versus the control group; †$P<0.05$ versus the MG group

MC3T3-E1 cells [19, 29, 32–34]. In the present study, microgravity modeled by the rotating wall vessel bioreactor suppressed differentiation of MC3T3-E1 cells marked by decreased ALP activity and Runx2 expression. In addition, incubation with HRM restored osteoblastic differentiation, indicating that microgravity-induced oxidative stress contributed to suppressed osteoblastic differentiation.

Osteoclasts are activated by binding of the RANKL [35] to its cognate receptor, RANK, whereas OPG, a soluble member of the tumor necrosis receptor superfamily, acts as a naturally occurring decoy receptor that competes with RANK for binding of RANKL [36, 37]. The balance of these two molecules plays a critical role in the control of osteoclastogenesis. In the current study, we found that modeled microgravity suppressed OPG and RANKL secreted from MC3T3-E1 cells, but increased the ratio of RANKL/OPG. Therefore, modeled microgravity might exacerbate the osteoclastogenesis through paracrine regulation of the release of OPG and RANKL from osteoblasts, at least in part. Treatment with hydrogen prevented the augmentation of the ratio of RANKL/OPG, indicating that microgravity-induced oxidative stress was also involved in this paracrine regulation.

Another interesting finding of our work was that modeled microgravity induced iNOS activation and NO formation in cultured osteoblasts, which was consistent with many previous in vitro and in vivo reports [38, 39]. The impact of NO on bone metabolism is bidirectional. Under normal conditions, NO derived from eNOS is one of the important local mediators and second messengers of systemic hormones including calcitonin gene-related peptide, parathyroid hormone, and sex steroids, particularly estrogen, which take part in the regulation of bone function [40]. But excess local production of NO derived from iNOS aggravates bone loss, and excess administration with exogenous NO donor induces cytotoxicity in osteoblastic cells [41]. Our result demonstrated that treatment with hydrogen could prevent microgravity-induced cell injury in osteoblasts through suppressing iNOS activation.

Previous studies demonstrated that ROS-induced Erk1/2 phosphorylation is involved in cell injury [42]. In the current study, modeled microgravity induced Erk1/2 phosphorylation and hydrogen treatment reversed it, indicating that there might be a microgravity–ROS–Erk1/2 pathway in the microgravity-induced cell injury.

Modeled microgravity aggravated RANKL-induced ROS formation in preosteoclast RAW264.7. Accumulating evidence indicated the crucial role for ROS in bone resorption and RANKL-induced osteoclast differentiation and osteoclastogenesis [43]. Modeled microgravity enhanced osteoclast differentiation and osteoclastogenesis and treatment with hydrogen restored it, indicating that microgravity exposure enhanced bone resorption through inducing oxidative stress.

In conclusion, consumption with hydrogen water alleviated microgravity-induced bone loss through abating oxidative stress, restoring osteoblastic differentiation, and suppressing osteoclastic differentiation. Oxidative stress plays an important role in the microgravity-induced bone loss. Molecular hydrogen could thus be envisaged as a nutritional countermeasure for spaceflight but remains to be tested in humans.

Conflicts of interest None.

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