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ORIGINAL PAPER

Hydrogen-rich saline prevents bone loss in diabetic rats induced by streptozotocin

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Abstract
Purpose As an antioxidant molecule, hydrogen has been received much more attention and reported to be used as the treatment strategy for various diseases. In this study, we hypothesize that systemic delivery of hydrogen saline water may improve the reservation of bone tissue in the tibias and femurs of osteoporotic rats caused by diabetes mellitus (DM), which is characterized by increased levels of oxidative stress and overproducing reactive oxygen species (ROS).
Methods The animals were divided into three groups of 12 animals and lavaged with normal saline (normal control and DM), or hydrogen saline water (DM + HRS). General status, blood glucose level, tibial and femoral mechanical strength, and micro-CT scans of the proximal tibia were recorded and analyzed.
Results After 12 weeks, the glucose level was significantly decreased in the DM + HRS group compared with that of the DM group. Micro-CT scans showed that bone volume/total volume, connectivity density, trabecular thickness, and trabecular number were significantly increased compared with the DM group. Mechanical results of energy, stiffness and elastic modulus in the DM + HRS group were significantly higher than in the other groups for the tibia and femur.
Conclusions The results indicate that the systemic delivery of hydrogen saline water, which is safe and well tolerated, preserves bone volume and decreases fracture risks in streptozotocin-induced diabetic status rats, whose bone structure or inherent material properties of bone tissues are changed.

Keywords Hydrogen · Diabetes · Osteoporosis

Introduction

Osteoporosis (OP) is a common disease which is characterized by the loss of bone mass, the deterioration of bone microstructure and increased fragility in elder people, and can be classified as primary and secondary OP. Primary OP is a common clinical injury, especially in menopausal women and elder people, and the incidence has become higher as the average lifespan of people increases. Secondary osteoporosis contains endocrine disease such as diabetes, induced by drug such as glucocorticoid and heparin, Huppert's disease. Among these diseases, diabetes mellitus (DM) is considered as an important protopathy for the development of osteoporosis besides menopause [1]. DM with related complications has increased worldwide and is becoming a major threat to human health and a tremendous burden in China. The fragility of bone is increased in patients with either type 1 (T1) DM or type 2 (T2) DM, which are caused by absolute or relative insulin insufficiency. Osteoporosis in diabetes is a metabolic bone disorder that may result in osteopenia, microstructural changes,

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decreased mechanical strength and increased fragility, even fractures, which is one of the main complications of DM receiving very little attention.

Most complications of DM, including osteoporosis, kidney failure, cardiovascular problems, and neuropathy, are associated with oxidative stress. High blood glucose increases the level of oxidative stress and produces more reactive oxygen species (ROS), including hydroxyl radicals (OH-), peroxynitirite anions (ONOO), hydrogen peroxide (H_2O_2) and superoxide (O_2-) and then exhausts endogenous antioxidants. Oxidative stress increases bone resorption through elevating the expression levels of the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), and leading to apoptosis of osteoblasts and the inhibition of bone formation [2]. Many other exogenous antioxidants, such as vitamin E, C and coenzyme Q, have been used to diminish the enhanced ROS to prevent occurrences of complications in diabetes. However, associated limitations of these agents, such as inefficiency and non-specificity, have urged people to discover other safer and more effective molecules to treat oxidative stress, such as alpha-lipoic acid (ALA) and hydrogen.

Hydrogen, which has no toxicity or smell and is present in many chemical compounds, is the lightest of all gas molecules. Recent biological and clinical studies have identified that hydrogen as an antioxidant has an anti-inflammatory effect and selectively reacts with hydroxyl radicals, which are the most cytotoxic ROS in cells, tissues and organs. Hydrogen with high permeability to cell membranes is mild, targets intracellular inflammatory factors effectively and does not disturb metabolic oxidation–reduction reactions or ROS involved in cell signaling. It has been proved to have a protective effect on oxidative stress-related disorders, such as neurovascular dysfunction, myocardial, liver, heart or intestinal injury, nephropathy, metabolic syndrome, uremia, rheumatoid arthritis, Alzheimer's disease and Parkinson's disease, and acute pancreatitis [3–6]. Hydrogen-rich water is safer, more convenient for administration and considered as a feasible preventative/therapeutic strategy to prevent astronauts from radiation-induced oxidative stress events [7]. As another antioxidant, alpha-lipoic acid antagonizes osteoporosis through decreasing ROS, promotes osteoblastic formation and prevents bone loss in ovariectomized rats [8]. Although there are reports that hydrogen has no effects on normal rats, it can alleviate microgravity-induced bone loss in rats [9, 10]. However, the effects of hydrogen on bone tissue of diabetic patients remains unknown and few studies report its function on osteoporosis. The aim of the study is to assess the hypothesis that systemic supplement of hydrogen-rich saline (HRS) can prevent bone loss in those diabetic rats.

Materials and methods

Animals and induction of DM

The animals (8-week-old SD rats) were purchased from Hebei Medical University Animal Center. The study was approved by the Ethics Board of the Third Hospital of Hebei Medical University and conducted in accordance with the institutional guidelines for the care and treatment of rats. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Hebei Medical University.

These rats were raised in a relatively stable ambient environment (temperature 24 ± 2 °C, humidity 45–55% and 12-hour light-dark cycle from seven a.m. to seven p.m., specific pathogen free) with free access to tap water and rodent chow diet, and were provided with nestlets and wood shavings for environment enrichment. The changes in appearance were monitored and solved immediately before induction. A total of 36 (12 animals per group) eight week-old male Sprague–Dawley rats (200 g ± 13 g) were used in this study. All rats were allowed for a one week acclimation period before interventions. The rats were randomly divided into normal control (NC, $n = 12$) group, diabetic model group (DM, $n = 12$) group, and hydrogen-rich saline treatment group (DM + HRS group, $n = 12$) by stratified randomization based on their baseline body weight.

Rats of the DM ($n = 12$) and DM + HRS ($n = 12$) groups were fasted for 12 hours and given intraperitoneal injection of streptozotocin (STZ; Sigma Aldrich, St. Louis, MO, USA) 65 mg/kg (dissolved in 0.1 mol/L sodium citrate buffer, PH4.3) to induce as diabetic rats. After three days of intraperitoneal injection, the blood glucose samples were measured with a Roche glucose meter through drawing blood from the caudal vein. Only STZ-treated rats whose fasting blood glucose concentration was ≥16.7 mmol/L, and urine sugar in the range +++ to ++++ were included and be considered as qualified DM models at both 24 hours and one week after STZ injection in the study. If they are not qualified, new SD rats were induced to make sure the number of rats in the DM and DM + HRS groups were 12 respectively. Plasma glucose was measured at eight and 12 weeks after inductions. Blood was collected (100–200 μL) from the abdominal aorta immediately prior to being euthanized. The sera and cells were centrifugated for 15 minutes at 5,500 RPM, 4 °C, and stored at −80 °C until assessment.

Administration of hydrogen-rich saline

For a substitution for STZ, citric acid buffer (Sigma Aldrich, St. Louis, MO, USA) in place of STZ was injected in rats of the NC group ($n = 12$) with an empty belly. After the accomplishment of induction of DM, the rats in the DM + HRS group ($n = 12$) were lavaged with hydrogen-rich saline (12 ml/kg, concentration, ≥0.6 mmol/L, ≥0.6 ppm; Beijing

Hydrovita, Beijing, China) daily for three months, while rats were lavaged with normal saline (12 mL/kg) in the NC and DM groups [11]. Saturated hydrogen-rich saline was stored at 4 °C in an aluminum bag with no dead volume under atmospheric pressure. Hydrogen-rich saline was freshly prepared every week to ensure a uniform concentration of 0.6 mmol/l. All rats were euthanized at 12 weeks after the last injection of HRS or normal saline, and bloods from their aorta abdominalis were harvested. The femur and tibia were harvested and stored at −20 °C until analysis.

Biochemical analysis

After beginning of administration, the general condition of the animals was observed. At eight and 12 weeks after the induction of diabetes, rats were euthanized for each group respectively and measure their blood glucose, serum creatinine with QuantiChrom Creatinine Assay Kits (Scr, BioAssay Systems, Hayward, CA, USA), 24 hours urine albumin, and blood urea nitrogen (BUN) were measured. Malondialdehyde (MDA) was also measured to evaluate the level of oxidative stress with a kit (Cayman).

Micro-CT scan

Three months after being euthanized, the tibias of six rats (n = 12) were removed and the relatively soft tissues were dissected away. Tibia bones of rats in the three groups were scanned using a cone beam-type desktop micro-computed tomography (micro-CT) scanner (μCT40; Scanco Medical, Bassersdorf, Switzerland)) with an isotropic voxel size of 10.5 μm (70 kV, 114 μA) and an acquisition of 500 projections per 180°. The noise of gray-scale images was suppressed using a three-dimensional (3-D) Gaussian filter with a protocol (sigma = 1.0, support = 1.0). All samples were scanned within two days after euthanasia. Proximal tibia was analyzed with established protocols (lower attenuation = 220 and upper attenuation = 1,000, μCT40 Evaluation Program v. 6.5–1; Scanco Medical). A total of 220 CT slices were obtained from a 2-mm region of interest (ROI). The ROI was chosen on two-dimensional (2-D) CT images using manually drawn contours. The percent bone volume/total volume (BV/TV, %), connectivity density (Conn, 1/mm^3), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, 1/mm), and trabecular separation (Tb.Sp, mm), structural model index (SMI: 0, plate; 3, cylindrical rod; 4, sphere) were calculated using the manufacturer's software of the micro-CT machine. Multiplanar reformations were used to obtain 3-D images. A BMD threshold (211 mg/cm^3) was defined to separate mineralized tissue from surrounding substances. After CT scan, all specimens were kept at −20 °C until conducting mechanical tests.

Biomechanical testing

The specimens were thawed overnight and immersed in 0.9% saline until testing at the room temperature of 25 °C. The specimens (n = 12, six rats) were fixed in a testing jig with their posterior surface resting on two lower supports bilaterally. The testing machine with the maximal load of 225 N (3520-AT; Bose, Eden Prairie, MN, USA) and built-in software was applied to assess the biomechanical property with three bending tests to confirm the functional recovery of the lower limbs. The rat tibia or femur was horizontally positioned on the fixture (15 mm span) of the machine. The compression was located in the center of whole tibia at a speed of 2 mm/min and applied in sagittal plane (Fig. 1). The sagittal and coronal widths of the fractured tibias or femurs were obtained using electronic sliding calipers. The measured parameters such as maximum load, stiffness (obtained from slope of the stress-strain curve), the Young's modulus and energy were recorded from the biomechanical tests.

Bone histomorphometry

Undecalcified tibias (n = 12, six rats) were fixed in 4% paraformaldehyde (PFA), dehydrated, and cleared in xylene, then infiltrated and embedded in methylmethacrylate (MMA) without decalcification. The diamond saw (Leica SP1600; Leica Instruments, Nussloch, Germany) and hand grinding were used to section the femur longitudinally (~50 μm thick). These undecalcified sections (by Stevenel's blue and Van Gieson's picrofuchsin for histomorphometric analysis) were analyzed with Image Pro software (version 5; Media Cybernetics, Silver Spring, MD, USA) to quantify the

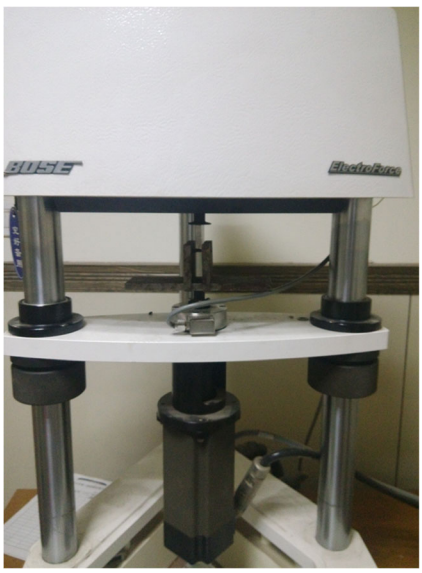

Fig. 1 The rat tibia or femur was horizontally positioned on the fixture of the machine. The compression was located in the center of whole tibia and applied in the sagittal plane

trabecular bone histomorphometric parameters such as mineral apposition rate (MAR), bone formation rate per bone surface (BFR/BS), mineralizing surface per bone surface (MS/BS). Two repeated measurements were performed for each section within the selected volume of interest (VOI). For histomorphometric analysis, the ROI was evaluated covering 2 mm proximal to the tubercle of the tibia in the captured 16× images. Cross-sections, 500-μm thick, were cut in an area within 2 mm of the original specimens, and ground to 30 μm thickness for histomorphometry. Four VOIs were chosen respectively to compare the differences in each group.

Statistical analysis

Data were checked for normality and homogeneity of variance and analyzed using SPSS Statistics (version 21.0; SPSS, Chicago, IL, USA). And the results were presented as means ± standard deviation (SD). Frequencies were used to express categorical data. The Kolmogorov-Smirnov test was used to examine the normal distribution, and for the homogeneity of variance, Levene's test was used. Analyses showed that all parameters obeyed normal distribution and homoscedasticity. One-way analysis of variance (ANOVA) was applied, with Fisher's protected least significant difference tests used to determine the statistically significant differences between the three groups. A p value of 0.05 or less was considered to represent a statistically significant difference.

Results

General status and biochemical analysis

There were no significant changes in diet, or urine, with normal fur and quick reaction to stimulation from the surroundings in the NC group. In contrast, polydipsia, polyphagia and urorrhagia were obvious in STZ-induced rats in the DM groups. Along with other indications, the successful establishments of diabetic models were observed in SD rats. After suffering STZ-induced hyperglycemia for 12 weeks in DM, rats treated with normal saline showed significant decreases in body weight and significant increases in blood glucose compared with age-matched non-diabetic controls. Although daily treatment with HRS for three months did not significantly attenuate the loss of body weight in diabetic rats, blood glucose level was decreased significantly in DM + HRS (Fig. 2). Compared with the NC group, the levels of serum creatinine, 24 hour urine albumin, BUN and MDA were significantly increased in the DM and DM + HRS groups at 8 and 12 weeks. After the treatment with HRS in the DM + HRS group, the levels of serum creatinine, 24 hours urine albumin BUN and MDA were significantly decreased compared with DM.

Micro-CT

After 12 weeks, 3-D reconstructed images illustrated that there was a well-organized structure of trabecular bone in the proximal tibia of rats of the NC group compared with that of DM group. The trabecular bone was protected and with smaller cavities or cores inside the intramedullary spaces in the DM + HRS group compared with that of the DM group (Fig. 3). There was a significant decrease in BV/TV, Conn, Tb.Th, Tb.N in DM compared with the NC group. SMI was higher in the DM group than in the NC group. The results from the present study showed that bone formation was significantly decreased in the DM group compared with the NC group. After the treatment with HRS, BV/TV, and Tb.N were significantly increased compared with that of DM ($p > 0.05$). There was not a significant increase of Conn and Tb.Th in DM + HRS ($p > 0.05$). Tb.Sp which was equal with marrow thickness and SMI was markedly decreased in DM + HRS compared with that of DM (Fig. 4).

Biomechanical testing

In concordance with our bone densitometry, ultimate load in NC group was significantly higher than that of the DM group at 12 weeks. The DM group showed significant decreases in stiffness, energy absorption and elastic modulus in comparison with the NC group at 12 weeks. The DM + HRS group showed that ultimate load was increased in comparison with the NC group. The parameters of energy showed a significant increase in the DM + HRS group compared with the NC group. In terms of stiffness, there was an obvious improvement in the DM + HRS group compared with the DM group. Moreover, the DM + HRS group showed a significantly higher elastic modulus than the DM group. There was no significant increase for the DM + HRS group in elastic modulus compared with the DM group (Table 1).

Bone histomorphometry

Quantitative comparisons of the dynamic histomorphometric parameters demonstrated that the DM group showed significantly lower MAR and BFR/BS than the NC group at 12 weeks post induction ($p < 0.05$). In comparison with the DM group, rats in the DM + HRS group exhibited significantly increased MAR and BFR/BS at 12 weeks ($p < 0.05$). In addition, no significant difference in the parameter of MS/BS was observed among the three groups (Table 2).

Discussion

Many studies have shown that hydrogen acts as a potential therapeutic antioxidant that suppresses anti-inflammatory

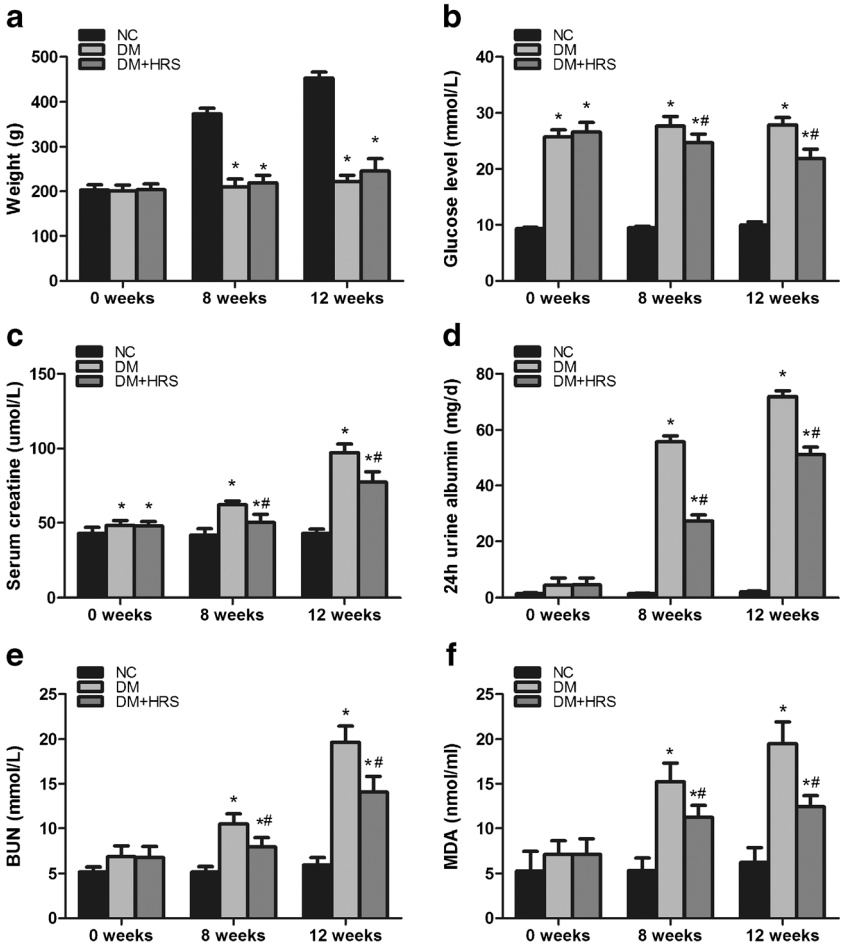

Fig. 2 Changes in weights, glucose levels and biochemical results in the three groups. *NC* normal control, *DM* diabetic mellitus, *DM + HRS* diabetic mellitus with hydrogen-rich saline administration. Each group was formed by ten rats. $^{*}p < 0.05$ compared to NC; $^{**}p < 0.01$ compared to NC; $^{\#}p < 0.05$ compared to DM; $^{\#\#}p < 0.01$ compared to DM

activities and protects many organs (especially against acute pancreatitis) from oxidative stress associated with overproduction of ROS, but there are no studies focused on the subsequent recovery of OP with the treatment of HRS in T1DM. Therefore, in this study, the research demonstrates that HRS supplementation for three months attenuates bone loss in a diabetic rat model. The beneficial effect of hydrogen-rich saline on bone is supported by the results from micro-CT scan and mechanical test. The microarchitecture of bone and mechanical strengths are improved after the consumption of HRS.

Diabetes with related complications threatens human health globally and may result in blindness, renal diseases, or even amputation. In this experiment, increased blood glucose, weight loss and pathological evidences observed in STZ-induced diabetic rats, which meant the successful establishment of T1DM model. In both types of diabetes, they all

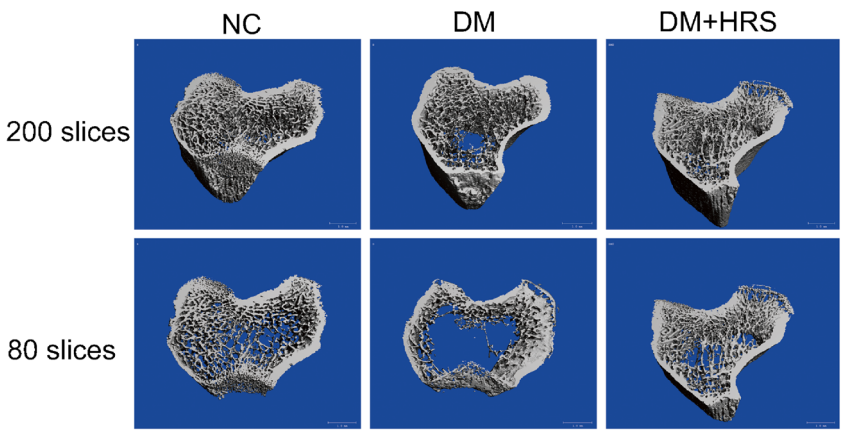

Fig. 3 Three-dimensional reconstructed images from micro-CT scans. The trabecular bone was rich and well organized in the NC group compared with that in DM with obvious hollow in the marrow cavity. After 12 weeks, the treatment with HRS attenuates the loss of trabecular bone

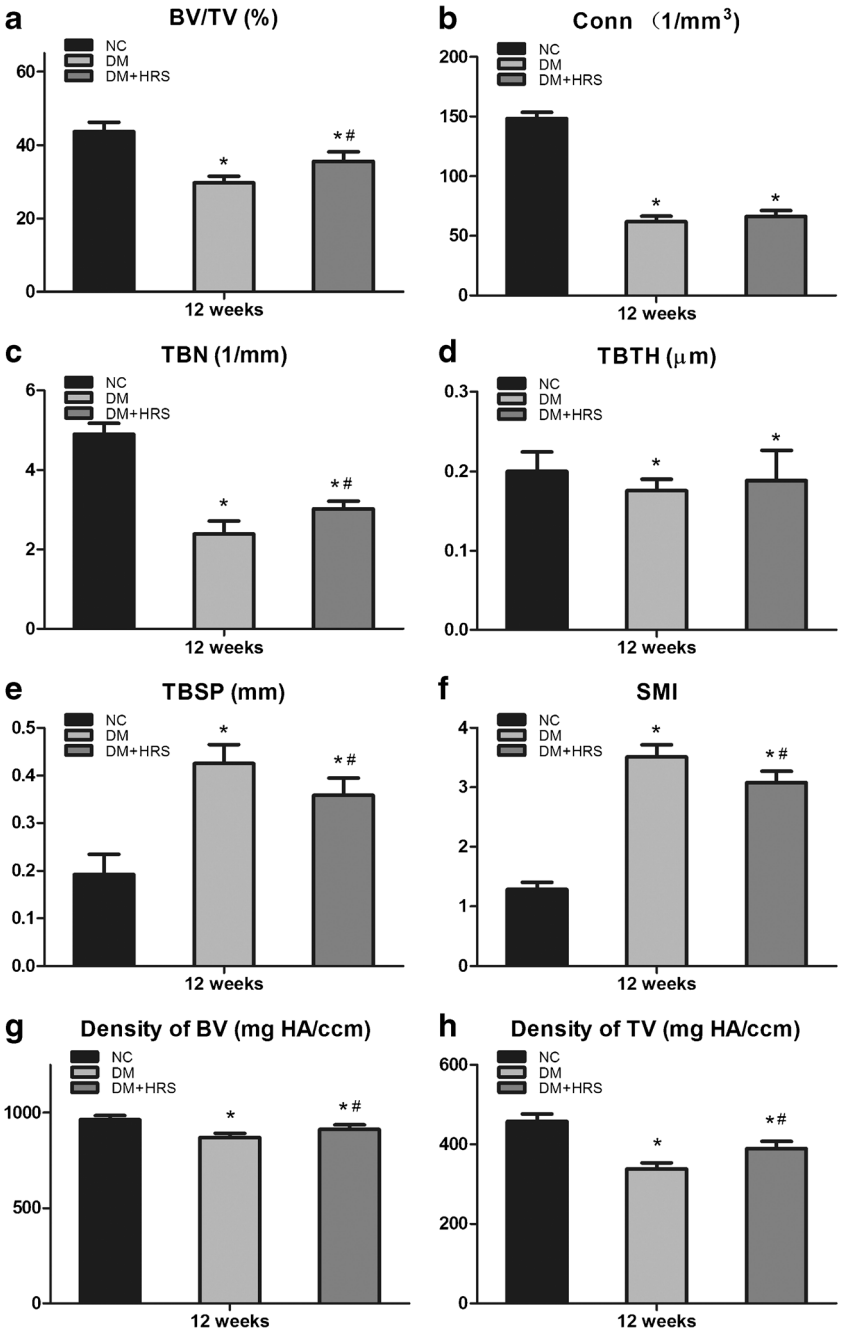

Fig. 4 Histomorphometry of the callus tissue by micro-CT scans. *NC* normal control, *DM* diabetic mellitus, *DM + HRS* diabetic mellitus with hydrogen-rich saline administration. Each group was formed by ten rats. $^{*}p < 0.05$ compared to NC; $^{**}p < 0.01$ compared to NC; $^{\#}p < 0.05$ compared to DM; $^{\#\#}p < 0.01$ compared to DM

exhibit inferior quality and strength, but the fracture risk is significantly higher in T1DM compared with that in T2DM, which is characterized by normal or even higher bone mineral density induced by hyperinsulinemia at the early stages [1, 12]. There is accumulating evidence that β-cell failure, cumulative hyperglycemic exposure, subsequently accelerated formation of advanced glycation end products (AGE), low levels of IGF1 which suppresses the function of osteoblasts and increased level of oxidative stress are contributed as main mechanisms to the development of OP in T1DM. Many other mechanisms, such as low tolerance and heredity, are also reported to be involved with bone fragility in diabetes rats [13]. The reduced insulin levels or insulin signaling in osteoblasts caused by diabetes in T1DM negatively affects bone and leads to reduced bone formation [14]. Besides a direct negative effect on osteoblasts and a positive effect on osteoclast under hyperglycaemic condition, osmotic diuresis caused by hyperglycaemia leads to increased urinary excretion of magnesium or calcium, impaired secretion of the calcium–parathyroid hormone which contributing to bone demineralization, and even the occurrence of hypoparathyroidism in DM [12, 14]. The increased glucose levels enhance

Table 1 Macroscopic biomechanical analysis of femurs and tibias at 12 weeks

	NC	DM	DM + HRS
Biomechanical properties of femur at 12 weeks			
Ultimate load (N)	113.94 ± 10.050	80.758 ± 4.9853**	98.578 ± 5.1376**
Energy absorption (mj)	106.92 ± 3.4327	78.435 ± 3.1025**	90.154 ± 3.9252** ##
Stiffness (N/mm)	178.87 ± 7.9694	140.96 ± 11.021**	160.74 ± 7.2293** #
Elastic modulus (Gpa)	2.8381 ± 0.0989	1.6430 ± 0.0920**	2.2805 ± 0.1119** ##
Biomechanical properties of tibia at 12 weeks			
Ultimate load (N)	99.907 ± 8.1388	68.798 ± 7.6627**	81.933 ± 6.8427** #
Energy absorption (mj)	87.923 ± 4.3514	62.426 ± 3.1531**	78.140 ± 5.9231** #
Stiffness (N/mm)	165.32 ± 12.553	87.354 ± 6.5590**	120.129 ± 5.7491** #
Elastic modulus (Gpa)	2.3680 ± 0.158.3	1.6950 ± 0.1502**	1.9864 ± 0.0945** ##

Values represent the mean ± SD. Each group was formed by six rats

NC normal control, *DM* diabetic mellitus, *DM + HRS* diabetic mellitus with hydrogen-rich saline administration

**$p < 0.01$ compared to NC. #$p < 0.05$ compared to DM; ##$p < 0.01$ compared to DM

glycation of bone matrix, and impair collagen turnover or matrix renewal [15]. Elevated level of AGE increases levels of superoxide production or oxidative stress in diabetic patients under hyperglycemic condition. The accumulation of AGEs in bone and their inhibition on enzymatic beneficial cross-links of collagen or formation of osteoblasts results in negative effects on bone formation and cause glycation of proteins or collagen [16]. In contrast to enzymatic crosslinks, nonenzymatic glycation or oxidation-induced reaction which is elicited by accumulated AGEs in protein contributes to the aging of macromolecules and subsequently causes fragility of bone [15]. Unlike T2DM, the insulin-like growth factor (IGF1) in T1DM which exerts an anabolic effect through relative receptor on osteoblasts is blunted by high glucose concentrations or AGEs. Peripheral vascular disease, which is commonly seen in diabetes, may also result in apoptosis and decreased density of osteocytes [17]. Oxidative stress caused by hyperglycemia is the main mechanism affected by HRS and the main discussion in our research. Nyman et al. [18] found that not only bone structure and architecture were affected, but also the relationship between strength and structure was negatively disturbed in T1DM, and resulted in reduction of tissue-level hardness, an increase of bone brittleness.

Oxidative stress represents an imbalance between the activity of antioxidants or antioxidant enzymes and the production of ROS. As we mentioned above, hyperglycaemia is an important reason for the increased oxidative stress levels in DM, and oxidative stress causes depletion of endogenous antioxidants and overproduction of ROS. The oxidative stress or its resultant overproduction of ROS has a direct negative effect on osteoblast function, and contributes to activation of osteoclasts by inducing RANK expression [14]. The hydroxyl radical is one of the strongest ROS and reacts indiscriminately with nucleic acids, lipids, and proteins. Meanwhile, there are deficiencies of endogenous detoxification systems for hydroxyl radicals in human body. As an antioxidant, hydrogen selectively reacts with hydroxyl radicals and peroxynitrite to attenuate oxidative stress levels, scavenge hydroxyl radicals, corrects the pro- or anti-oxidative imbalance and forms a harmless end product [19]. In our study, as we expected, BV/TV, TH.N in the DM + HRS group was significantly improved compared with that of non-treated diabetic rats, and the level of glucose was reduced, although the actual mechanism was still unclear. However, the "gold standard" strategy for T1DM remains subcutaneous insulin infusion. Hashimoto et al. [20] reported that HRS did not affect blood pressure or blood glucose levels. However, Feng et al. [11] found that HRS upregulated the anti-oxidative enzyme activities after a trial to study neurovascular dysfunction in the retina. The balance between ROS and the activity of antioxidant defense systems may contribute indirectly to the smooth level of blood glucose in STZ-induced diabetic rats. It has been reported that electrolyzed-reduced water (ERW, high dissolved hydrogen), which produces hydrogen near the anode, exerts beneficial effects on β-

Table 2 Effects of hydrogen-rich saline on trabecular bone histomorphometry in rats

Group	NC	DM	DM + HRS
MAR (μm/day)	1.61 ± 0.16	1.11 ± 0.19**	1.31 ± 0.13** ##
BFR/BS ($\mu m^3/\mu m^2$ per day)	0.31 ± 0.12	0.21 ± 0.13**	0.27 ± 0.18** #
MS/BS (%)	23.3 ± 2.6	12.2 ± 5.0**	17.5 ± 3.1**

Values represent the mean ± SD. Each group was formed by ten rats

**$p < 0.01$ compared to NC; #$p < 0.05$ compared to DM; ##$p < 0.01$ compared to DM

cell function in diabetic mice (T1DM) induced by STZ [21, 22]. As a potent scavenger of reactive oxygen species, hydrogen concentration is maintained with sodium chloride in HRS and may has similar effects on β-cells. Kajiyama et al. [23] observed that HRS normalized the glucose tolerance in patients with impaired glucose tolerance. Although the model is different from our research (T1DM), we are still assured of the positive effect with systemic delivery of HRS on blood glucose level. In coincidence with our results, Amitani et al. [24] also found that hydrogen was effective in improving glycemic control in an STZ-induced TIDM rat model without producing hypoglycaemia.

An increase of AGE and ROS may aggravate the damage to blood vessels and DM related complications [25, 26]. HRS blocks the pathways of oxidative stress-induced cellular damage by quenching or lowering of free radicals. More and more evidences indicate the important role of oxidative stress in the progression of osteoporosis [27, 28]. Guo et al. [10] found that HRS consumption prevented osteoporosis through the ablation of oxidative stress induced by estrogen withdrawal. Although the potential mechanism of hydrogen reacting with ROS has been reported to eliminate the powerfully toxic (ROS) hydroxyl radical selectively, the underlying molecular mechanisms of HRS in diabetic-related osteoporosis remain to be elucidated. Beside the anti-oxidative effect, Shi et al. [29] and Ishibashi et al. [30] confirmed that HRS blocked the degradation of IκB, which combined with NF-κB in the normal state, and prevented the activation of NF-κB in an acute renal injury model or rheumatoid arthritis. The activated NF-κB binds with DNA, and then upregulates the expression of IL-6, which belongs to the pro-inflammatory cytokines. IL-6 also plays a pivotal role in bone metabolism, and it accelerates the formation of osteoclast and bone loss [31]. With the treatment of HRS, IL-6 mRNA expression and osteoclastgenesis are suppressed in DM + HRS. In another respect of bone formation, hydrogen water is reported to alleviate TNF-α (another pro-inflammatory factor which decreases osteoblastic bone formation through suppressing proliferation, inducing apoptosis, and inhibiting differentiation of osteoblast) induced injury of osteoblasts [32]. And in other experiments, plasma TNF-α level which inhibits osteoblastic bone formation is also decreased in ovariectomized rats and this ascertains its positive effect on bone metabolism through TNF-α [10]. So we conclude that the nuclear factor-κB (NF-κB) signaling pathway is one of the mechanisms of HRS on bone [33]. Beside disturbances to the WNT and NF-κB signaling pathway, Li et al. [34] also reported that the phosphoinositide 3-kinase (PI3K)/AKT, c-Jun N-terminal kinase (JNK)/mitogen-activated protein kinase (MAPK) signaling pathways were activated in diabetes-related osteoporosis [1]. After activating phosphatidylinositol-3-OH kinase (PI3K), atypical protein kinase C (aPKC), and AMP-activated protein kinase (AMPK) under conditions of severe insulin deficiency, hydrogen promotes glucose uptake into skeletal muscle by stimulating Glut4 translocations [24]. Beside a radical scavenging effect, hydrogen also influences free radical chain reaction of unsaturated fatty acid and lipid peroxidation on cell membranes or intracellular mitochondrial function in bone and may be the second master regulation for intracellular signaling and gene expression [32, 35]. Above all, hydrogen is likely to have multiple master regulators, which induce a diverse array of downstream mechanisms, and minimize bone loss caused by diabetes.

Beside protection of bone tissue, hydrogen water prevents early neurovascular dysfunction resulting from inhibition of oxidative induced by STZ. Hydrogen water also exhibits therapeutic effects on 6-hydorxydopamine-induced Parkinson's disease in rats [35]. For lung injury, acute myocardial infarction, and ischemia-reperfusion injuries of different organs, the positive effects of hydrogen on them are also prominent. Gas inhalation and oral hydrogen intake are traditional delivery methods; local delivery is also reported to reduce oxidative stress effectively [36]. There are a few studies that reported the negative side effects of HRS, which may be explained as its mild antioxidant effects, and low possibility of disturbing to cell signaling or physiological oxidation-reduction reactions [37]. Different from a biologic hydrogen donor such as NADPH or NADH which plays an essential role in aerobic respiration, photosynthesis, electron transfer and other antioxidant supplements with strong reductive reactivity that increase mortality, possibly by affecting essential defensive mechanisms, H_2 is mild enough not to disturb metabolic oxidation reduction reactions or to disrupt ROS involved in cell signaling. This is advantageous for medical procedures, as it means that the use of H_2 should not have serious unwanted side effects.

The limitation of this study is the deficiency of histopathological observations. Micro-CT scans and mechanical tests of tibia and femur are sufficient to support the positive effect of HRS on the microarchitectures of diabetic bone. Passive diffusion of hydrogen in the stomach and the diffusion from circulating venous flow into bone tissue are impossible to identify and may be different for each rat, and this may have affected on our results, so the intravenous or local delivery of HRS may exhibit better results compared with the oral delivery used in our study. Fracture healing is complicated, and the therapeutic effect of HRS on inflammatory response, cartilage and mineralized woven bone is a more interesting project to be studied.

In conclusion, diabetes with a high level of oxidative stress deteriorates bone material properties in terms of collagen post-translational modification such as enzymatic immature and mature cross-links and non-enzymatic AGEs formation. HRS, which is safe and well tolerated, plays a protective role in bone volume loss and decreases the rate of fracture risks in STZ-induced status, whose bone structure or inherent material

properties of bone tissues are changed by diabetes. However, it still cannot be substituted for insulin, and other assisted treatment such as supplementation of calcium and vitamin D should be conducted simultaneously to ameliorate the complications caused by diabetes. Further researches are necessary to describe the specific mechanisms underlying the therapeutic effects of hydrogen. Moreover, fracture healing in diabetic rats may be negatively affected because diabetes delays the regenerative processes of connective tissues including bone, and future works focusing on fracture healing in a diabetic model or other different models are interesting and will be studied soon.

Author contributions Conceived and designed the experiments: ZYH. Funding acquisition: ZYH. Project administration: JLG, PCW. Experiment performance: JLG, WCD, JL. Formal analysis: GJL, WCD. Methodology: YZZ. Writing original draft and editing: GJL, ZYH, YZZ.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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