Effects of drinking hydrogen-rich water in men at risk of peripheral arterial disease: a randomized placebo-controlled trial

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Abstract

Aims Smoking, hypertension, hyperlipidemia, and diabetes are considered to increase the incidence of peripheral arterial disease (PAD). They can activate endogenous free radicals, cause inflammation and oxidative stress, and lead to endothelial cell dysfunction. Hydrogen (H_2) has been proven to decrease oxidative stress, improve cell function, and reduce chronic inflammation. The purpose of this research was to validate the role of H_2 in individuals who are at risk of PAD.

Methods Sixty subjects were randomly assigned to placebo (PBO) group or H_2-rich water (HRW) group and drank either bottled pure water or H_2-rich water (245 mL/time, 3 times/d) for ten weeks.

Results The pulse wave velocity was ameliorated in the HRW group with no significant change in the ankle-brachial index. The serum total cholesterol of the HRW group was significantly reduced compared to the placebo group. In addition, compared to baseline, the levels of lipoprotein(a) was decreased, the malondialdehyde content was reduced, the superoxide dismutase activity was increased, and the expression of intercellular cell adhesion molecule-1 was decreased significantly in the HRW group. The oxidized phospholipid of 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphatidylcholine level in the HRW group were significantly reduced compared to the placebo group. Finally, H_2 significantly improved the antioxidant, antiinflammatory, and antiapoptotic abilities of high-density lipoprotein (HDL).

Conclusions Drinking HRW can improve vascular sclerosis indicators, improve dyslipidemia, reduce vascular oxidative stress and inflammation, and improve HDL function. H_2 may be used to prevent and relieve PAD caused by major risk factors such as smoking, hypertension, hyperlipidemia, and diabetes.

Introduction

Atherosclerotic diseases, including peripheral arterial disease (PAD), coronary artery disease, and cerebral artery disease are the leading causes of death worldwide. PAD is an abnormal narrowing of the arteries, and mainly includes disease of the aortoiliac, femoropopliteal, and infrapopliteal arterial segments. The symptomatic manifestations of PAD include leg pain, intermittent claudication, pain at rest, gangrene when the limb is severely ischemic, and even amputation[1]. Currently, there are more than 202 million patients with PAD worldwide, and it is predicted that up to 45 million patients with PAD will die from coronary or cerebrovascular disease over a 10-year period[2]. Smoking, hypertension, hypercholesteremia, and diabetes are the four major risk factors for PAD [3]. Smoking is a particularly strong risk factor for PAD with an obvious dose-response relationship, and heavy smokers are four times more likely to develop PAD than nonsmokers[4]. Diabetes, hypertension, and hypercholesterolemia are also highly associated with PAD, with an approximately two- to three-fold increased risk. The incidence of PAD is also increased with age[5,6].

Planned exercise and lifestyle improvements are practical ways to reduce the risk and delay the progress of PAD. Currently, PAD is mainly treated with antiplatelet therapy, anticoagulant therapy, statins,

antihypertensive therapy, and medications to improve circulatory flow[7]. These therapies are mainly aimed at the cause of PAD, and applied to patients with symptomatic PAD; however, such drugs are usually accompanied by side effects. At present, 17β-estradiol [8], ginsenoside Rb3[9], recombinant human Relaxin-2[10] and active substances from red wine [11] have shown promise in improving vascular damage caused by smoking. However, their curative effects have certain limitations. Patients with typical PAD symptoms account for only 20%, and approximately 50% of patients are asymptomatic[12]. People generally lack awareness of the diagnosis and treatment of PAD, and are unlikely to choose long-term treatment to slow the progression of PAD. Therefore, for asymptomatic patients with PAD, it is of great significance to choose a safe treatment with no side effects in order to prevent and alleviate the progression of the disease.

Hydrogen (H_2) is a bioactive gas that has beneficial effects in diseases such as metabolic syndrome [13,14], type 2 diabetes [15], chronic liver inflammation [16] ,and focal brain and ischemia/reperfusion injury [17]. the mechanisms of which are thought to be related to its antioxidative, antiinflammatory, and antiapoptotic properties. In addition, it has been previously demonstrated that H_2 has a protective effect on endothelial cells. Indeed, a previous report showed that H_2-saturated water could promote the recovery of blood perfusion in a mouse PAD model by increasing angiogenesis and decreasing the level of oxidative stress [18]. However, the effect of H_2 in individuals at risk of PAD has not yet been demonstrated. In this study, we performed a randomized placebo-controlled trial to characterize the effect of H_2-rich water drinking on PAD in men at risk of PAD, and its effects on oxidative stress and inflammatory factors. This is the first randomized controlled trial of H_2 on risk of PAD.

Results

Subject's baseline characteristics

The selection process is shown in Fig. 1. 59 subjects participate in the study, and one subject withdrew at week 10 due to his work outside. The specific baseline clinical indicators are shown in Table 1. The study subjects were divided into the placebo (PBO) group or the H_2-rich water (HRW) group at random. The study subjects each drank three bottles of placebo water or H_2-rich water (245 mL/bottle) per day for 10 weeks. Blood samples were collected at the beginning and after the 10-week trial.

Table 1
Baseline level of general information of the two groups.

variable		PBO (n = 29)	HRW (n = 30)	P value
		Number of cases (percentage)	Number of cases (percentage)	
Age				0.31
	40–55	16 (53.3%)	12 (40%)	
	55–65	9 (30%)	11 (36.6%)	
	≥ 65	5 (16.6%)	7 (23.3%)	
BMI				0.79
	27-29.9	10 (33.3%)	11 (36.6%)	
	≥ 30	20 (66.6%)	19 (63.3%)	
Drink alcohol				0.61
	Often	16 (53.3%)	18 (60%)	
	occasionally	8 (26.6%)	7 (23.3%)	
	No	6 (20%)	5 (16.6%)	
Activity				0.66
	Often	15 (50%)	16 (53.3%)	
	occasionally	9 (30%)	10 (33.3%)	
	No	6 (20%)	4 (13.3%)	
Smoking index				0.12
	< 400	4 (13.3%)	9 (30%)	
	≥ 400	26 (86.6%)	21 (70%)	
Disease condition				
	hypertension	10	9	
	Diabetes	14	12	
	Hyperlipidemia	8	8	
Systolic blood pressure				0.19

HRW, H_2-rich water group. PBO, placebo group. BMI, body mass index.

variable		PBO (n = 29)	HRW (n = 30)	P value
		Number of cases (percentage)	Number of cases (percentage)	
	< 140	16 (53.3%)	21 (70%)	
	≥ 140	14 (46.6%)	9 (30%)	
Diastolic blood pressure				0.10
	< 90	17 (56.6%)	23 (76.6%)	
	≥ 90	13 (43.3%)	7 (23.3%)	

HRW, H_2-rich water group. PBO, placebo group. BMI, body mass index.

Breath hydrogen measurement following administration of H_2-rich water

As shown in Fig. 2, after drinking H_2-rich water (650–700 μmol/L, 245 mL), the exhaled H_2 concentration rose rapidly over the course of 5 min and reached the peak in the 10th min, with a value of approximately 7 ppm. Subsequently, the concentration gradually decreased and returned to baseline in the 60th min. There was no significant difference between the results of males and females (data not shown).

Effect of H_2 on PWV and ABI

Table 2 depicts the changes in ABI and PWV after 10 weeks of H_2-rich water intervention. The low value of ABI (the minimum value of the measured ABI of the left and right lower limbs) and the ABI of the left and right lower limbs were not significantly different between the HRW group and the PBO group). The low value of PWV (the minimum value of the measured PWV of the left and right lower limbs) and the PWV of the left lower limb decreased significantly after drinking H_2-rich water ($P < 0.05$). The PWV of the right lower limb also showed a decreasing trend after drinking H_2-rich water ($P > 0.05$).

Table 2
Effect of H2 on PWV and ABI

measure	HRW group		P	PBO group		P	P§
	0-week	10-week		0-week	10-week		
Left lower limb ABI	1.17 ± 0.10	1.22 ± 0.14	0.08	1.17 ± 0.11	0.16 ± 0.14	0.90	0.21
Right lower limb ABI	1.11 ± 0.11	1.16 ± 0.15	0.11	1.15 ± 0.15†	1.14 ± 0.17	0.64‡	0.72
ABI low value	1.10 ± 0.12	1.12 ± 0.16	0.47	1.14 ± 0.10†	1.11 ± 0.17	0.98‡	0.55
PWV left, cm/s	1583.5 ± 269.90	1430.16 ± 228.53	0.01*	1421 ± 412†	1520 ± 315 †	0.19‡	0.39‡
PWV right, cm/s	1561.13 ± 272.93	1502.53 ± 258.41	0.17	1439 ± 421†	1454 ± 313.5†	0.08‡	0.79‡
PWV low value, cm/s	1583.5 ± 269.90	1391.56 ± 201.88	0.0004*	1427 ± 326†	1386 ± 267.5†	0.56‡	0.50‡

P§represents the 10-week comparison between the hydrogen-rich water group and the placebo group. † Non-normal data is represented by median and interquartile range and the remaining results are shown as mean ± SD. ‡Statistical analysis was performed by nonparametric tests for nonparametric data, and Student's t test for normally distributed data. *P < 0.05.

Effect of H_2 on lipid profiles

After 10 weeks of intervention, the TC level in the HRW group was significantly lower than that in the PBO group (P < 0.05, Table 3). The serum Lp(a) levels were significantly decreased after 10 weeks of H2 treatment in the HRW group (P < 0.05, Table 3). In addition, the levels of TG, VLDL, and LDL-C showed a slight decreasing trend in the HRW group after 10 weeks of intervention compared with the HRW group before intervention or the PBO group (Table 3). No obvious changes in Apo A□, Apo B, and HDL-C levels were observed (Table 3).

Table 3
Effect of H2 on lipid profiles, oxidative and inflammatory biomarkers

measure	HRW group		P	PBO group		P	P§
	0-week	10-week		0-week	10-week		
TC, mmol/L	5.24 ± 1.21	5.34 ± 0.82†	0.82‡	5.39 ± 0.77	5.69 ± 0.84	0.004*	0.047*
TG, mmol/L	1.19 ± 0.64†	1.53 ± 1.18†	0.22‡	1.30 ± 0.65†	1.42 ± 0.64†	0.82‡	0.43‡
Lp(a), mg/L	2.06 ± 0.50	1.98 ± 0.54	0.01*	1.98 ± 0.36	1.99 ± 0.38	0.50	0.55
LDL-C, mmol/L	1.21 ± 0.27	1.23 ± 0.27	0.70	1.22 ± 0.20	1.24 ± 0.21	0.27	0.72
VLDL, mmol/L	0.85 ± 0.43†	0.86 ± 0.23	0.22 ‡	0.83 ± 0.3†	0.86 ± 0.30	0.79‡	0.98
HDL-C (mmol/L)	1.44 ± 0.32	1.41 ± 0.35	0.70	1.30 ± 0.28	1.32 ± 0.28	0.72	0.95
ApoB, g/L	1.24 ± 0.21	1.24 ± 0.21	1.00	1.22 ± 0.20†	1.23 ± 0.24†	0.72‡	0.90‡
ApoA□, g/L	1.48 ± 0.23	1.43 ± 0.21	0.34	1.38 ± 0.33†	1.46 ± 0.27	0.85‡	0.60
MDA, mmol/L	3.93 ± 1.68	3.12 ± 0.69	0.03*	3.38 ± 1.39	3.32 ± 0.85	0.86	0.98
SOD activity, U/mL	12.47 ± 1.84	13.39 ± 0.88†	0.002* ‡	12.71 ± 1.84 †	12.98 ± 1.91†	0.06‡	0.44‡
PAZPC,relative peak area	——	0.08 ± 0.02	——	——	0.09 ± 0.03	——	0.04
PGPC,relative peak area	——	0.05 ± 0.02	——	——	0.06 ± 0.02	——	0.27
PONPC,relative peak area	——	0.0056 ± 0.0004	——	——	0.0059 ± 0.0008†	——	0.09‡
POVPC,relative peak area	——	0.0045 ± 0.002	——	——	0.0046 ± 0.001	——	0.85

P§represents the comparison between the hydrogen-rich water group and the placebo group at 10-week.*P < 0.05. †Non-normal data is represented by median and interquartile range and the remaining results are shown as mean ± SD. ‡Statistical analysis was performed by nonparametric tests for nonparametric data, and Student's t test for normally distributed data.

measure	HRW group		P	PBO group		P	P§
	0-week	10-week		0-week	10-week		
ICAM-1, pg/mL	5.32 ± 0.39	5.30 ± 0.37	0.003*	5.11 ± 0.85†	5.10 ± 0.83†	0.16‡	0.42‡
MMP-1, pg/mL	2.67 ± 0.32	2.66 ± 0.30	0.82	2.69 ± 0.31	2.64 ± 0.27	0.35	0.85
CCL1, pg/mL	1.35 ± 0.35†	1.49 ± 0.25†	0.19‡	1.48 ± 0.20†	1.35 ± 0.35†	0.33‡	0.26‡

P§represents the comparison between the hydrogen-rich water group and the placebo group at 10-week.*P < 0.05. †Non-normal data is represented by median and interquartile range and the remaining results are shown as mean ± SD. ‡Statistical analysis was performed by nonparametric tests for nonparametric data, and Student's t test for normally distributed data.

Effect of H_2 on oxidative and inflammatory biomarkers

The serum levels of MDA decreased and the activity of SOD increased in the HRW group after 10 weeks of intervention (P < 0.05, Table 3). The levels of oxidized phospholipids also decreased, especially PAzPC (P < 0.05, Table 3). The HRW group demonstrated significant attenuation of the inflammatory biomarker of ICAM-1 (P < 0.05, Table 3). The concentration of MMP-1 and CCL1 was not significantly different following intervention (Table 3).

H_2 improves the oxidation and the functional properties of HDL

H_2 significantly reduced thiobarbituric acid-reactive substances generated by LDL oxidation (Fig. 3A), indicating that H_2 can improve HDL antioxidant function. We detected cell viability by CCK-8 to verify the antiapoptotic function of HDL after H_2 intervention. We found that H_2 inhibited endothelial cell apoptosis induced by ox-LDL (Fig. 3B). Ox-LDL can induce HUVECs to increase the adhesion of monocytes to endothelial cells. Following incubation with HDL, we found that the adhesion of monocytes in the HRW group was significantly reduced after H_2 intervention (Fig. 3C), showing that H_2 can enhance the antiinflammatory effect of HDL.

Discussion

We performed a randomized placebo-controlled trial and found that H_2 can relieve vascular sclerosis, regulate lipid metabolism disorder, improve antioxidant capacity, and reduce inflammation in people who are at risk of PAD. Since ischemia/reperfusion injury that occurs during PAD is accompanied by an increase in reactive oxygen species (ROS) formation, antioxidant therapy may be a viable countermeasure [19]. H_2, as a novel antioxidant, has been shown to have roles in various diseases, and a

previous report has demonstrated the therapeutic effect of H_2 in PAD in mice [18]. Therefore, it is important to further verify the effect of H_2 in patients with PAD.

We measured the H_2 concentration in the exhaled gas of patients in order to study the *in vivo* kinetics of H_2 after drinking H_2-rich water. Our results demonstrated that it took 10 min for H_2 to reach the peak, and it gradually returned to baseline in 60 min. These results were consistent with previous reports that showed that the breath H_2 concentration reaches the maximum 10–15 minutes after intake, and then decreases gradually, returning to baseline levels 45–150 min after drinking H_2-rich water[20–22]. The peak concentration in the current study was approximately 7 ppm after drinking 245 mL of H_2-rich water; this is lower than previous reports that found peak breath hydrogen levels of 30–60 ppm after drinking 200–300 mL of H_2-rich water[20–22].

PWV and ABI are becoming increasingly important in the assessment of arterial stiffness. ABI refers to the pressure ratio of the left and right upper arms to the left and right ankles, and has a normal range of 1.0–1.4. PWV is the most widely used measure of arterial stiffness, and an increase in the propagation speed of the PWV in the artery is associated with an increase in arterial stiffness. The fluctuating conduction velocity between two heartbeats is used to judge the elasticity of arterial wall[23]. The normal PWV is less than 1400 cm/s, and our study showed that H_2 had a significant impact on PWV following 10-week intervention.

The lipid-lowering effect of H_2-rich water has been verified in patients with potential metabolic syndrome [24]. In our study, the TC level was lower in the HRW group than the PBO group, and the 10-week H_2 intervention reduced the Lp(a) level in individuals at risk of PAD. Furthermore, our previous study also showed the reducing effect of H_2-rich water on TC in patients with potential metabolic syndrome or hypercholesterolemia [13,25]. In addition, studies have shown that the serum TC levels were significantly decreased in ethanol-induced fatty liver and nonalcoholic fatty liver mice after H_2-rich water treatment [26,27]. It is well known that a high cholesterol level is one of the most important risk factors for PAD. Therefore, elucidation of the mechanism by which H_2 decreases serum cholesterol will provide solid evidence for the application of H_2 in PAD therapy.

Lp(a) is a cholesterol-rich, LDL-like particle, with specific apolipoprotein (a) and apolipoprotein B-100. The Lp(a) levels of plasma independently predict atherosclerotic cardiovascular disease (CVD) and PAD, although its mechanism of action in atherosclerosis remains unclear [28]. Therapy to lower the plasma levels of Lp(a) has gained much attention in recent years, and our results reveal that H_2-rich water can reduce the level of plasma Lp(a). Lp(a) is regarded as a preferential carrier of oxidized phospholipids (OxPLs) in human plasma [29], and we demonstrated that H_2-rich water can reduce the levels of oxidized phospholipids, especially PAzPC. Moreover, a previous study showed that H_2 can suppress the autoxidation of linoleic acid and PAPC in a pure chemical system[30]. The mechanism by which H_2 decreased the formation of OxPLs *in vivo* is worthy of further study.

PAD is an atherosclerotic disease of arterial vessels, in which the cycles of ischemia and reperfusion induced by PAD leads to increased mitochondrial ROS[19]. Smoking is one of the main risk factors for PAD, and Smokers with hypertension, hyperlipidemia, and diabetes will increase the incidence of PAD. Cigarette products are known to activate the production of oxidative free radicals in the body [31]. In our study, H_2, as a novel antioxidant, could significantly reduce MDA and significantly increase the activity of SOD. MDA is the product of lipid peroxidation and a marker of oxidative stress, while SOD is an antioxidant enzyme that can remove ROS from the body [32]. Studies have shown a reduction in serum MDA and the increase in SOD activity in patients with potential metabolic syndrome [33] or type 2 diabetes [20] after H_2 treatment. Previous reports have also shown that H_2 can inhibit formation of MDA in ethanol/acetaminophen-induced fatty liver [34] or liver ischemia reperfusion injury in mice [35], and reduce MDA and increase SOD activity to relieve oxidative stress induced by chronic intermittent hypoxia in rats [36]. Thus, H_2 may reduce the formation of lipid peroxides in people at risk PAD by improving antioxidant enzyme activity.

ICAM-1 plays key roles in immune-mediated and inflammatory processes; it can be induced by interleukin-1 and tumor necrosis factor, and expressed by the vascular endothelium, macrophages, and lymphocytes. ICAM-1 is also involved in local plaque formation, and has been shown to be an independent predictor of the development and progression of PAD [37]. Previous studies have shown that H_2 can inhibit the expression of ICAM-1 and reduce the inflammatory response in different animal models, including a pressure ulcer mouse model [38], sepsis mouse model [39], ,and noise-induced hearing loss guinea pig model [40]. In our study, the reduced expression of serum ICAM-1 may have led to the alleviation of PAD.

HDL is known to have vasoprotective actions and antiatherogenic effects[41]. The underlying mechanism is mainly related to its function in promoting cholesterol efflux from macrophage foam cells and stimulating endothelial cell NO production to improve endothelial cell function[41,42]. HDL also plays roles in the reduction of inflammation and oxidative stress [43], and is beneficial to endothelial cells by protection of cytokine-induced monocyte adhesion [44]. It has been shown that H_2 can improve HDL function by enhancing the cholesterol efflux ability mediated by HDL, preventing LDL oxidation, and reducing ox-LDL-induced endothelial cell apoptosis and monocyte adhesion to endothelial cells [25,33]. In this study, we isolated HDL from different groups before and after intervention and tested the functions *in vivo*. Our results showed that H_2 can improve the antioxidant, antiinflammatory, and antiadhesion effects of HDL, which may improve oxidation and inflammation, and reduce vascular damage in individuals at risk of PAD.

This study shows the effects of H_2 on relieving vascular sclerosis by antioxidation and antiinflammatory mechanisms, as well as improving HDL function. It is likely that H_2 exerts antioxidant and antiinflammatory effects by improving SOD activity and HDL function; this, in turn, decreases the levels of MDA, OxPLs, ICAM-1, CCL-1, and even Lp(a), thereby alleviating artistic stiffness as measured by PWV. At the same time, H_2 exerts a lipid-lowering effect by reducing plasma TC, which also functions to lower the risk of PAD .

This study has several limitations. First, the number of participants is small, and an expanded sample is needed to verify the results. Second, the dose-effect of H_2-rich water was not studied, and a further study is needed to determine the best dose. Third, the intervention only lasted for 10 weeks, and the effect of long-term intervention needs further verification. Finally, besides the antioxidative and antiinflammatory properties, the molecular mechanisms of H_2 need further study, especially its regulatory effects on lipid metabolism disorder and its effect on Lp(a).

In conclusion, our data show that H_2-rich water can improve vascular sclerosis indicators and lipid disorders, and reduce oxidative stress and inflammatory factor infiltration. H_2 may be used in adjunctive therapy for alleviating PAD.

Methods

Subjects and study design

The study was a 10-week, randomized, placebo-controlled trial. The study protocol was authorized by the Ethics Committee of Shandong First Medical University (NO.2019121, Date 08/10/2019). This study was registered in the Chinese Clinical Trial Registry (www.chictr.org.cn, Registration number Chi CTR 2000035232, Date: 04/08/2020). All participants who were eligible and agreed to participate in the randomized assignment were required to sign a written informed consent before participating in the study. This study followed CONSORT guidelines. All methods were performed in accordance with the relevant guidelines and regulations for research involving humans. Sixty-three subjects over 40 years old were enrolled from Zhoudian community (Tai'an, China). The enrollment conditions were as follows: Current smoker or quit smoking within the past 10 years, ankle-brachial index (ABI) < 1.0 or smoking index (smoking intensity × duration of smoking) ≥ 200, with or without risk factors for diabetes, high blood pressure, and hyperlipidemia, and able to complete the questionnaire independently or with the help of the researcher.

H_2-rich water and placebo water

Bottled H_2-rich water, in which the nano-scale hydrogen bubbles are physically mixed with pure water, was purchased from Beijing Huoli Qingyuan Co., Ltd. (China). The placebo was pure water, which was consistent with H_2-rich water in terms of appearance, dosage, and packaging. The water was drunk by subjects within 15 min of opening the sealed cap. The H_2 concentration of the H_2-rich water was between 650 to 700 μmol/L when the bottle was opened, as measured by the H_2 sensor (Unisense, Aarhus, Denmark) in our laboratory.

Breath hydrogen measurement after H_2-rich water administration

We recruited 10 participants (5 males and 5 females) met the following criteria: 20–30 years old, without intestinal disease, hypoglycemia, or major diseases, and not taking intestinal flora drugs. After fasting for 12 h, the participant drank a bottle of H_2-rich water over the course of 1 min. The exhaled breath was

collected every 5 min thereafter, and the H_2 concentration was measured using a sensor gas chromatograph (SGHA-P1; FIS Co. Ltd., Hyogo, Japan).

Analysis of vascular stiffness and elasticity index

Peripheral arterial stiffness and elasticity were assessed by automatic ABI and pulse wave velocity (PWV) measurement using a noninvasive vascular screening device (BP-203RPEIII, Omron, Japan) with participants in the supine position after resting for 10 min. The ABI refers to the pressure ratio of the left and right upper arms to the left and right ankles, and PWV refers to the speed at which the blood from the heartbeat travels to the periphery in the form of waves, forming a pulse wave and propagating in the arteries.

Serum lipid analysis

Serum lipoprotein(a) (Lp[a]) was measured by the latex immunoturbidimetric method, and total cholesterol (TC), triacylglycerols (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein (VLDL-C), apolipoprotein A (Apo A), and Apo B were measured by enzymatic methods on a chemical autoanalyzer (Hitachi Co, Tokyo, Japan).

Serum oxidative stress and inflammatory factors

Serum levels of malondialdehyde (MDA) were determined following spectrophotometric measurement of thiobarbituric acid-reactive substances (TBARS) using a commercial kit (Nanjing Jiancheng Biochemistry, China). The activity of superoxide dismutase (SOD) was tested according to the manufacturer's instructions (Nanjing Jiancheng Biochemistry, China). Serum concentrations of intercellular cell adhesion molecule-1 (ICAM-1), matrix metalloproteinase-1 (MMP-1) and Chemokine (C-C Motif) Ligand 1 (CCL1) were measured by Luminex kits (Univ-bio, Shanghai, China).

Oxidized phospholipids in serum

Lipids were extracted from plasma using a methyl-tert-butyl ether method with dimyristoylphosphatidylcholine (14:0/14:0 PC) as an internal standard. Lipid extracts were analyzed using a Shimadzu LC-20 AD binary pump system interfaced with an ABI 4000QTrap mass spectrometer (Sciex, Framingham, MA, USA). Chromatographic separation was performed using a Waters Symmetry C18 column (3.5 μm, 2.1 mm i.d. × 100 mm) at 40 °C with a flow rate of 0.3 mL/min. The injection volume was 10 μL; the mobile phase comprised solvent A (acetonitrile/water, 60:40, v/v) and solvent B (2-propanol/acetonitrile, 90:10, v/v), with both solvents containing 10 mM ammonium acetate and 0.1% acetic acid. Isocratic elution was performed for 16 min with 95% B. Detection of oxidized phospholipids was accomplished during the multiple reaction monitoring mode with positive-ion detection using m/z 184 as the product ion. The parent ions of 1-palmitoyl-2-(5-oxo-valeroyl)-sn-glycero-3-phosphatidylcholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphatidylcholine (PGPC), 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphatidylcholine (PAzPC), and 1-palmitoyl-2-(9-oxo-nonanoyl)-sn-glycero-3-phosphatidylcholine (PONPC) were 594.4, 610.4, 666.5, and 650.5, respectively.

Antioxidant properties of HDL

HDL was isolated from the pooled serum by ultracentrifugation (n = 3 samples for each group, each comprising the serum of 4–5 subjects) as described [45]. LDL (100 μg/mL) from healthy people and HDL (200 μg/mL) isolated from each group were incubated with freshly prepared $CuSO_4$ (10 μmol/L) at 37 °C for 2 h. The extent of LDL oxidation was assessed by measuring the level of MDA via a spectrophotometric method according to the manufacturer's instructions (Nanjing Jiancheng Biochemistry, China).

Endothelial cell - monocyte adhesion assay

The monocyte adhesion assay was slightly modified as described previously[46]. Human umbilical vein endothelial cells (HUVECs) were cultured at 37 °C in a humidified 95% air-5% CO_2 atmosphere, grown to 70–80% confluence in 96-well plates, and stimulated with ox-LDL (100 μg/mL) in the presence or absence of HDL (100 μg/mL) for 24 h. THP-1 monocytes at a density of 2×10^5 were labeled with 10 μmol/L 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) at 37 °C for 1 h in RPMI-1640 medium and rinsed with serum-free RPMI-1640 medium. HUVECs in 96-well plates were washed three times and incubated with 100 μL THP-1 cells for 1 h. Then, each well was rinsed three times with PBS to remove unbound THP-1 cells. THP-1 cells bound to HUVECs were visualized with a fluorescent microscope (Nikon, Japan) at 4 fields per × 100 high-power-field well. Experiments were performed at least three times and the selection of high-power fields to count separate wells was performed at random.

Cell viability determined by CCK-8 assay

HUVECs were seeded in 96-well plates and pretreated with or without HDL (100 μg /mL) for 6 h and stimulated with ox-LDL (100 μg/mL) for 18 h. The viability of the HUVECs was measured by CCK-8 assay (Med Chem Express, USA), and the absorbance was measured at 450 nm using a microplate spectrophotometer system (Tecan, Sweden). The percentage viability was calculated using the following formula: HUVECs viability % = $(OD_{sample} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\%$[33].

Statistical analysis

Descriptive statistics were used to compare the baseline characteristics of the subjects in the two groups (means ± SD). Statistical analysis was performed by Student's t test for normally distributed data and by nonparametric tests for nonparametric data. The SPSS program (version 22.0) was used for all statistical analyses, and all data were plotted with GraphPad Prism 8. P-values < 0.05 were considered significant.

Declarations

Conflict of Interest:

The authors have no conflicts of interest to report.

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Author contributions

S.Q., Y.X, B.L., and Q.G., participated in study conception and design. Q.G., B.L., J.X. M.Z., X.Z., M.W., and M.Z. performed the acquisition of data. Q.G., B.L., and J.X. participated in analysis and interpretation of data. B.L. and Q.G. drafted the manuscript and S.Q. helped in critical review of the manuscript. S.Q. obtained the funding. S.Q. and Y.X. supervised the study. All the authors have read and approved the submitted manuscript.

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Figures

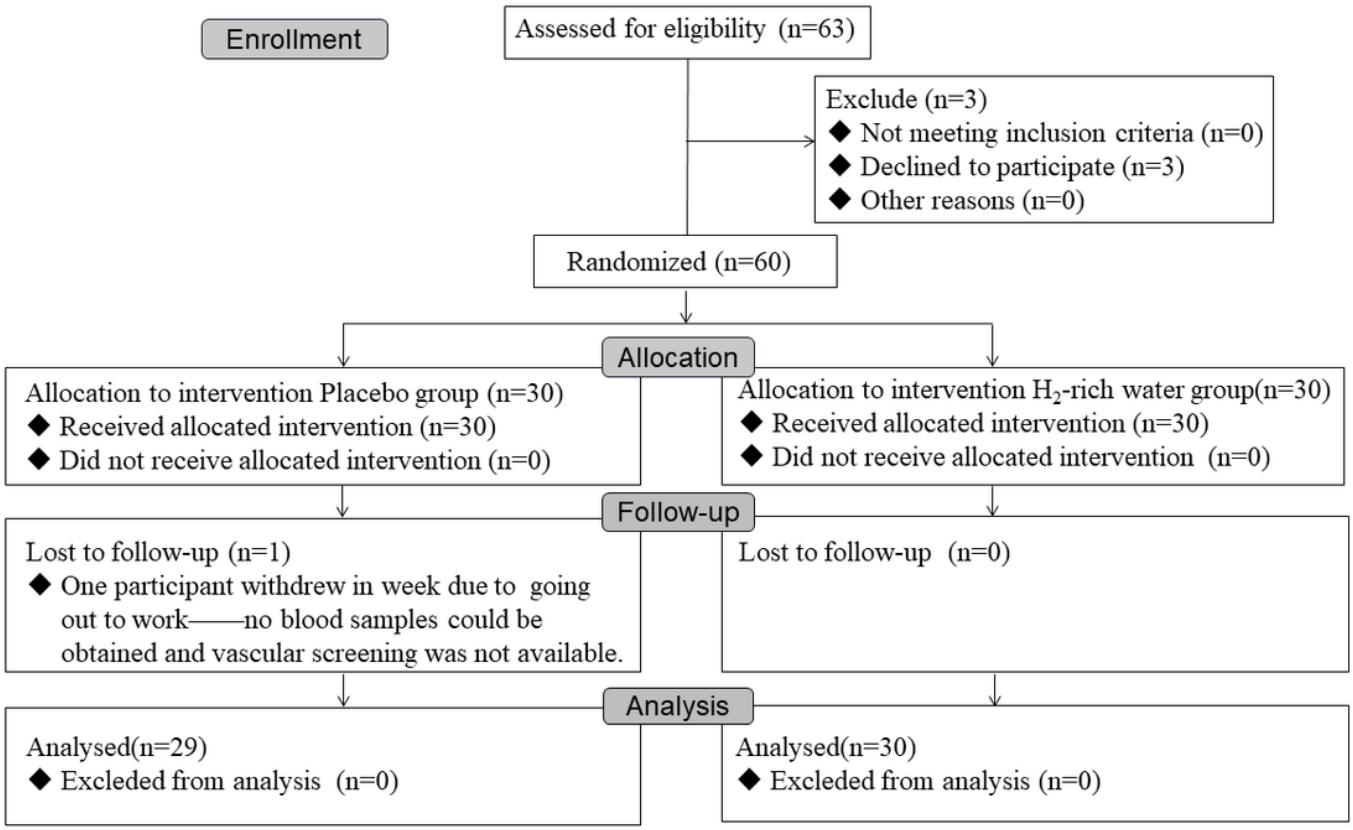

Figure 1

Flow diagram of the study subjects

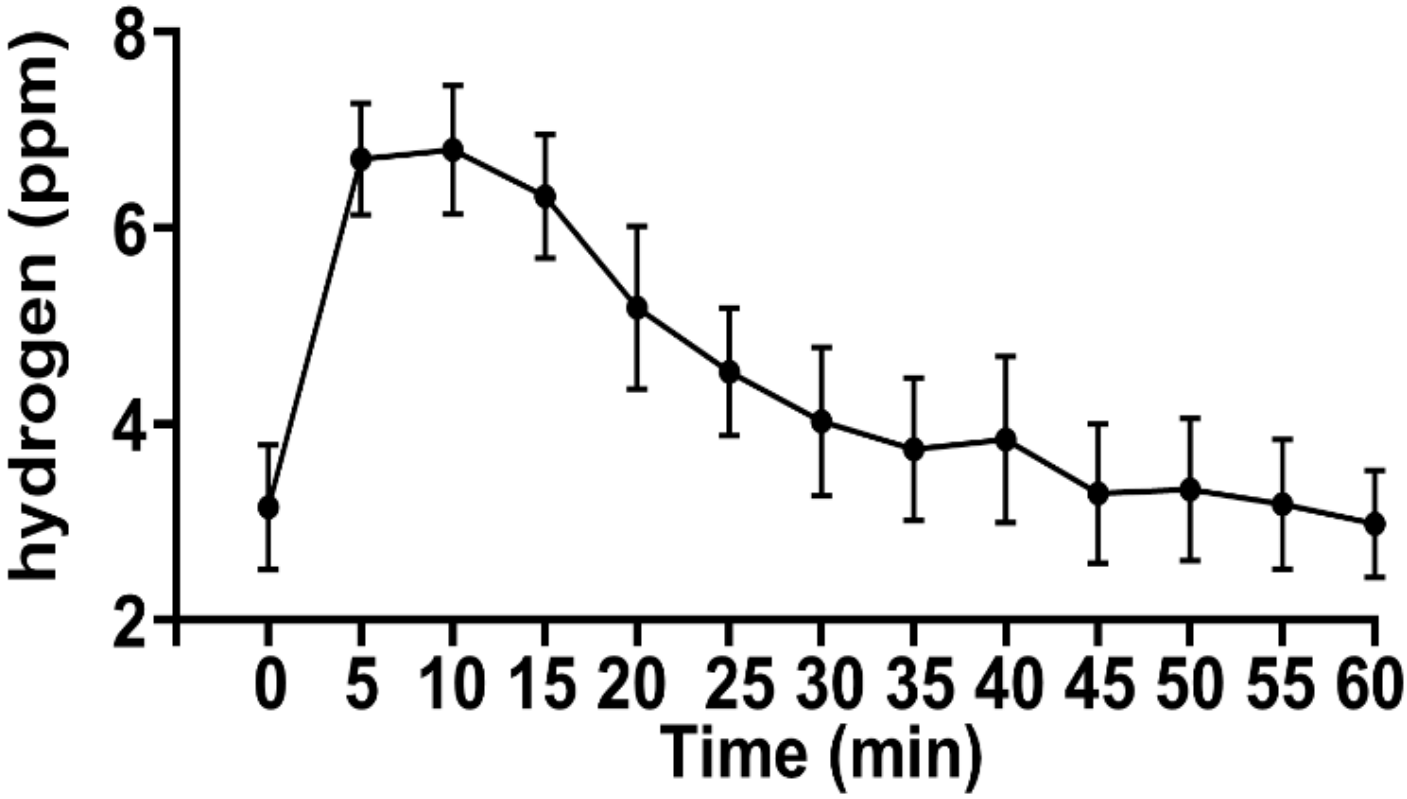

Figure 2

Exhaled H2 concentration after drinking hydrogen-rich water.

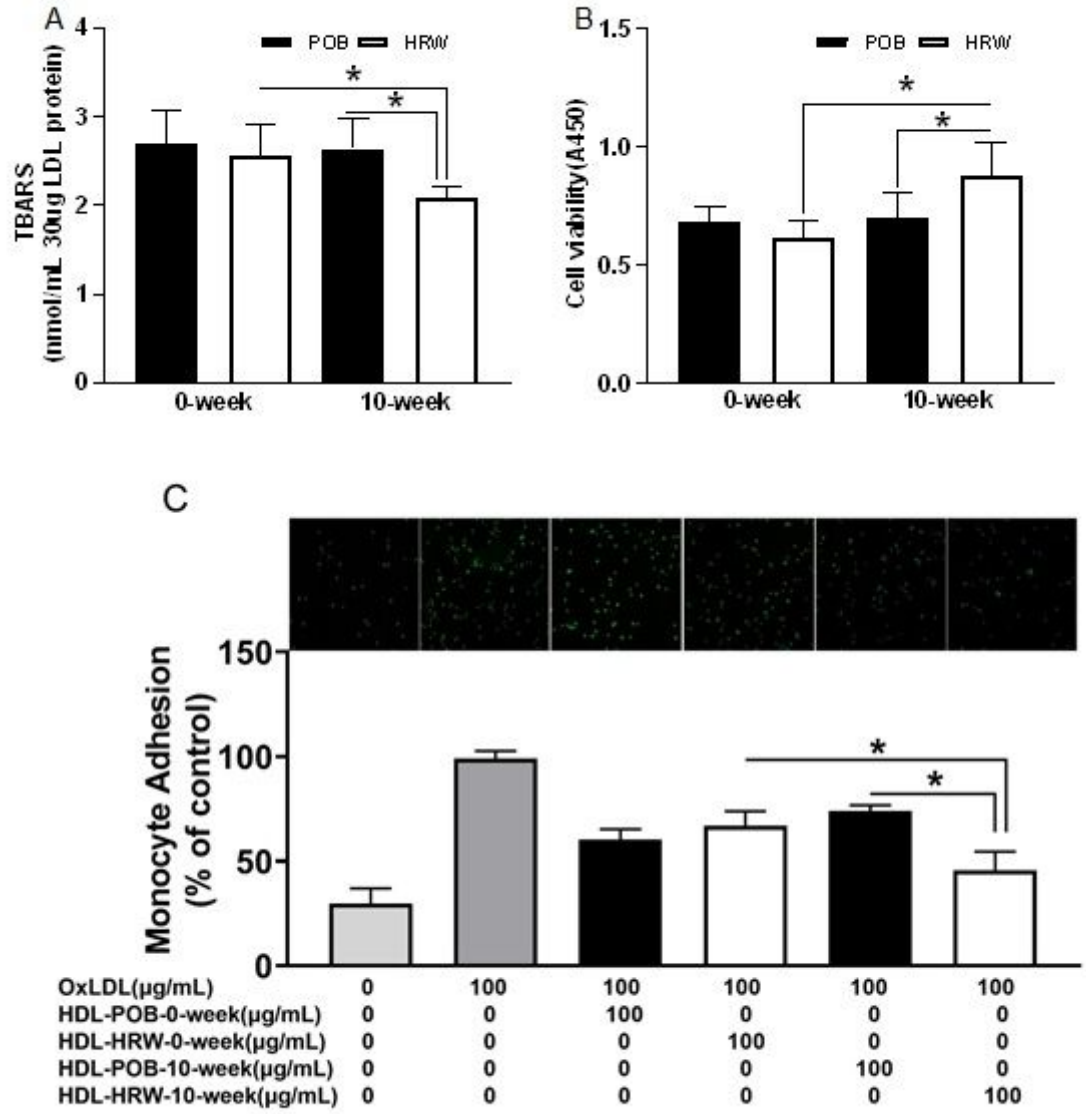

Figure 3

Effect of H2 on the functional of HDL particle. The results are shown as mean ±SEM. (A)TBARS (nmol/mL 30 μg LDL protein), (B) Cell viability, and (C) Monocyte Adhesion. *, P <0.05.