Hydrogen-rich water decreases serum low-density lipoprotein cholesterol levels and improves high-density lipoprotein function in patients with potential metabolic syndrome

Guohua Song [§ 1], Min Li [§ 2], Hui Sang [§ 3], Liying Zhang [2], Xiuhong Li [4], Shutong Yao [3], Yang Yu [1], Chuanlong Zong [1], Yazhuo Xue [* 2], Shucun Qin [* 1]

1 Key Laboratory of Atherosclerosis in Universities of Shandong and Institute of Atherosclerosis, TaiShan Medical University, ShanDong, China

2 Institute of Nursing, TaiShan Medical University, ShanDong, China

3 Department of Pathophysiology, Institute of Basic Medicine, TaiShan Medical University, ShanDong, China

4 TaiAn mental diseases hospital, ShanDong, China

§ Equal contribution to this study

Abbreviated title: H_2 decreases LDL-C level and improves HDL function in patients

Correspondence to *Shucun Qin, Institute of Atherosclerosis, Taishan Medical University, No. 2 Yingsheng East Road, Shandong 271000, P. R. China, E-mail: shucunqin@hotmail.com, Telephone: 86-538-6237252, Fax: 86-538-6225275
*Yazhuo Xue, Institute of Nursing, TaiShan Medical University, ShanDong, China, E-mail: yzxue@tsmc.edu.cn, Telephone: 86-538-8216180, Fax: 86-538-6238121

Abstract

We have found hydrogen (dihydrogen; H_2) has beneficial lipid-lowering effects in high-fat diet-fed Syrian golden hamsters. The objective of this study was to characterize the effects of H_2-rich water (0.9-1.0 L/day) on the content, composition, and biological activities of serum lipoproteins on 20 patients with potential metabolic syndrome. Serum analysis showed that consumption of H_2-rich water for 10 weeks resulted in decreased serum total-cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C) levels. Western blot analysis revealed a marked decrease of apolipoprotein B100 (apoB100) and apoE in serum. Besides, we found H_2 significantly improved high-density lipoprotein (HDL) functionality assessed in four independent ways, namely (i) protection against LDL oxidation, (ii) inhibition of TNF-α induced monocyte adhension to endothelial cells, (iii) stimulation of cholesterol efflux from macrophage foam cells, (iv) protection of endothelial cells from TNF-α induced apoptosis. Further, we found consumption of H_2-rich water resulted in an increase in antioxidant enzyme superoxide dismutase and a decrease in thiobarbituric acid-reactive substances in whole serum and LDL. In conclusion, supplementation with H_2-rich water appear to decrease serum LDL-C and apoB levels, improve dyslipidemia injured HDL functions, and reduce the oxidative stress and may have a beneficial role in prevention of potential metabolic syndrome.

Keywords: Apolipoprotein B, anti-oxidative property, metabolic syndrome

Introduction

Hydrogen (dihydrogen; H_2), as the lightest and most abundant chemical element, is considered a novel antioxidant that can reduce oxidative stress (1). Since then, hydrogen gas has come to the forefront of therapeutic medical gas research. Accumulated evidence in a variety of biomedical fields using clinical and experimental models for many diseases proves that H_2, administered either through gas inhalation or consumption of an aqueous H_2-containing solution, can act as a feasible therapeutic strategy in different disease models. For example, supplementation with H_2-rich water was demonstrated to have a beneficial role in prevention of type 1, type 2 diabetes and insulin resistance (2-3), chronic liver inflammation (4), acute oxidative stress, and focal brain ischemia/reperfusion injury (1). In addition, we have reported that consumption of H_2-saturated saline for 8 weeks prevent atherosclerosis in apolipoprotein E-knockout (apoE-/-) mice (5) and administration of H_2-saturated saline for 4 weeks decreases serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels in high-fat diet-fed hamsters (6). However, the lipid-regulating effect of H_2 in humans has not yet been demonstrated. Therefore, the aim of this study was to characterize the effects of H_2 on the content and composition of serum lipoproteins in patients with potential dyslipidemia.

Metabolic syndrome is characterized by a constellation of metabolic and anthropometric abnormalities, which include excess weight, insulin resistance, hyperglycemia, hypertension, low concentration of high-density lipoprotein cholesterol (HDL-C) and dyslipidemia (7-9). Metabolic disease remains a serious concern in the world and people with metabolic syndrome are at increased risk of developing cardiovascular disease, stroke, and type 2 diabetes (9-10). It is known that the functions and concentrations of serum HDL have strong inverse correlations with risk of metabolic syndrome and atherosclerotic cardiovascular disease (11-13). HDL is known to undergo dramatic modification in structure and composition under the concerted actions of inflammation and oxidative stress (14-15). As a result, HDL particles progressively lose normal biological activities and acquire altered properties. It is well known that H_2 is an electron donor and therefore has a high reducing ability, and the beneficial effects of H_2 on different disease model are mostly dependent on its anti-oxidative, anti-inflammation and anti-apoptotic properties (16). Therefore, it is

possible that dyslipidemia injured HDL function might be improved by H_2 treatment in animals or patients with hyperlipidemia or metabolic syndrome. We have previously demonstrated that administration of H_2 -saturated saline markedly improves the functional properties of the HDL particle in mice and golden hamsters (5-6). However, whether the same effects of H_2 could be observed in humans has not yet been determined. Here, we aimed to characterize the effects of H_2 on functional properties of the HDL particle on 20 patients with potential metabolic syndrome.

Methods

Subjects

The study protocol was approved by the Ethics Committee of TaiShan Medical University. We recruited 20 subjects ≥43 years, males (n = 12) and females (n = 8), from existing patient databases or by advertisement. In this study, subjects were required to have one or more of the following conditions: prehypertension (diastolic blood pressure of 80–89 mmHg and systolic blood pressure of 139 mmHg or lower), pre-diabetes (fasting serum glucose from 5.2 to 6.9 mmol/L), TC > 5.18 mmol/L and/or LDL-C > 2.59 mmol/L, body mass index (BMI) between 25.0 and 34.9 kg/m^2, waist circumference of ≥ 100 cm for males and ≥ 88 cm for females. All participants provided written informed consent to participate before enrollment in the study.

Study Design and Preparation of H_2 Water

The patients consumed 0.9-1.0 L/day of H_2-rich pure water for 10 weeks. A plastic shelled product (Premium FDR, Friendear, Tokyo, Japan) consisting of metallic magnesium (99.9% pure) and natural stones in 500 ml of polypropylene water bottles (NongFu Spring, HangZhou, China) was used to produce H_2. The product was capable of generating H_2 when placed in drinking water by the following chemical reaction; $Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2$. The H_2 water stick was placed into the sealed water bottle for 12 hours before consumption by the patients. The sealed cap of the bottle protects the H_2 from escaping thus preserving the H_2 ions and molecules in the water. The water was drank by patients within 15 min after opening the sealed cap and the H_2 concentration was maintained between 0.2 and 0.25 mM and pH between 7.8 and 8.2 measured by a H_2 sensor (Unisense, Denmark) for continuous 15 min after opening the sealed cap. Subjects were instructed to reuse the

magnesium sticks by transferring the sticks to a new bottle of water after use. In summary, subjects were expected to consume 450-500 ml of H_2 rich water 2 times/day for a total minimum consumption of 900 ml to a maximum consumption of 1000 ml. The blood samples were collected at baseline (0 week) and after 10 weeks of drinking H_2 water.

Serum Analysis

a) Serum lipids

Blood samples were obtained in the morning after an overnight fast. Serum glucose levels were measured by the glucose oxidase method. Serum TC, high-density lipoprotein cholesterol (HDL-C), LDL-C, and triacylglycerols (TG) were measured by enzymatic methods on a chemical autoanalyzer (Hitachi Co, Tokyo, Japan). Lipoprotein profiles were obtained by fast protein liquid chromatography (FPLC), using Superpose 6 10/300GL column (17). Briefly, 100 µl of fasting serum was applied to a Superose 6 column, and the samples were eluted in a mobile phase (0.15 M NaCl, 0.01 % NaN_3, and 2 mM EDTA, pH 7.5) at a rate of 0.3 ml/min in 60 fractions of 500 µl. Lipid composition of fractions corresponding to VLDL, LDL, and HDL were quantified by enzymatic assays using commercially available kits for TC (BioSino, Beijing, China).

b) Measurement of serum oxidative stress and oxidizability

Serum levels of malondialdehyde (MDA), a marker for oxidative stress, were determined by a spectrophotometric measurement of thiobarbituric acid-reactive substances (TBARS) according to the manufacturer's instructions (Nanjing Jiancheng Biochemistry, China). The activity of superoxide dismutase (SOD), which acts as antioxidant and protect cellular components from being oxidized by reactive oxygen species, was measured by a commercial kit (Nanjing Jiancheng Biochemistry, China) according to the manufacturer's instructions. The activity of paraoxonase-1 (PON1), an antioxidant enzyme associated with HDL, was measured by adding serum to 1 ml of Tris-HCl buffer (100 mM, pH 8.0) containing 1 mM $CaCl_2$ and 1 mM of phenylacetate (Sigma) as described previously (18). The rate of phenyl acetate hydrolysis was determined spectrophotometrically (Uvikon 930 spectrophotometer, Kontron) at 270 nm. PON1 activity was expressed in international units (U) per milliliter of serum. When measuring PON-1 activity in lipoproteins, the activity was expressed in international U per gram of protein. The contents of the biologically

active oxidized lipids in serum, including 12-hydroxy eicosatetraenoic acid (12-HETE), 13-hydroxy octadecadienoic acid (13-HODE), prostaglandin (PG) and 8-iso-prostaglandin F2α(8-iso-PGF2α), were determined by ELISA (Bluegene, Shanghai, China) according to the manufacturer's instructions.

c) Measurement of serum inflammatory factors

Serum concentrations of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), were determined by ELISA kits (Bluegene, Shanghai, China) according to the manufacturer's instructions.

Western Blots

For serum apolipoprotein measurement, 0.2 μl of serum was denatured at 90 °C for 10 min and then subjected to western blot analysis using anti-apoB, apoE and apoAI antibody (Abcam). The proteins were visualized and quantified using a chemiluminescence method (Pierce) and Quantity One (Bio-Rad) software program.

Measuring anti-oxidant properties of HDL

The serum of every 5-6 patients was pooled and fasted serum lipoproteins were fractionated by ultracentrifugation at 40,000 rpm in a Beckman Optima LE-80K into VLDL (density less than 1.006 g/ml), LDL (density = 1.006-1.063 g/ml) and HDL3 (density = 1.125-1.21 g/ml) as described previously (19). Fractions were dialyzed in PBS at 4°C and LDL (100 μg protein/ml) was incubated with freshly prepared $CuSO_4$ (10 μ mol/L) in the presence or absence of the isolated HDL (200 μg protein/ml). After incubation at 37°C for 2 h, the extent of LDL oxidation was assessed by measuring of thiobarbituric acidreactive substances (TBARS) formation (20) via a spectrophotometric method according to the manufacturer's instructions (Nanjing Jiancheng Biochemistry, China).

Endothelial Cell - Monocyte Adhesion Assay

Monocyte adhesion assays were performed under static conditions as previously described (21) with minor modification. Human umbilical vein endothelial cells (HUVECs) were grown to confluence in 24-well plates and pretreated with or without HDL (100 μg protein/ml) for 18 hours and stimulated with TNF-α (10 ng/ml) or LDL (100 μg protein/ml) for 6 hours. THP-1 cells were labeled with a fluorescent dye, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM),

by incubation with 10 µmol/L BCECF-AM at 37°C for 1 hour in RPMI-1640 medium and were subsequently washed with EBM-2. Confluent HUVECs in 24-well plates were washed 3 times, and labeled THP-1 cells ($2x10^5$ cells per 200 µL) were added to each well of HUVECs. THP-1 cells were allowed to adhere to HUVECs by incubation at 37°C for 60 minutes, and unbound THP-1 cells were removed by washing (3 times, 5 minutes). THP-1 cells bound to HUVECs were counted under fluorescent microscope. The numbers of adherent leukocytes were determined by counting 4 fields per ×100 high-power-field well using fluorescent microscopy (Nikon, Japan) and photographed. Four randomly chosen high-power fields were counted per well. Experiments were performed in duplicate or triplicate and were repeated at least 3 times. The person counting the adherent monocytes was unaware of the treatment.

HDL Induced Cholesterol Efflux Assay

Cholesterol efflux experiments were performed as described by Smith et al (22). Acetyl LDL (AcLDL) was prepared according to the methods of Basu et al (23). RAW264.7 macrophages at 50 % confluence were cholesterol loaded and labeled in 1 ml of RGGB (RPMI 1640 supplemented with 50 mM glucose, 2 mM glutamine, and 0.1 % BSA) containing [^{3}H]cholesterol (1 µCi/ml) and AcLDL (100 µg protein/ml) for 30 min. Then, macrophages were washed twice with 0.1% BSA-PBS, and equilibrated with RGGB for 24 h. On the following day, the medium was then replaced with RGGB containing 200 µg protein/ml of HDL. After 12 h of incubation, the culture was centrifuged to remove cell debris and 100 µl of the medium was removed for determination of radioactivity. At the end of the chase period, the macrophages were dissolved in 0.4 ml of 0.1 M sodium hydroxide, and the radioactivity per aliquot was measured. The percentage cholesterol efflux was calculated by dividing the media-derived radioactivity by the sum of the radioactivity in the media and the macrophages.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

HUVECs were grown to confluence in 96-well plates and pretreated with or without HDL (100 µg protein/ml) for 18 hours and stimulated with TNF-α (15 ng/ml) for 6 hours. Cells treated with medium only served as a negative control group. After removing the supernatant of each well and washing twice by PBS, 10 µl of MTT solution (5 mg/ml in PBS) and 100 µl of medium were then introduced. After

incubation for another 4 h, the resultant formazan crystals were dissolved in dimethyl sulfoxide (150 µl) and the absorbance intensity measured by a microplate reader (Tecan, Sweden) at 490 nm with a reference wavelength of 620 nm. All experiments were performed in quadruplicate, and the relative cell viability (%) was expressed as a percentage relative to the untreated control cells.

LDL oxidation assay

LDL was isolated by ultracentrifugation from the serum. The MDA content of LDL was determined by the measurement of TBARS and expressed as nmol per mg protein. For determining LDL-mediated inflammation, 100 µg protein/ml of LDL was added to the cultured RAW264.7 macrophages and bone marrow-derived macrophages. After incubation for 24 hr, quantitation of the secreted proinflammatory cytokines, including IL-6 and TNF-α, were performed from aliquots of conditioned medium by ELISA (Bluegene, Shanghai, China) according to the manufacturer's instructions. Bone marrow-derived macrophages in mice were obtained as described previously (24).

Statistical Analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) test with the GraphPad Prism programme ver.4.0. Results are expressed as means ± SD. P values less than 0.05 were considered significant.

Results

Subject characteristics

The baseline demographics of subjects are presented in Table 1. Subjects enrolled in the study included those who had TC >5.18 mmol/L (n = 17), LDL-C >2.59 mmol/L (n = 18), BMI 25-34.9 (n = 17), and/or smokers (n = 10). All subjects showed mean normal clinical levels of baseline biometric parameters, clinical chemistry and hematology. All smokers were occasional smokers.

Effect of H_2 on serum lipoprotein profiles, and serum levels of anti-oxidative and inflammatory biomarkers

The serum lipid levels of each individual were presented in Table 2. And the

distribution of lipid levels was shown in Figure 1. We can see that serum TC and LDL-C levels were significantly decreased after 10 weeks of H_2 treatment in all of the 20 patients with potential metabolic syndrome. Among 20 patients, 18 had decreased and 1 had increased (patient 14) TC and LDL-C levels, and 1 was not altered after H_2 consumption (patient 8).

In addition, among the 20 patients, 10 were smokers and 10 were non-smokers. As shown in Table 2, H_2 treatment decreases serum TC and LDL-C levels not only in smokers but also in non-smokers. And it seems that the lipid-lowering effects of H_2 on smokers were better than that on non-smokers although there is no significant difference. Besides, Serum levels of HDL-C and glucose were not altered by consumption of H_2 (Table 2, Figure 1C, D). Interestingly, serum TG levels have a trend to be decreased by H_2 treatment in 10 smokers although there is no significant difference (Table 2), which causes a slight decrease in TG levels in all of the 20 patients (Table 2 and Figure 1E).

Moreover, serum lipoprotein profile by FPLC further confirmed the decrease in serum LDL-C levels in H_2-treated subjects and revealed that serum VLDL-C and HDL-C remained unchanged after intake of H_2 water (Figure 2).

Changes in biomarkers of oxidative and inflammatory status after 10 weeks of consumption of H_2 water are shown in Figure 3. Serum levels of MDA, one of the most frequently used indicators of lipid peroxidation, was decreased significantly (Figure 3A) and the activity of SOD, which act as antioxidants and protect cellular components from being oxidized by reactive oxygen species, was increased after H_2 water consumption (Figure 3B). However, the activity of PON-1, an antioxidant enzyme associated with HDL, was not altered in both serum and HDL3 fractions by H_2 water (Figure 3C and 3D). In addition, we detected serum concentrations of several biologically active oxidized lipids, including 12-HETE, 13-HODE, PG and 8-iso-PGF2α. The data in Figure 3E and 3F showed that 13-HODE and 8-iso-PGF2α levels were improved following the H_2 treatment, however 12-HETE and PG levels were not altered by H_2. Besides, there was no significant effect of intake of H_2 water on serum levels of inflammatory biomarkers, including TNF-α and IL-6 (Figure 3G and 3H).

H_2 treatment decreases apoB and apoE protein levels in serum

Serum LDL and HDL are particles composed of a variety of lipids and protein

components, it is thus necessary to clarify which content of the lipoprotein could be affected by H_2 treatment. Consistent with the difference observed for LDL-cholesterol, the major proteins on LDL, apoB100, were significantly decreased by consumption of H_2 water in patients with potential metabolic syndrome (Figure 4). The apoE protein which is mainly present in VLDL and LDL particles was also lowered with the treatment of H_2 (Figure 4). Besides, the major proteins on HDL, apoAI, were not altered after intake of H_2 water (Figure 4), which is consistent with the changes observed for HDL-C. These data suggested that H_2 could decrease the expression of the major protein constituent of LDL and VLDL.

H_2 improves the oxidation and the functional properties of the HDL particle

HDL is known to undergo dramatic modification in structure and composition under the concerted actions of inflammation and oxidative stress (14-15). Recent evidence indicates that H_2 acts as a therapeutic medical gas in a variety of disease models by exerting antioxidant and anti-inflammatory effects (3, 25-26). Therefore, it is possible that administration of H_2 might improve the functional quality of HDL particle. Firstly, H_2 water supplementation tended to decrease the MDA content in HDL3 (Figure 5A), suggesting H_2 improves the oxidation of HDL particle. Secondly, the biological effect of H_2 on the anti-oxidative functionality of HDL was tested, namely, the protection of LDL particles from oxidation. As showed in Figure 5B, H_2 treatment significantly inhibited the formation of TBARS.

Thirdly, the effect of H_2 on the anti-inflammatory properties of HDL was tested, including protection of cytokine induced monocyte adhesion to endothelial cells and stimulation of endothelial NO production, which has been suggested as an important endothelial-atheroprotective effect of HDL. As shown in Figure 5C, after incubation of HUVECs for 6 hr with TNF-α, adhesion of monocytes to HUVECs was significantly increased and pre-incubation of HUVECs with HDL3 isolated from patients after 10 weeks of drinking H_2 water (HDL-H_2-A), markly reduced TNF-α induced adhension of monocyte to HUVECs compared with those of HDL3 isolated at baseline (0 week) (HDL-H_2-B). These data suggested the anti-inflammatory function of HDL was improved by H_2 water. We also determined the effects of HDL-H_2-A as compared with HDL-H_2-B on endothelial NO production. Unfortunately, the NO production was not altered significantly after intake of H_2 water (data not shown), which might be attributable to the sensitivity of Griess

method.

Fourthly, the ability of the isolated HDL particles to elicit efflux from cholesterol-loaded macrophages was tested. As showed in Figure 5D, HDL particles isolated from the serum after H_2 treatment exhibited dramatically higher efflux properties compared with the HDL particles isolated from the serum before H_2 treatment, indicating the cholesterol efflux ability mediated by HDL particle was increased by H_2. Lastly, the biological effect of H_2 on the anti-apoptotic functionality of HDL was determined. As shown in figure 5E, H_2 treatment significantly inhibited TNF-α induced endothelial cell apoptosis. These data indicate that, dyslipidemia injured HDL functions, including the ability to protect against LDL oxidation, the ability to inhibit cytokine induced monocyte adhension to endothelial cells, the ability to stimulate cholesterol efflux from macrophage foam cells and the ability to protect endothelial cell apoptosis were markedly improved by administration of H_2 water in patients with potential metabolic syndrome.

H_2 reduces the oxidation of LDL and LDL-mediated inflammation

The oxidation of LDL plays an important role in atherogenesis and may influence lipid metabolism. In this study, we determined the effects of H_2 water on the oxidation of LDL and LDL-mediated inflammation. As shown in Figure 6A, the MDA content of the isolated LDL was reduced by H_2-treatment, suggesting H_2 reduces the oxidation of LDL. Nextly, for determining LDL-mediated inflammation, 100 μg protein/ml of the isolated LDL was added to the cultured RAW264.7 macrophages and bone marrow-derived macrophages for 24 hr, as shown in figure 6B and C, the secretion of TNF-α and IL-6 by macrophages has a trend to be reduced by H_2-treatment. Furthermore, we tested the effects of H_2 on LDL-induced monocyte adhesion to endothelial cells. As shown in figure 6D, consumption of H_2 water significantly decreased LDL-induced monocyte adhesion to endothelial cells. These data revealed that H_2 reduces LDL-mediated inflammation properties in culture.

Discussion

In the previous study, we have found that H_2 has beneficial lipid-lowering effects in high-fat diet-fed Syrian golden hamsters (6). However, it remains unknown whether H_2 has effects on lipid and lipoprotein metabolism in humans. The key finding of our present study is that the novel anti-oxidant chemical element, H_2,

appear to alleviate lipid metabolism disorder, including hyperlipidemia and defective HDL function, in patients with potential metabolic syndrome. Our results were not consistent with that of human studies in type 2 diabetes mellitus, they showed significant decreases in modified LDL-C levels and no effect on total and LDL-cholesterol (3). The inconsistency might be attributable to the difference in pathological conditions, the dosage of administration, or the intervention period. Despite this possibility, the discrepancy might be explained by the fact that modified LDL, like oxidized LDL was largely existed in the hyperlipidemia model, which is used in our study.

Subanalysis was conducted on 20 subjects and we found the serum TC levels of 17 subjects with hyperlipidemia (TC >5.18 mmol/L) was decreased by intake of H_2 water for 10 weeks, and the serum TC levels of the remained 3 subjects without hyperlipidemia (TC < 5.18 mmol/L) was not significantly altered by H_2 treatment, even the TC level of 1 subject was slightly increased after treatment (Patient 14 in Table 2). The data gave us a clue that H_2 might exert lipid-lowering effects in the patients with hyperlipidemia and has little effect on the population without hyperlipidemia.

It is well known that high cholesterol level is one of the important risk factors for atherosclerosis. Therefore, the elucidation of the mechanism by which H_2 decreases serum cholesterol will provide solid evidence for the application of H_2 in cardiovascular disease therapy. Serum LDL and VLDL are particles composed of a variety of lipids and protein components, it is thus necessary to clarify which content of the lipoprotein could be affected by H_2 treatment. Firstly, LDL is a contributing factor to the development of atherosclerosis. Apo B100 is the major protein present in LDL particles, and like LDL cholesterol, the serum apoB level has been positively correlated with risk for atherosclerotic disease. In the present study, we found H_2 treatment not only decreased serum LDL cholesterol levels, but also remarkably lowered apoB100 and apoE protein levels in serum. The data gave us a clue that H_2 might regulate the metabolism of LDL-C by decreasing the synthesis of apoB or increasing the catabolism of LDL-C. Secondly, like LDL cholesterol, VLDL cholesterol is considered a type of "bad" cholesterol because elevated levels are associated with an increased risk of coronary artery disease (27). We found that apoB and apoE, the major protein in VLDL, was lowered by H_2 although cholesterol analysis by FPLC revealed no changes of VLDL-C after H_2 treatment. The

inconsistencies in protein and cholesterol levels might be explained by the suspection that the functional target of H_2 might be apolipoprotein expression, but not cholesterol. Taken together, the data indicated that molecular H_2 dissolved in water might have beneficial regulating effect on lipid abnormality in patients with metabolic syndrome, especially in patients with hyperlipidemia. And this effect is partially related with its regulation of lipid and protein contents of LDL and VLDL. Further experiments are needed to identify the mechanisms by which H_2 regulate the lipid and protein contents of lipoprotein particles and improve serum lipoprotein profile. Previous studies have demonstrated that administration of H_2-rich saline improves insulin sensitivity (3) that, in our view, this could partly contribute to the improved lipid metabolism in our study. Furthermore, liver cells sense the reduced levels of hepatic intracellular cholesterol and seek to compensate by synthesizing LDL receptors to draw cholesterol out of the circulation (28). Future studies on hepatic HMG-CoA reductase and LDL receptors may open a novel window to elucidate the mechanism by which H_2 action on lipoprotein regulation.

HDL are known to protect against the development of atherosclerosis and are widely documented as a 'negative risk factor' for coronary heart diseases (29). The anti-atherogenic activity of HDL is principally attributable to a variety of anti-oxidative, anti-inflammatory, and anti-apoptotic properties and the reverse transport of cholesterol (30). In the present study, we found H_2 treatment appear to improve the functionality of HDL3 without altering HDL-C serum levels. It is known that the beneficial effects of H_2 on different disease model are mostly dependent on its anti-oxidative, anti-inflammation and anti-apoptotic properties (16). Therefore, it is possible that the protective effect of H_2 on HDL function in our study is attributable to, at least in part, the anti-oxidative and anti-inflammatory properties of H_2. Besides, a number of therapeutic strategies are being developed to target HDL-C in an attempt to inhibit the progression or induce regression of atherosclerosis and reduce cardiovascular events nowadays. Therefore, the protective effect of H_2 on HDL function in the present study might provide a further evidence for H_2 application in vascular disease therapy. Moreover, in the present study, we did not observe any unwanted side effects of H_2, including headache, diarrhea and vomiting (data not shown), suggesting a less toxicity and adverse effects of H_2.

The oxidation of LDL plays an important role in atherogenesis and may influence lipid metabolism. In this study, our data revealed that H_2 appear to reduce

the oxidation of LDL and LDL-mediated inflammation changes and further support our prediction that H_2 might improve lipid metabolism in patients with hyperlipidemia by inhibiting LDL-mediated inflammation changes. Previous studies have demonstrated that administration of H_2 reduces atherogenesis in apoE-/- mice (5) that, in our view, this could partly contribute to the observed protective effects of H_2 on the oxidation of LDL and LDL-mediated inflammation changes in our study.

Indeed, our data gave us a clue that H_2 might have the potential to be used as a novel lipid-regulating agent with the advantage of no toxicity compared with other commonly used lipid-regulating drugs which have side-effects on the liver and kidney. Further understanding of the mechanisms underlying the signaling pathways involved in the capacity of H_2 to influence lipid and cellular metabolism is required to fully exploit intake of H_2 gas as a therapeutic strategy.

In addition, there are several limitations to the present study. First, the number of subjects recruited is small and it`s hard to draw a solid conclusion particularly having smokers among them. It should, however, be emphasized that we analyzed the data in smokers and non-smokers separately and the response to H_2 in smokers seem to be stronger than that in non-smokers, but the difference did not reach the significance. It would be useful to enlarge the sample size and examine the reaction to H_2 in smokers due to the anti-oxidative property of H_2. Another limitation of the study was that this was not a double-blinded randomized controlled trial comparing H_2 rich water to placebo and comparing experimental endpoints from H_2-treated metabolic syndrome with those measured in untreated healthy individuals, it is therefore possible that during the 10 weeks of the study the subjects made alteration in their lifestyles that could have affected the parameters studied. Accordingly, it`s hard for us to draw a solid conclusion and it limit the conclusions to stating that H_2 treatment appear to regulate lipid metabolism in patients.

In conclusion, our data show that in vivo administration of H_2 water appear to decrease serum TC and LDL-C levels and improve HDL functions in patients with potential metabolic syndrome, suggesting that H_2 may be used as a newer pharmacological agent to treat or control lipid metabolism disorder.

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Disclosure statement

The authors declare that there are no conflicts of interest in our manuscript.

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Figure legends

Figure 1. Effect of H_2 on the levels of serum lipids and glucose. (A) Serum total cholesterol (TC), (B) Low-density lipoprotein cholesterol (LDL-C), (C) High-density lipoprotein cholesterol (HDL-C), (D) Glucose, (E) Triglyceride (TG) in patients with potential metabolic syndrome before and after 10 weeks H_2 consumption were determined by enzymatic method. Data (means ± SD, n = 20) were expressed as mmol per liter. H_2-B: before drinking H_2-rich water; H_2-A: after drinking H_2-rich water. * $P < 0.05$, **$P < 0.01$

Figure 2. FPLC cholesterol profiles using pooled serum samples of n = 5 patients per group showing cholesterol content (mg/dl) of serum lipoprotein fractions.

Figure 3. Effect of H_2 on serum levels of anti-oxidative and inflammatory biomarkers. (A) serum concentrations of MDA, (B) SOD activity in serum, (C and D) PON-1 activity in serum and HDL3 fraction, (E) serum concentrations of 12-HETE and 13-HODE, (F) serum concentrations of 8-iso-PGF2α and PG, (G) serum concentrations of TNF-α, (H) serum concentrations of IL-6. n = 20, * $P < 0.05$, ** $P < 0.01$

Figure 4. Effect of H_2 on serum levels of apoB, apoE and apoAI protein. (A) Effect of H_2 on serum apoB, apoE and apoAI protein levels by western blots. (B) Densitometric quantitation of western blot data (n= 4-5) by Quantity One software. * $P < 0.05$

Figure 5. H_2 appear to improve the oxidation and the functional properties of the HDL particle. The serum of every 5-6 patients was pooled and HDL3 was isolated by ultracentrifugation from the serum. (A) The MDA content in HDL3 particle was shown. HDL function was determined as (B) protection of LDL against oxidation, (C) inhibition of TNF-α induced THP-1 monocytes adhesion to endothelial cells, (D) stimulation of cholesterol efflux from macrophage foam cells, and (E) protection of endothelial cells from TNF-α induced apoptosis. Assays were performed as detailed in Methods. n = 3-4 of pooled serum sample, * $P < 0.05$, ** $P < 0.01$

Figure 6. H_2 appear to reduce the oxidation of LDL in patients and LDL-mediated inflammation in macrophages. The serum of every 5-6 patients was pooled and LDL was isolated by ultracentrifugation from the serum. (A) The MDA content of the isolated LDL. (B) 100 μg protein/ml of the isolated LDL was added to the cultured RAW264.7 macrophages. Supernatants were harvested at 24 h for the measurement of TNF-α and IL-6 by ELISA assay. (C) 100 μg protein/ml of the isolated LDL was added to the cultured bone marrow-derived macrophages and cytokines in medium was measured after 24 hr incubation. (D) HUVECs were stimulated with LDL (100 μg protein/ml) for 6 hours and THP-1 cells, labeled with a fluorescent dye BCECF-AM, were added to HUVECs. After adhereing, THP-1 cells bound to HUVECs were counted under fluorescent microscope as described in Methods. The results are expressed as means ± SD (n = 3-4 of pooled serum sample). * $P < 0.05$

Table 1. Characteristics of subjects, biometrics, and glucose for all patients

variable	Before drinking H_2 water	After drinking H_2 water
Patients number	20	
Age (years)	55.8 ± 10.6	
Gender-Male	12/20	
Gender-Female	8/20	
Mean Systolic BP (mmHg)	134 ± 16.6	128.8 ± 16.8
Mean Diastolic BP (mmHg)	82.6 ± 11.5	80.1 ± 11.5
Height (cm)	164.8 ± 8.5	164.8 ± 8.5
Weight (kg)	74.1 ± 13.4	72.0 ± 10.5
Waist Circumference (cm)	99.7 ± 8.5	94.6 ± 6.6
BMI (kg/m^2)	27.3 ± 2.6	26.5 ± 2.1
Fasting Glucose (mmol/L)	5.7 ± 0.6	5.8 ± 1.0
Alcohol Use		
Daily	3/20	
Weekly	3/20	
Occasional	14/20	
Tobacco Use		
Yes	10/20	
None	10/20	

Table 2. Effect of H_2 on the levels of serum lipids and glucose in each individual patient

Patients	Gender	Smoking	TC (mM)	LDL-C (mM)	HDL-C (mM)	TG (mM)	Glucose (mM)	TC (mM)	LDL-C (mM)	HDL-C (mM)	TG (mM)	Glucose (mM)
					H_2-B					H_2-A		
1	M	N	6.96	4.2	1.34	2.24	5.93	6.15	3.14	1.2	2.26	6.33
2	F	N	5.91	3.88	1.35	1.71	5.67	5.77	3.73	1.37	1.04	6.52
3	F	N	6.4	3.88	1.82	1.07	5.72	5.83	3.29	1.6	0.94	6.36
4	F	N	6.23	4.01	1.15	2.01	5.52	5.32	2.94	0.83	4.05	5.39
5	F	N	6.92	4.44	1.5	1.68	5.18	5.42	3.46	1.39	1.72	5.18
6	F	N	6.47	4.47	1.42	0.87	5.52	5.6	3.78	1.33	1.6	5.69
7	F	N	8.26	4.69	2.01	1.66	5.54	6.62	3.8	1.71	0.93	5.59
8	M	N	5.12	3.29	1.11	0.83	5.02	5.17	3.38	1.23	0.71	5.06
9	F	N	5.66	3.7	1.34	0.61	5.12	5.29	3.28	1.35	1.24	4.57
10	M	N	7.21	5.1	1.48	2.21	5.01	6.54	4.12	1.5	2.23	5.06
11	F	Y	6.33	4.08	1.1	2.23	5.74	5.47	3.36	1.11	1.68	6.04
12	M	Y	6.74	4.15	1.02	3.55	5.02	5.65	3.4	1.21	1.23	5.07
13	M	Y	8.22	5.21	1.56	2.56	5.98	6.36	4.19	1.42	3.09	5.47
14	M	Y	3.96	1.6	1.2	4.95	5.61	4.33	1.82	1.08	2.66	5.74
15	M	Y	7.44	3.63	1.05	4.83	4.36	5.01	2.43	1.05	5.32	4.73
16	M	Y	5.91	3.51	1.14	2.77	7.2	5.82	3.41	1.12	2.22	5.8
17	M	Y	6.3	3.28	1.49	3.88	7.3	4.74	2.16	1.37	3.82	7.01
18	M	Y	7.05	4.97	1.27	1.36	5.93	5.54	3.6	1.1	1.18	6.26
19	M	Y	4.09	2.55	0.72	1.62	5.23	3.36	1.82	1.26	0.67	4.95
20	M	Y	7.28	4.54	1.28	1.51	5.31	5.41	3.61	1.21	1.16	6.87
Mean of non-smokers			6.51 ± 0.88	4.17 ± 0.53	1.45 ± 0.28	1.49 ± 0.60	5.42 ± 0.32	5.77 ± 0.52*	3.49 ± 0.36#	1.35 ± 0.24	1.67 ± 1.00	5.58 ± 0.65
Mean of Smokers			6.33 ± 1.38	3.75 ± 1.10	1.18 ± 0.24	2.93 ± 1.33	5.77 ± 0.92	5.17 ± 0.85*	2.98 ± 0.84#	1.19 ± 0.13	2.30 ± 1.45	5.79 ± 0.77
Mean of all patients			6.42 ± 1.13	3.96 ± 0.86	1.32 ± 0.29	2.21 ± 1.24	5.60 ± 0.69	5.47 ± 0.75**	3.24 ± 0.68#	1.27 ± 0.20	1.99 ± 1.25	5.68 ± 0.71

M : male; F : female; N : non-smokers; Y : smokers; H_2-B: Before drinking H_2-rich water; H_2-A: After drinking H_2-rich water; * $p < 0.05$ compared with TC levels before drinking H_2-rich water; ** $p < 0.01$ compared with TC levels before drinking H_2-rich water; # $p < 0.05$ compared with LDL-C levels before drinking H_2-rich water

Figure 1

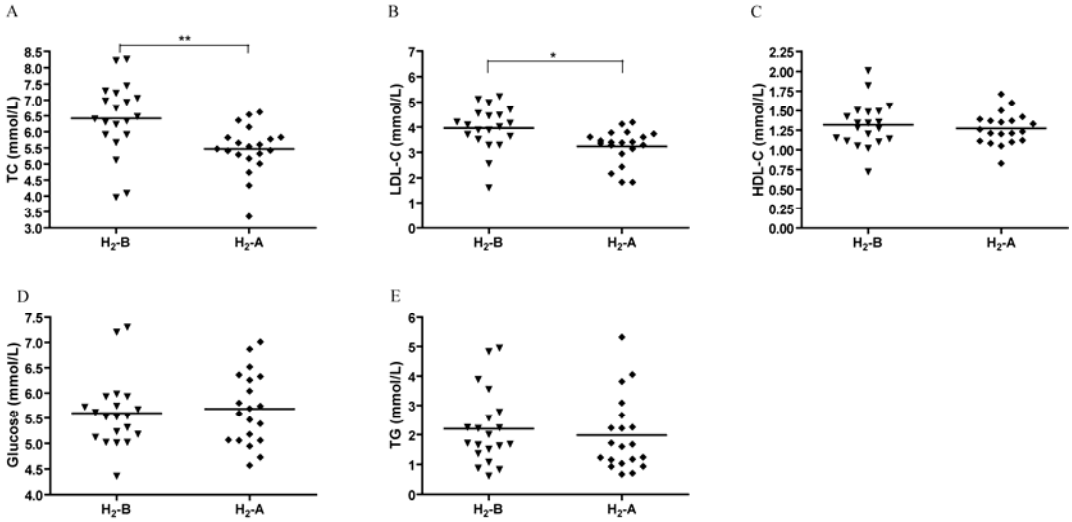
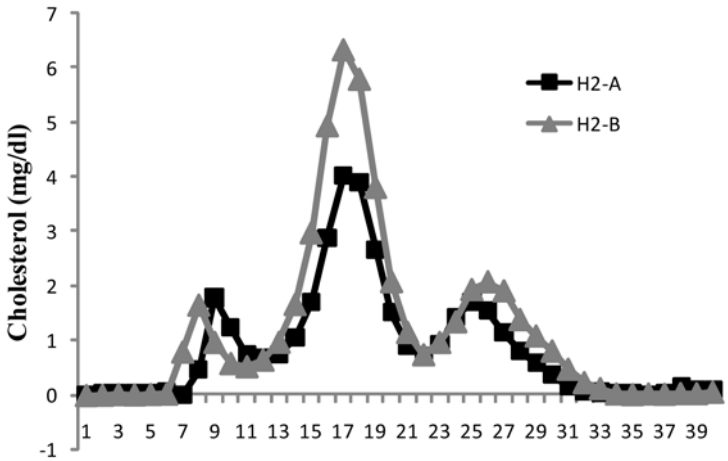


Figure 2



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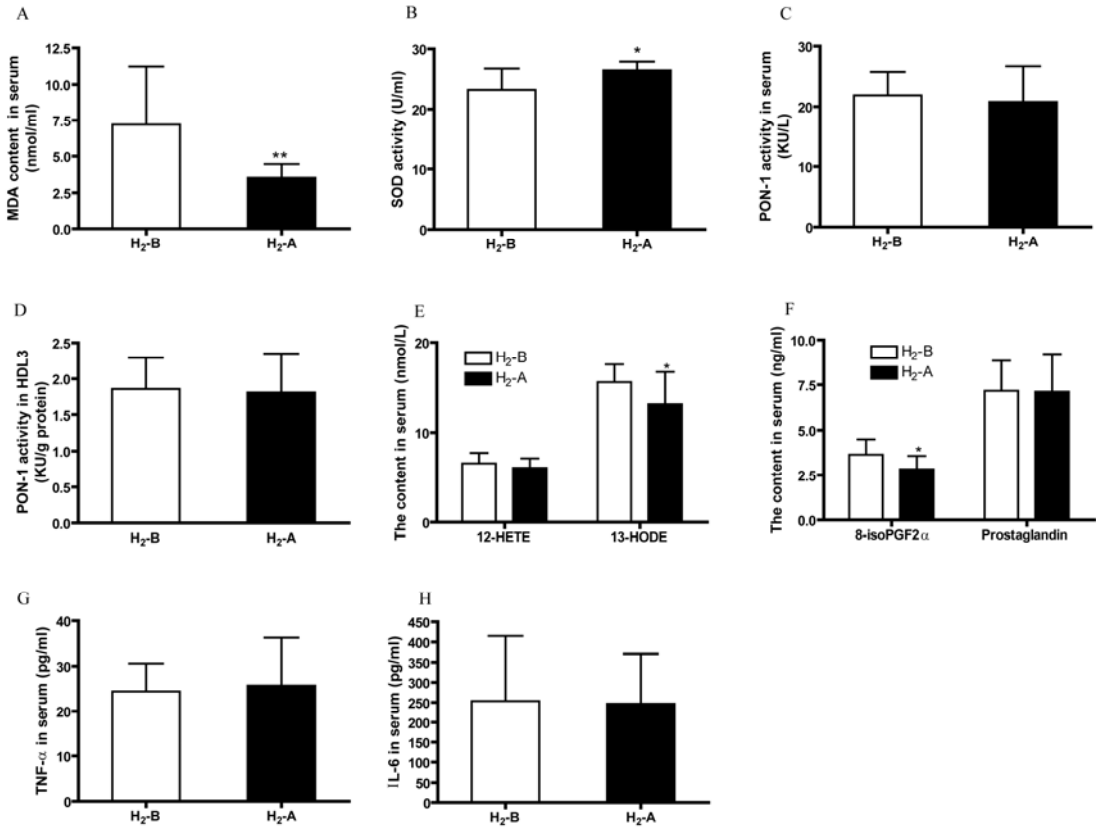

Figure 4

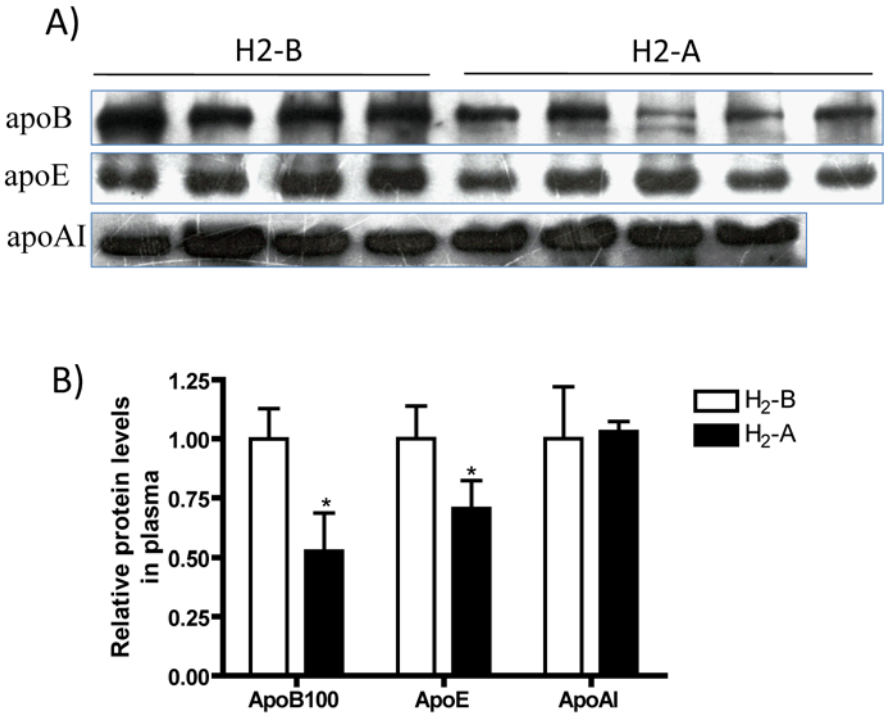

Figure 5

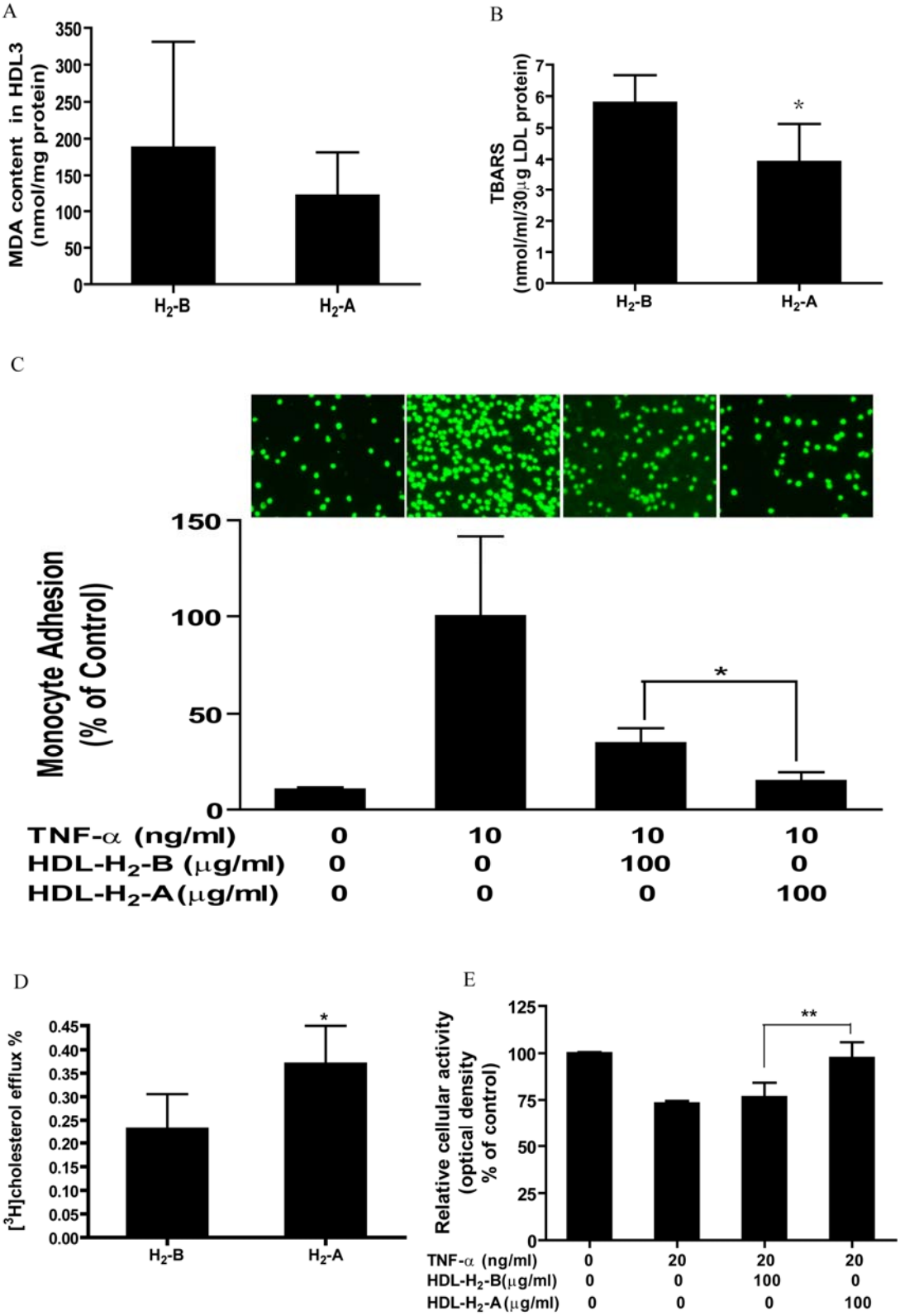

Figure 6