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Administration of hydrogen-saturated saline decreases plasma low-density lipoprotein cholesterol levels and improves high-density lipoprotein function in high-fat diet–fed hamsters

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ABSTRACT

Hydrogen (dihydrogen; H_2) has an antiatherosclerotic effect in apolipoprotein (apo) E knockout mice. The goals of this study were to further characterize the effects of H_2 on the content, composition, and biological activities of plasma lipoproteins in golden hamsters. Plasma analysis by enzymatic method and fast protein liquid chromatography showed that 4-week intraperitoneal injection of hydrogen-saturated saline remarkably decreased plasma total cholesterol and low-density lipoprotein (LDL) cholesterol levels in high-fat diet–fed hamsters. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of apolipoproteins from ultracentrifugally isolated plasma lipoproteins revealed a marked decrease of apo B100 and apo B48 in LDL. A profound decrease of apo E level in very low-density lipoprotein was also observed. Besides, we determined the functional quality of high-density lipoprotein (HDL) particles isolated from H_2-treated and control mice. H_2 significantly improved HDL functionality assessed in 2 independent ways, namely, (1) stimulation of cholesterol efflux from macrophage foam cells by measuring HDL-induced [^{3}H]cholesterol efflux and (2) protection against LDL oxidation as a measure of Cu^{2+}-induced thiobarbituric acid reactive substances formation. Administration of hydrogen-saturated saline decreases plasma LDL cholesterol and apo B levels and improves hyperlipidemia-injured HDL functions, including the capacity of enhancing cellular cholesterol efflux and playing antioxidative properties, in high-fat diet–fed hamsters.

1. 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

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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 2 diabetes mellitus and insulin resistance [2], chronic liver inflammation [3], acute oxidative stress, and focal brain ischemia/reperfusion injury [1]. In addition, Ohsawa et al [4] reported that the oral consumption of hydrogen water for 6 months prevents atherosclerosis in apolipoprotein E knockout (apo E–/–) mice fed a chow diet, mainly through decreasing the oxidative stress level in vivo. However, the effects of H_2 on plasma lipids and plasma lipoprotein levels still remained elusive. Therefore, the purpose of this study was to characterize the effects of H_2 on the content and composition of plasma lipoproteins in hamsters fed a hyperlipidemic diet.

In addition, the functions and concentrations of plasma high-density lipoprotein (HDL) have strong inverse correlations with risk of atherosclerotic cardiovascular disease [5,6]. High-density lipoprotein is known to undergo dramatic modification in structure and composition under the concerted actions of inflammation and oxidative stress [7,8]. As a result, HDL particles progressively lose normal biological activities and acquire altered properties. It is well known that hydrogen is an electron donor and therefore has a high reducing ability. It has been reported that the beneficial effects of hydrogen on different disease model are mostly dependent on its antioxidative, anti-inflammation, and antiapoptotic properties [9]. Therefore, we hypothesized that hyperlipidemia-injured HDL function against atherosclerosis might be improved by hydrogen treatment in animals or patients with hyperlipidemia or other cardiovascular disease. The effects of H_2 on the functional properties of the HDL particle in golden hamsters were determined.

2. Methods

2.1. *Preparation of hydrogen-saturated saline*

The hydrogen-saturated saline was obtained from the Department of Diving Medicine, Second Military Medical University, Shanghai, China. It was prepared as previously described [10]. Briefly, it was generated by using a self-designed hydrogen-saturated water-producing apparatus in which hydrogen was dissolved in saline, which has been degasified, for 2 hours under high pressure (0.4 MPa) to the supersaturated level. The saturated hydrogen-saturated saline was stored under atmospheric pressure at 4°C in an aluminum bag with all the air removed. Hydrogen-saturated saline was freshly prepared every week to ensure a constant hydrogen concentration of more than 0.6 mmol/L.

2.2. *Animals and experimental design*

Forty-five male Syrian golden hamsters were purchased from the Vital River Company, Beijing, China. All experiments were approved by the laboratory animals' ethical committee of Taishan Medical University and followed national guidelines for the care and use of animals. The 45 male hamsters were randomly allocated into 5 groups: regular chow diet group with vehicle saline (n = 5, CD), high-fat diet group with vehicle saline (HFD, n = 10), HFD group with low dosage of H_2-saturated saline (HFD + H_2-L, 1 mL/[kg d], n = 10), HFD with middle dosage of H_2 (HFD + H_2-M, 2.5 mL/[kg d], n = 10), and HFD with high dosage of H_2 (HFD + H_2-H, 5 mL/[kg d], n = 10). Eight-week-old hamsters were fed with a regular CD or HFD (15% lard and 0.2% cholesterol) [11,12] for a total of 8 weeks. After diet feeding for 4 weeks, vehicle (saline as control) or H_2-saturated saline was intraperitoneally injected once daily for 4 weeks.

2.3. *Plasma lipid analysis*

After 4 weeks of H_2 treatment and chloral hydrate anesthesia, blood was collected from the abdominal aorta of the hamsters without dietary exposure for 12 hours. Concentrations of plasma total cholesterol (TC), triglyceride (TG), HDL cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were determined by enzymatic methods (BioSino, Beijing, China). Lipoprotein profiles were obtained by fast protein liquid chromatography (FPLC) using Superose 6 10/300 GL column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) [13]. Briefly, 100 μL of fasting plasma was applied to a Superose 6 column; and the samples were eluted in a mobile phase (0.15 mol/L NaCl, 0.01% NaN_3, and 2 mmol/L EDTA, pH 7.5) at a rate of 0.3 mL/min in 60 fractions of 500 μL. Lipid composition of fractions corresponding to very low-density lipoprotein (VLDL), LDL, and HDL were quantified by enzymatic assays using commercially available kits for TC (BioSino).

2.4. *Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of apolipoproteins*

Fasted plasma lipoproteins were fractionated by ultracentrifugation at 40 000 rpm in a Beckman Optima LE-80K (Beckman Coulter, Inc. Brea, CA, USA) into VLDL (density less than 1.006 g/mL), LDL (density = 1.006-1.063 g/mL), and HDL (density = 1.063-1.21 g/mL) as described previously [14]. Fractions were dialyzed in 150 mmol/L NaCl and 0.3 mmol/L EDTA at 4°C, lipoproteins (VLDL, LDL, and HDL) containing equal amounts of cholesterol were loaded on a 4% to 15% sodium dodecyl sulfate (SDS) polyacrylamide gradient gel, and the apolipoproteins were stained by Coomassie Brilliant Blue as described by Jiang et al [14]. Stained gels were scanned and analyzed with Quantity One (Bio-Rad, Hercules, CA, USA) software program.

2.5. *HDL-induced cholesterol efflux assay*

Cholesterol efflux experiments were performed as described by Smith et al [15]. Low-density lipoprotein and HDL were separated by sequential ultracentrifugation, and acetyl LDL was prepared according to the methods of Basu et al [16]. RAW264.7 macrophages at 50% confluence were cholesterol loaded and labeled in 1 mL of RGGB (RPMI 1640 supplemented with 50 mmol/L glucose, 2 mmol/L glutamine, and 0.1% bovine serum albumin) containing [^{3}H]cholesterol (1 μCi/mL) and acetyl LDL (100 μg/mL) for 30 min. Afterward, macrophages were washed twice with 0.1% bovine serum albumin–

phosphate-buffered saline and equilibrated with RGGB for 24 hours. On the following day, the medium was then replaced with RGGB containing 200 μg/mL of HDL. After 12 hours of incubation, the culture medium 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 mol/L 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.

2.6. HDL antioxidation capability measurement

Low-density lipoprotein (100 μg/mL) in phosphate-buffered saline was incubated with freshly prepared $CuSO_4$ (10 μmol/L) in the presence or absence of the isolated HDL (200 μg/mL). After incubation at 37°C for 2 hours, the extent of LDL oxidation was assessed by measuring of thiobarbituric acid reactive substances (TBARS) formation [17] via a spectrophotometric method according to the manufacturer's instructions (Nanjing Jiancheng Biochemistry, Nanjing, China).

2.7. Statistical analysis

Statistical analysis was performed by one-way analysis of variance test with the GraphPad Prism program version 4.0 (GraphPad software Inc., San Diego, CA, USA). Results are expressed as means ± SD. *P* values less than .05 were considered significant.

3. Results

3.1. Administration of H_2 lowers plasma TC and LDL-C levels

High-fat diet containing 15% lard and 0.2% cholesterol was used for producing the hyperlipidemic model in hamsters. As shown in Fig. 1, HFD induced marked increase in plasma TC, LDL-C, HDL-C, and TG levels by 4.2-, 2.5-, 2.7-, and 3.9-fold, respectively, when compared with regular CD, which indicated a successfully prepared hyperlipidemic model in hamsters. The results after 4 weeks of H_2 treatment showed that plasma TC and LDL-C levels were significantly decreased by 28.3% and 34.3%, respectively, compared with those of HFD control (Fig. 1A, B). Plasma HDL-C levels, however, were not affected by H_2 treatment. In addition, plasma TG levels had a trend to be decreased by H_2, although there was no significant difference among the 4 HFD-fed groups ($P > .05$). Moreover, plasma lipoprotein profile by FPLC further confirmed the decrease in plasma LDL-C levels in H_2-treated animals and revealed that plasma VLDL cholesterol (VLDL-C) levels had a trend to be lowered by H_2 (Fig. 2). There is no impact of H_2 treatment on the plasma HDL profile (Fig. 2).

3.2. H_2 downregulates apo B and apo E protein levels in plasma

Consistent with the difference observed for LDL-C, the major proteins on LDL, apo B100 and apo B48, were significantly decreased by H_2 treatment in animals fed an HFD (Fig. 3). The

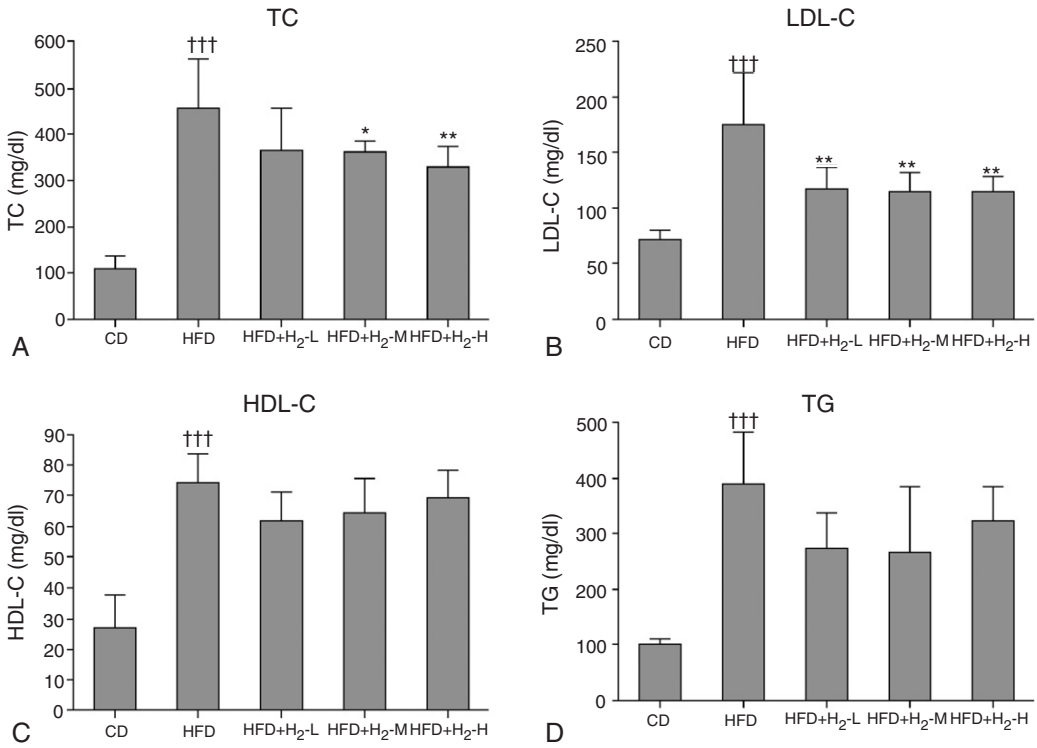


Fig. 1 – Effect of H_2 on the levels of plasma lipids. A, Plasma TC, (B) LDL-C, (C) HDL-C, and (D) TG in hamsters after 4 weeks of H_2 treatment were determined by enzyme method. Data (means ± SD, n = 10 each in 4 HFD groups, n = 5 in CD group) were expressed in milligrams per deciliter. †††$P < .001$ vs CD group; *$P < .05$, **$P < .01$ vs HFD group.

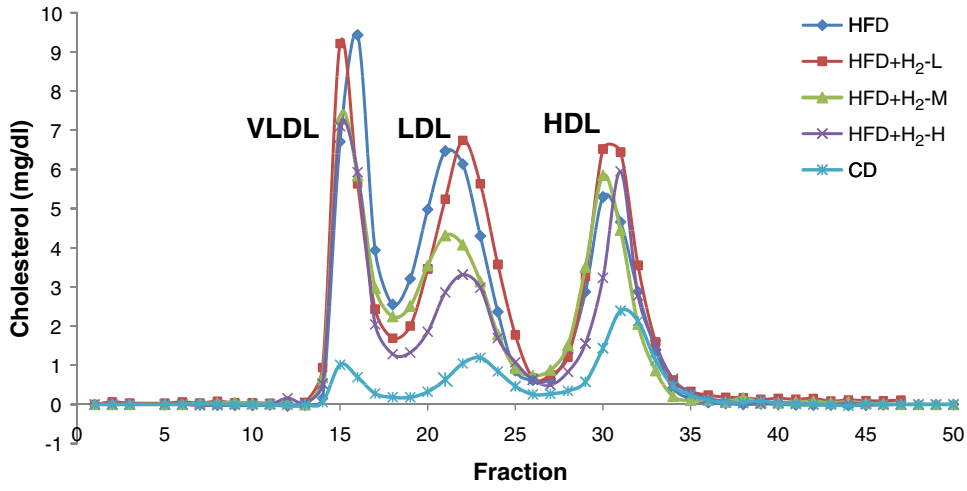
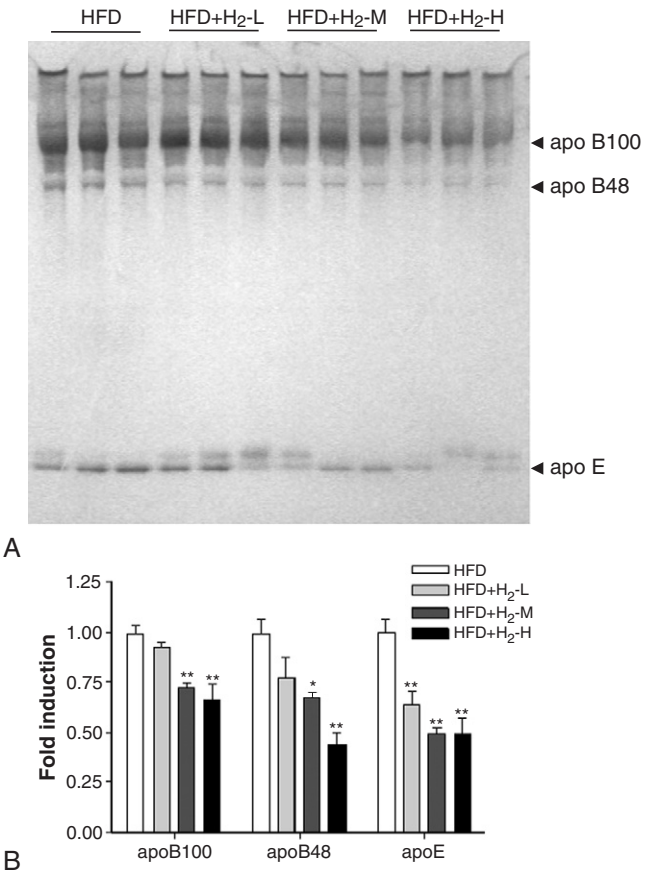


Fig. 2 – The FPLC cholesterol profiles using pooled plasma samples of n = 2 to 3 hamsters per group showing cholesterol content (milligrams per deciliter) of plasma lipoprotein fractions.

apo E protein in LDL particles was also downregulated with the treatment of H_2 (Fig. 3). Besides, SDS polyacrylamide gel electrophoresis (PAGE) of the isolated VLDL particles showed that H_2 significantly decreased the content of apo E in VLDL (Fig. 4). These data suggested that H_2 could downregulate the expression of the major protein constituent of LDL and VLDL, which is consistent with the data from plasma lipoprotein profile by FPLC.



3.3. *Administration of H_2 improves the functionality of HDL particle in hyperlipidemic hamsters*

High-density lipoprotein is known to undergo dramatic modification in structure and composition under the concerted actions of inflammation and oxidative stress [7,8]. 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 [2,4,18]. Therefore, it is possible that administration of H_2 might improve the functional quality of HDL particle. Firstly, the ability of the isolated HDL particles to elicit efflux from cholesterol-loaded macrophages was tested. As shown in Fig. 5A, HFD treatment reduced the ability of HDL to elicit efflux compared with the regular CD group, indicating that the cholesterol efflux ability mediated by HDL particle was injured by hyperlipidemia. In addition, HDL isolated from H_2-treated hamsters exhibited dramatically higher efflux properties compared with vehicle-treated HFD group (Fig. 5A). Next, the biological effect of H_2 on the antioxidative functionality of HDL was tested, namely, the protection of LDL particles from oxidation. As shown in Fig. 5B, compared with the CD group, the formation of TBARS has a trend to increase after HFD feeding; and different dosage of H_2 treatment significantly inhibited the formation of TBARS. These data indicate that hyperlipidemia-injured HDL functions, including the ability to stimulate cholesterol efflux from macrophage foam cells and the ability to protect against LDL oxidation, were improved by administration of H_2 in hamsters. However, the major proteins on HDL

Fig. 3 – The SDS-PAGE analysis of apolipoproteins from ultracentrifugally isolated plasma lipoproteins. Plasma LDL (density = 1.006-1.063 g/mL) was separated by preparative ultracentrifugation as described. A, The SDS-PAGE was performed on 4% to 15% SDS polyacrylamide gradient gel, and the apolipoproteins were stained by Coomassie Brilliant Blue as described. B, A representative SDS-PAGE result from 3 independent experiments is shown. Densitometric quantitation of SDS-PAGE data (n = 3) by Quantity One software. *P < .05, **P < .01 vs HFD group.

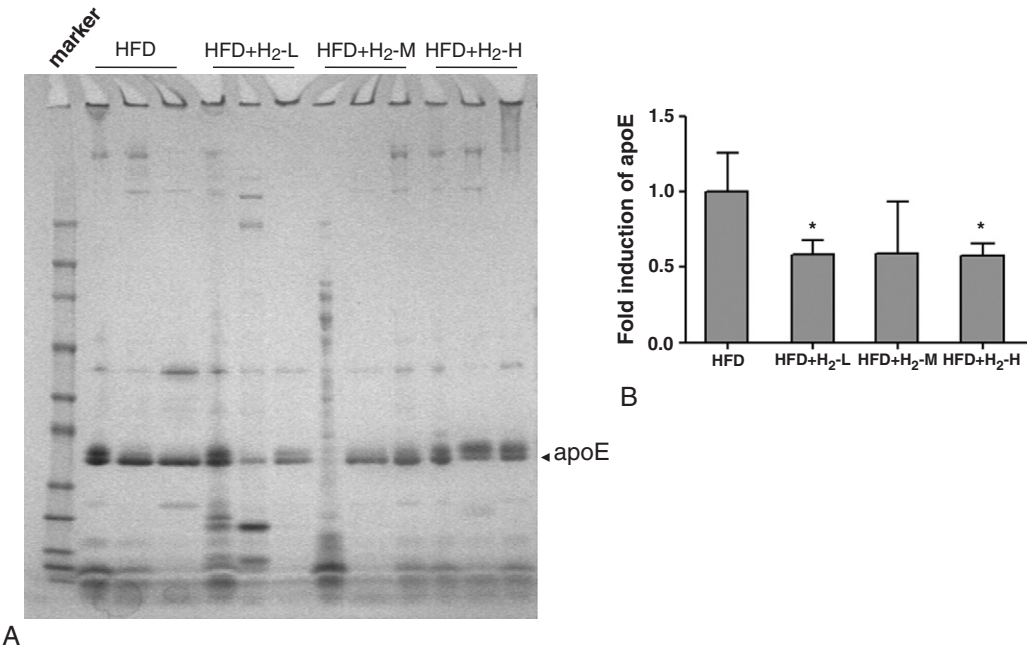


Fig. 4 – The SDS-PAGE analysis of apolipoproteins from ultracentrifugally isolated plasma lipoproteins. Plasma VLDL (density less than 1.006 g/mL) was separated by preparative ultracentrifugation as described. A, The SDS-PAGE was performed on 4% to 15% SDS polyacrylamide gradient gel, and the apolipoproteins were stained by Coomassie Brilliant Blue as described. B, A representative SDS-PAGE result from 3 independent experiments is shown. Densitometric quantitation of SDS-PAGE data (n = 3) by Quantity One software. *P < .05 vs HFD group.

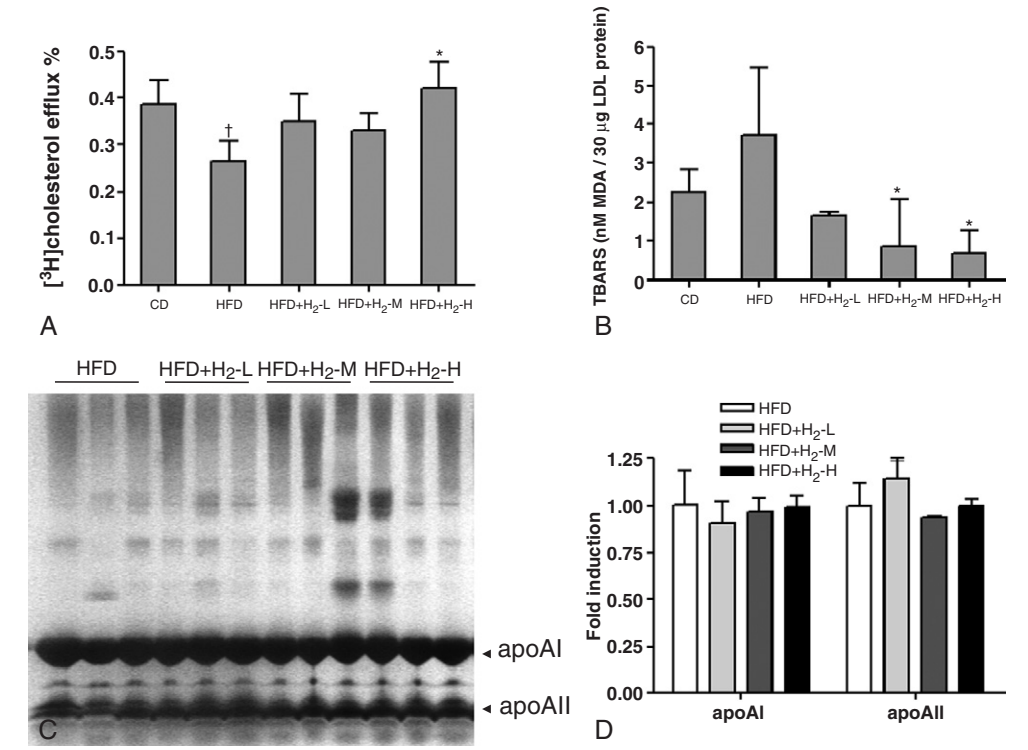


Fig. 5 – Administration of H_2 improves the functional properties of the HDL particle. The HDL function was determined as cholesterol efflux (A) and protection of LDL against oxidation (B) in vitro. Assays were performed as detailed in methods. C, Plasma HDL (density = 1.063-1.21 g/mL) was separated by preparative ultracentrifugation as described. The SDS-PAGE was performed on 4% to 15% SDS polyacrylamide gradient gel, and the apolipoproteins were stained by Coomassie Brilliant Blue as described. D, Densitometric quantitation of SDS-PAGE data (n = 3) by Quantity One software. Data are presented as means ± SD; n = 10 hamsters per group. †P < .05 vs CD group; *P < .05 vs HFD group.

particle, apo A-I and apo A-II, were not significantly altered by H_2 treatment in animals fed an HFD (Fig. 5C, D), consistent with the data from plasma lipoprotein profile by FPLC.

4. Discussion

It has been reported that consumption of hydrogen water prevents atherosclerosis in apo E–/– mice, mainly through decreasing the oxidative stress level in vivo [4]. However, it remains unknown whether H_2 has effects on lipid and lipoprotein metabolism in vivo. The key finding of our present study is that the novel antioxidant chemical element, H_2, alleviates dietary (ie, HFD)-induced lipid metabolism disorder, including hyperlipidemia and defective HDL function, in golden hamsters. The male Syrian golden hamster is a widely used model in studying cholesterol metabolism in response to dietary fat manipulations and has many similar profiles in lipid metabolism compared with humans [19,20]. In our study, consumption of HFD that contains 15% lard and 0.2% cholesterol for 4 weeks induced an increase in plasma TC and LDL-C levels as previously described [21]; and treatment with H_2 markedly improved the impaired plasma lipid profile (downregulating plasma TC and LDL-C levels), suggesting that H_2 could regulate lipid metabolism to alleviate the hyperlipidemia in hamsters. In addition, treatment with H_2 has a trend to decrease plasma TG concentrations; but H_2 showed no effect on the gain in body mass in the HFD-fed hamsters (data not shown). It would have important clinical significance to the population with hyperlipidemia if H_2 was proven to decrease plasma TC and improve HDL quality in humans. Our results were not consistent with those of human studies in type 2 diabetes mellitus; they showed significant decreases in modified LDL-C levels and no effect on TC and LDL-C [2]. The inconsistency might be attributable to the difference in species and pathological conditions, the route of administration, or the intervention period. Despite this possibility, the discrepancy might be explained by the fact that modified LDL, like oxidized LDL, largely existed in the hyperlipidemia model, which is used in our study.

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 plasma cholesterol will provide solid evidence for the application of H_2 in cardiovascular disease therapy. Plasma 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. Apolipoprotein B100 and apo B48 are the major proteins present in LDL particles; and like LDL-C, the plasma apo B level has been positively correlated with risk for atherosclerotic disease. In the present study, we found that H_2 treatment not only decreased plasma LDL cholesterol levels but also remarkably downregulated apo B100, apo B48, and apo E protein levels in the LDL particles. Secondly, like LDL-C, VLDL-C is considered a type of "bad" cholesterol because elevated levels are associated with an increased risk of coronary artery disease [22]. We found that apo E, the major protein in VLDL, was lowered by H_2; and the cholesterol analysis by FPLC revealed a trend for VLDL-C to be decreased by H_2. Taken together, the data indicated that molecular H_2 dissolved in saline has beneficial regulating effect on HFD-induced lipid abnormality in hamsters; 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 regulates the lipid and protein contents of lipoprotein particles and improves plasma lipoprotein profile. Previous studies have demonstrated that administration of H_2-rich saline improves insulin sensitivity [2]; in our view, this could partly contribute to the improved lipid metabolism in our study. Furthermore, it is possible that inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase and blocking the pathway for synthesizing cholesterol in the liver might be other possible pathways. 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 [23]. The following studies on hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase and LDL receptors may open a novel window to elucidate the mechanism by which H_2 acts on lipoprotein regulation.

As we know, besides cholesterol and apo E, VLDL contains the highest amount of TG compared with other lipoprotein types. In this study, we found that plasma TG has a trend to be decreased by H_2; thus, further experiments need to be performed to determine the effects of H_2 on TG metabolism.

High-density lipoprotein is known to protect against the development of atherosclerosis and is widely documented as a "negative risk factor" for coronary heart diseases [24]. The antiatherogenic activity of HDL is principally attributable to the reverse transport of cholesterol, whereby HDL stimulates the efflux of cholesterol from peripheral cells to the liver. Moreover, the HDL particle itself has a variety of antioxidative properties. In addition, HDL is known to undergo dramatic modification in structure and composition under conditions with inflammation, oxidative stress, and cardiovascular diseases [7,8]. As a result, HDL particles progressively lose normal biological activities and, importantly, lose its atheroprotective effects. In the present study, the functionality of HDL particles, including the ability to stimulate cholesterol efflux from macrophage foam cells and the ability to protect against LDL oxidation, was impaired by HFD; and H_2 treatment significant improved hyperlipidemia-injured biological activities of HDL (Fig. 5) without altering HDL-C plasma levels. It is known that the beneficial effects of H_2 on different disease models are mostly dependent on its antioxidative, anti-inflammation, and antiapoptotic properties [9]. 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 antioxidative and anti-inflammation 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 find any changes from hepatic and renal sections after H_2 treatment (data not shown), suggesting less toxicity and adverse effects of H_2.

Indeed, our data gave us a clue that H_2 has the potential to be used as a novel lipid-regulating agent with the advantage of less toxicity compared with other commonly used lipid-regulating drugs that have adverse effects on the liver and kidney. When translated into clinical practice, inhalation of H_2 gas must be most frequently applied in the treatment of

patients with hyperlipidemia because of the convenience. 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 inhalation of H_2 gas as a therapeutic strategy. In addition, in our study, the number in each group is not big; and neither sample size nor power calculations were done. Therefore, we cannot exclude the possibility that some of the nonsignificant findings in the results might reflect a lack of power to declare an otherwise real significant difference. However, the reason we took only 5 animals fed chow diet as a control group is that we aimed to make sure that HFD is a successful way to make a hyperlipidemia model in hamsters; and the result showed us that we reached this goal because all animals fed with the HFD caught hyperlipidemia.

In conclusion, our data show that in vivo administration of H_2 dissolved in saline to a saturated level effectively decreases plasma TC and LDL-C levels and improves HDL functions in HFD-fed hamsters, suggesting that H_2 may be used as a newer pharmacological agent to treat or control HFD-induced lipid metabolism disorder. However, clinical tests will be needed to determine the relevance of H_2-saturated saline to exert lipid-regulating effects.

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Conflict of Interest

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

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