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# Consumption of hydrogen water prevents atherosclerosis in apolipoprotein E knockout mice

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ABSTRACT

Oxidative stress is implicated in atherogenesis; however most clinical trials with dietary antioxidants failed to show marked success in preventing atherosclerotic diseases. We have found that hydrogen (dihydrogen; $H_2$) acts as an effective antioxidant to reduce oxidative stress [I. Ohsawa, M. Ishikawa, K. Takahashi, M. Watanabe, K. Nishimaki, K. Yamagata, K. Katsura, Y. Katayama, S, Asoh, S. Ohta, Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals, Nat. Med. 13 (2007) 688–694]. Here, we investigated whether drinking $H_2$-dissolved water at a saturated level ($H_2$-water) *ad libitum* prevents arteriosclerosis using an apolipoprotein E knockout mouse (apoE$^{-/-}$), a model of the spontaneous development of atherosclerosis. ApoE$^{-/-}$ mice drank $H_2$-water *ad libitum* from 2 to 6 month old throughout the whole period. Atherosclerotic lesions were significantly reduced by *ad libitum* drinking of $H_2$-water ($p = 0.0069$) as judged by Oil-Red-O staining series of sections of aorta. The oxidative stress level of aorta was decreased. Accumulation of macrophages in atherosclerotic lesions was confirmed. Thus, consumption of $H_2$-dissolved water has the potential to prevent arteriosclerosis.



Atherosclerosis is a multifactorial and long-lasting process, and atherosclerosis and related cardiovascular diseases represent a state of inflammation and heightened oxidative stress characterized by the accumulation of macrophages and oxidized products of low-density lipoprotein in affected blood vessels [1–3]. Oxidation of low-density lipoprotein is considered an early event; however, most clinical trials supplying a single dietary antioxidant have not resulted in great success in preventing atherosclerotic diseases [1,4–7].

We have reported that molecular hydrogen is an efficient antioxidant by gaseous rapid diffusion into tissues and cells [8]. This finding was soon confirmed by several laboratories [9–12]. Moreover, consumption of water with dissolved molecular hydrogen to a saturated level (hydrogen water) prevents stress-induced cognitive decline in mice [13], and the superoxide formation in mice [14]. A clinical trial showed the decrease in modifying low-density lipoprotein by drinking hydrogen water [15].

Here, we show that consumption of hydrogen dissolved in water has the potential to prevent atherosclerosis using apolipoprotein E knockout (apoE$^{-/-}$) mice, which show impaired clearing of plasma lipoproteins and which develop atherosclerosis in a short time [16,17].

## Materials and methods

*Animals.* Apolipoprotein E-deficient mice (apoE$^{-/-}$) were purchased at the age of 2 months from Taconic. The care and treatment of experimental animals were in accordance with institutional guidelines. This study was approved by the Animal Care and Use Committee of Nippon Medical School.

*Hydrogen water administration.* Molecular hydrogen ($H_2$) was dissolved in water under high pressure (0.4 MPa) to a supersaturated level using hydrogen water-producing apparatus (ver. 2) produced by Blue Mercury Inc. (Tokyo, Japan). The saturated hydrogen water was stored in an aluminum bag. Hydrogen water was freshly prepared every week, which ensured that a concentration of more than 0.6 mM was maintained. We confirmed the hydrogen content with a hydrogen electrode (ABLE). Each day, hydrogen water from the aluminum bag was placed in a closed glass vessel (70 mL) equipped with an outlet line containing two ball bearings, which kept the water from being degassed. This vessel ensured that the hydrogen concentration was more than 0.4 mM after one day. Hydrogen water degassed by gentle stirring was used for control

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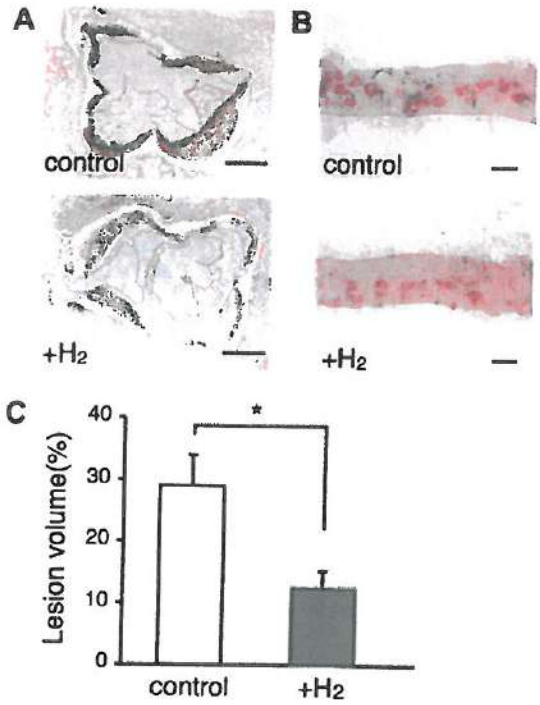


**Fig. 1.** Consumption of hydrogen water decreased atherosclerotic lesion. ApoE$^{-/-}$ mice drank water containing hydrogen (+$H_2$) or degassed water (control) for 6 months from the age of 2 months old. Representative microscopic pictures of horizontal sections of the proximal aorta attached to the heart (A) and vertical sections of the distal aorta (2 mm from the heart) (B) are shown by Oil-Red-O staining. Scale bar; 100 μm (for A) and 1 mm (for B). (C) Lesion volume was estimated by Oil-Red-O staining of a series of 30 sections (mean value ± SEM, $n = 10$, $p = 0.0069$).

animals; the complete removal of hydrogen gas was confirmed with a hydrogen electrode.

*Quantification of atherosclerotic lesions in the aorta.* The proximal aorta attached to the heart was used to prepare cross-sections. After fixation with 4% paraformaldehyde, cryosections (8 μm) were cut from the site where the aorta valve cups appear at the aorta root. All other sections were collected and stained with Oil-Red-O [17]. The volume of stained lipid (%) was calculated from eight sections for each mouse. The distal aorta (2 mm from the heart) was fixed with 4% paraformaldehyde, opened longitudinally using microscissors and stained with Oil-Red-O.

*Immunocytochemistry.* After fixation of the proximal aorta with 4% paraformaldehyde, cross-sections (6 μm) were cut with a cryostat, incubated with either an antibody against mouse macrophage (MOMA-2, AbD Serotec), anti-iNOS (BIOMOL), and anti-4-hydroxyl-2-nonenal (HNE) antibody (JaICA, Japan) [19–21]. After washing, the sections were then exposed to a biotinylated second antibody and avidin–peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories Inc.). Sections were developed with DAB as a substrate. One section from each mouse was stained with hematoxylin and eosin (HE).

*Statistical analysis.* We performed statistical analysis using StatView software (SAS Institute) by applying an unpaired two-tailed Student's *t*-test and ANOVA followed by Fisher's exact test.

## Results

It is easy to consume molecular hydrogen by drinking water containing dissolved molecular hydrogen (hydrogen water). Thus, we examined whether consumption of hydrogen water prevents atherosclerosis using apoE$^{-/-}$ mice. Mice drank nearly the same volume of hydrogen water as control water [4.3 ml/day/mouse(0.1(SD)(hydrogengroup) vs. 4.0 ml/day/mouse(0.1(SD)(controlgroup)]. The amount of food eaten per mouse was also the same in both groups [3.56 ± 0.3 g/day (hydrogen group) vs. 3.28 ± 0.6 g/day (control group)]. After 6 months, we removed the aorta to stain with Oil-Red-O staining. As expected, atherosclerotic lesions were found in 6-month-old apoE$^{-/-}$ mice. In contrast, in mice that had drunk hydrogen water, the volume of atherosclerotic lesion was

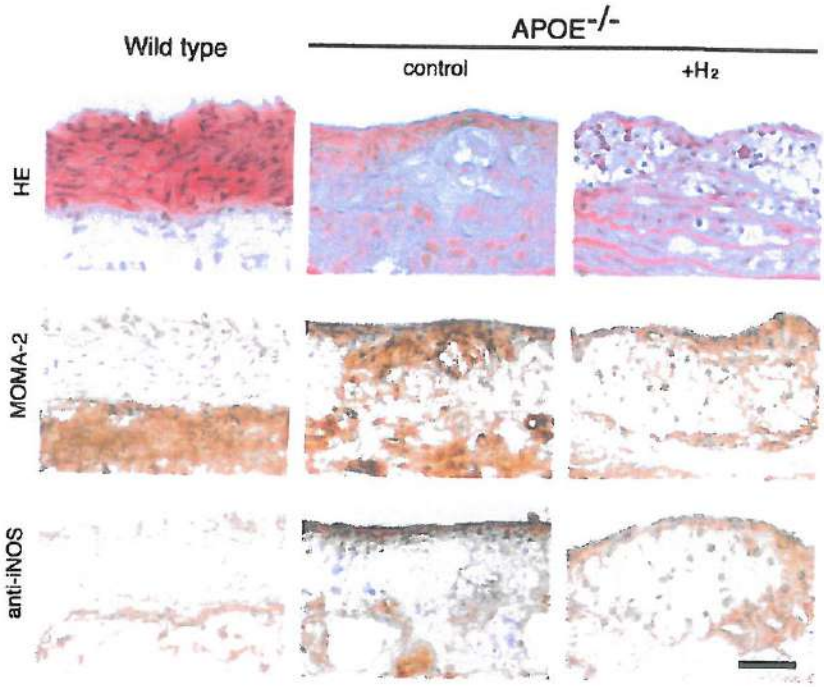


**Fig. 2.** Representative histochemical or immunostaining of the aorta. ApoE$^{-/-}$ mice drank hydrogen or control water throughout the 6-month period from 2 months old. The proximal aorta attached to the heart was sectioned and stained with HE staining, anti-MOMA-2 immunostaining and anti-iNOS immunostaining. Scale bar: 250 μm.

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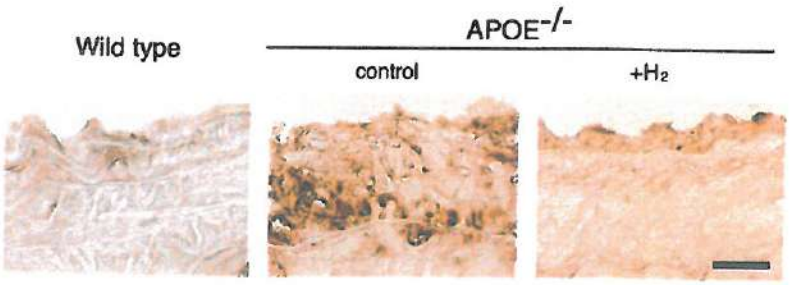


Fig. 3. Representative immunostaining of the aorta. ApoE$^{-/-}$ mice drank hydrogen or control water throughout the 6-month period from 2 months old. The proximal aorta attached to the heart was sectioned and stained with anti-HNE immunostaining. Scale bar: 250 μm.

significantly reduced (Fig. 1). We confirmed that the lesion was derived from macrophage accumulation by staining the sections with anti-MOMA-2 and iNOS antibodies, both of which are macrophage markers [19,20]. (Fig. 2). Moreover, to evaluate the oxidative stress level, we stained the sections with anti-HNE antibody [21]: HNE is an oxidative stress marker (Fig. 3).

These findings suggest that continued consumption of hydrogen water decreased the oxidative stress level and prevented the formation of atherosclerosis, at least in model mice.

## Discussion

Clinical evidence as well as experimental results strongly suggests the major contribution of oxidative stress to atherogenesis [1–3]. Thus, dietary consumption of an efficient antioxidant is believed to prevent atherosclerosis; however, the trials have not resulted in great success [1,4–7,22]. Moreover, recent studies have suggested that excessive antioxidant increased the mortality and rates of cancer, because it may interfere with essential defensive mechanisms [23–25]. This may be because low levels of ROS, such as superoxide anion and hydrogen peroxide, function as signaling molecules to regulate apoptosis, cell proliferation, and differentiation [26,27]. The strategy of combining different compounds improved to oxidative status to enable dose reduction of each compound to below the threshold of its side effects [28].

We have found that molecular hydrogen selectively reduces hydroxyl radicals, but not superoxides and hydrogen peroxides that play physiological roles [8]; thus, we suggest that the side effects of hydrogen must be small, different from other antioxidants. Inhalation of hydrogen gas does not influence physiological parameters such as body temperature, blood pressure, pH, and $pO_2$ in the blood, as shown previously [8,10]. Hydrogen has already been used for humans to prevent decompression sickness in divers at the level of 2 MPa partial pressure of hydrogen, suggesting that 16 mM hydrogen in blood could be safe [29]. When hydrogen water was placed in the stomach, hydrogen was detected in the blood, indicating the incorporation of hydrogen into the body by drinking [13]. Hydrogen diffuses very rapidly into cells, and high efficacy is expected [8,10].

When the preventive level of atherosclerotic lesions in this study is compared with the previous data that apoE$^{-/-}$ mice was used, the efficacy of hydrogen water seems to be greater than folic acid [30], vitamin E [31], iron [32], and α-lipoacid [33]. It is easy to drink hydrogen water daily. We propose that regular consumption of molecular hydrogen dissolved in water has the potential to prevent atherosclerosis. This is the first report that hydrogen water suggests to prevent a lifestyle-related disease. Clinical tests will be needed to elucidate the relevance of hydrogen water to prevent atherosclerosis.

## Uncited reference

[18].

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