Something in the Water

Hydrogen Water & Autoimmune Disease

Live LONGER Feel BETTER
DEFEATING DEMENTIA, DEPRESSION & DIABETES
We all want to drink the best water we can. Full books have been written about water – fourth phase water, alkaline water, Hydrogen water. And as with all things health related – there is never a consensus about what’s best. Someone will always disagree. We believe that Hydrogen enriched water is THE best kind – and that Alkaline water actually manifests improvements in people due more to the Hydrogen effect than anything else.

Hydrogen is #1 on the periodic table because it is a tiny element. Being so small allows Hydrogen to get into membranes, joints, brain, gut, organs, lungs, eyes, ears, etc. Molecular or diatomic hydrogen is 2 atoms of hydrogen. It is also referred to as H₂.

In Hydrogen Water systems, H₂ gas is dissolved in the water. The water is the delivery vehicle for the H₂ gas which has benefits. Molecular hydrogen (H₂) is why water electrolysis was developed in 1800. H₂ is a selective antioxidant only reacting with ROS (reactive oxygen species). ROS damages cells. The most cytotoxic (cell damaging) ROS (free radicals) are Hydroxyl Radicals (HO*). When H₂ combines with 2 hydroxyl radicals, 2 water molecule are formed. H₂ has therapeutic benefits for every organ in the body. Our bodies were designed to create hydrogen gas in the gut through the normal fermentation and digestion of food. The problem is that many people have issues that prevent their gut from working correctly. 60% of the immune system is based on gut health. If the gut is compromised and the diet does not have beneficial fiber to be converted into hydrogen gas, issues occur.

Some Benefits of Molecular Hydrogen:

- Reduction of oxidative stress and Inflammation.
- Regulation of over 200 Biomolecules in the body.
- Stimulates gastric ghrelin to increase cognitive function.
- Stimulation of anaerobic microflora in the intestinal tract.
- Shown to help with Rheumatoid Arthritis and joint issues.
- 700 studies showing therapeutic effects with 170 human disease models.

Molecular hydrogen has benefits. Peer reviewed articles and studies have shown molecular hydrogen to reduce oxidative stress and inflammation which lead to many diseases. Studies have been conducted on 170+ human diseases and conditions including Rheumatoid Arthritis, Diabetes, Colon Cancer, Autoimmune, Parkinson’s, Alzheimer’s, Autism, Bipolar, Schizophrenia, & IGT. In addition to helping with existing conditions, molecular hydrogen can support the body to avoid susceptibility to diseases and conditions. It is truly one of the best anti-aging tools you can use.

In 1800, Anthony Carlisle, a surgeon in London, discovered water electrolysis because he wanted to make a hydrogen generator. Dr. Carlisle wanted a way to produce hydrogen gas easily because he learned in 1798 that hydrogen had antioxidant properties. Traditional electrolysis devises convert water (H₂O) to hydrogen gas (H₂) and hydroxide ions (OH⁻) at the negative side (cathode), and oxygen gas (O₂) and hydrogen ions (H⁺) at the positive side (anode). Traditional water electrolysis machines have standard membranes that separates the alkaline OH⁻ ions from the acidic H⁺ ions if you are separating the water streams. A new method of electrolysis was designed to only produce H₂ without changing the pH of the source water. In this method, the water is not separated into alkaline and acid streams. Proton Exchange Membranes (PEM) are used instead of standard membranes. The advantage of the PEM is that it creates its own conductivity in water and can produce H₂ gas even in pure water with no minerals as in reverse osmosis or distilled water. The benefits do not come from the pH of the water. The pH change comes if you separate the water streams. It is the H₂ gas that can be dissolved in the water that provides the therapeutic benefits.

**Typically, electrolysis systems that separate the water streams into alkaline and acid water are notable to dissolve H₂ gas in the water longer than a few weeks.** This is because the positively charged minerals naturally want to bond to the negatively charged cathode (see graphic above). If minerals build up on the cathode, H₂ gas will not be dissolved in the water because the hydrogen bubbles will be too large to be dissolved. The H₂ gas will go into the atmosphere and the benefits with it. In some H₂ systems, the patented technology changes the polarity of the electrodes every time the machine is used. This makes it impossible for minerals to build up. This is the only system that guarantees H₂ gas will always be dissolved in the water. With other electrolysis systems, minerals will build up within 2-3 weeks of use and the benefits will be gone.

II. Benefits of Echo® Hydrogen Enriched Water™

Everyone is talking about how free radicals are damaging our cells. What most people don’t know is that many free radicals are beneficial to health. It is only the cell damaging (cytotoxic) oxygen radicals that we need to scavenge. H₂ converts these cell damaging radicals into water molecules.

**Athletic Performance:**

Hydrogen Enriched Water has helped many athletes increase performance. It better empowers you to function at optimal efficiency by ridding the cells of Hydroxyl Radicals allowing the mitochondria to produce energy more efficiently. It reduces fatigue by lessening lactate buildup in muscles. Recovery times can be cut in half. When a person is properly hydrated with hydrogen-enriched water, they perform at peak levels for longer periods of time.
Detoxification and Weight Loss:
H2 water supports healthy cleansing and weight loss. When the toxins and wastes are flushed, the burden on the body is lessened. Water can also help to clean out the intestines and colon. People feel more hydrated, have more success with their weight loss programs, experience more productive sleep, wake up more alert, have fewer allergy symptoms, and generally feel more energy throughout the day. H₂ stimulates gastric Ghrelin that increases cognitive function.

Immune Boost:
The effectiveness of the immune system and the digestive system are directly linked to the level of hydration. Being properly hydrated is one of the best ways to increase your immune system and prevent sickness and disease. 60-75% of your body is water and it should be no surprise that the type of water you drink can directly influence the way you feel. Hydrogen enriched water™ can stimulate anaerobic microflora to naturally restore gut health. The health of your gut is directly related to the strength of your immune system and the susceptibility to disease and sickness.

Intrinsic Energy and Frequencies:
Many people understand that energy and frequencies are all around us. Radio frequencies, cell phone frequencies, Infrared energies, EMF’s, etc. There are good and bad energies and frequencies. Some use energies to benefit individuals in need. The Echo® Water System has hundreds of beneficial energies and frequencies. They can protect you from harmful frequencies and energies. They can also balance chakras and help the body heal. Individuals that are intuitive can feel them. Others say that the water just feels good to them. Professionals in energy medicine, Cranial Sacral Therapists, Reiki Masters, etc. love and recommend H2 water because of these beneficial energies and frequencies.

Proper Hydration:
Because H2 Water tastes so great, you will be drinking more water. A study conducted by the University of Utah, showed the more water you drink the better. In the study, subjects consumed either 4, 8 or 12 glasses of water daily. On the fifth day before rising, their hydration status was monitored and a computer measured how many calories they had burned in a resting state. The groups who drank 8 and 12 glasses of water daily were sufficiently hydrated, whereas subjects who drank only four glasses showed definite signs of dehydration. The well-hydrated subjects reported better concentration and more energy. They burned more calories at rest than the group who drank only 4 glasses.
results were in line with previous University of Utah findings that the ability to burn calories can decline by about 2% per day when people are dehydrated. Metabolic rate and digestion are increased by being properly hydrated.

**Increased Cognitive Function - Ghrelin: Help with Neurological Conditions**

Studies show that H₂ gas stimulates Ghrelin secretions. Ghrelin is known as the hunger hormone in the body. Ghrelin affects many things in the body including cognitive function, hunger, weight regulation, anti-inflammatory function. This is accomplished in the hippocampus, hypothalamus, and the brain stem. Specific studies have shown that water with H₂ gas can be helpful with neurologic issues like Parkinson’s, Alzheimer’s, Bipolar, Schizophrenia, and Autism.

**III. Study references:**

1. [www.synergyscience.com/research.html](http://www.synergyscience.com/research.html)
2. [www.synergyscience.com/research.html](http://www.synergyscience.com/research.html)

**Disclaimer:**

There are hundreds of studies showing molecular hydrogen to have therapeutic benefits. Please visit [www.synergyscience.com/research.html](http://www.synergyscience.com/research.html) or [www.hydrogenstudies.com](http://www.hydrogenstudies.com) to read additional clinical studies which are continually being added. The studies below are shared for educational purposes only. They are not shared to indicate any expected outcome for anyone with a similar or same disease or pathology. The results achieved in the studies should not be taken as an indicator of results you will experience. The study shows potential outcomes. There are no protocols, drugs, natural methods, or techniques that are 100% effective. Every individual is different and diseases are complex.

![Diagram](image)

**Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals**

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Acute oxidative stress induced by ischemia-reperfusion or inflammation causes serious damage to tissues, and persistent oxidative stress is accepted as one of the causes of many common diseases including cancer. We show here that hydrogen (H₂) gas has potential as an antioxidant in preventive and therapeutic applications. We induced acute oxidative stress in cultured cells by three independent methods. H₂ selectively reduced the hydroxyl radical, the most cytotoxic of reactive oxygen species (ROS), and effectively protected cells; however, H₂ did not react with other ROS, which possess physiological roles. We used an acute rat model in which oxidative stress damage was induced in the brain by focal ischemia and reperfusion. The inhalation of H₂ gas markedly suppressed brain injury by buffering the effects of oxidative stress. Thus H₂ can be used as an effective antioxidant therapy, owing to its ability to rapidly diffuse across membranes, it can reach and react with cytotoxic ROS and thus protect against oxidative damage.
Hydrogen–water enhances 5-fluorouracil-induced inhibition of Colon Cancer


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ABSTRACT

Oxidative stress is involved in cancer development. Hydrogen (H2) is a potent antioxidant and exhibits anti-inflammatory and potentially anticancer-like activities. This study aimed to investigate the role of H2 in combination with 5-fluorouracil (5-FU) in cancer treatment both in vitro and in vivo using the colon 26 cell line. The survival rate was determined using the Kaplan–Meier survival test, and cell viability was assessed using cell viability imaging kit and the MTT assay, and activation of the cell apoptosis pathway (Phosphorylated adenosine monophosphate activated protein kinase (p-AMPK), Apoptosis-inducing factor (AIF) and Caspase3) were characterized by western blots. Hydrogen water administration improved the survival of mice with colon 26-induced cancer. Furthermore, hydrogen water enhanced cell apoptosis in cancer cells, resulting in a marked increase in the expression of p-AMPK, AIF and Caspase 3 in colon 26 cells. Hydrogen water also increased the inhibitory effect of 5-FU on colon 26 cells with respect to cell survival rate and anticancer functions. Additionally, high-content hydrogen water exhibited stronger antioxidant and anticancer activity than did the natural hydrogen water. In conclusion, high-content hydrogen water can inhibit colon cancer, particularly in combination with 5-fluorouracil.

Supplementation of hydrogen-rich water improves lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance


Oxidative stress is recognized widely as being associated with various disorders including diabetes, hypertension, and atherosclerosis. It is well established that hydrogen has a reducing action. We therefore investigated the effects of hydrogen-rich water intake on lipid and glucose metabolism in patients with either type 2 diabetes mellitus (T2DM) or impaired glucose tolerance (IGT). We performed a randomized, double-blind, placebo-controlled, crossover study in 30 patients with T2DM controlled by diet and exercise therapy and 6 patients with IGT. The patients consumed either 900 mL/d of hydrogen-rich pure water or 900 mL of placebo pure water for 8 weeks, with a 12-week washout period. Several biomarkers of oxidative stress, insulin resistance, and glucose metabolism, assessed by an oral glucose tolerance test, were evaluated at baseline and at 8 weeks. Intake of hydrogen-rich water was associated with significant decreases in the levels of modified low-density lipoprotein (LDL) cholesterol (ie, modifications that increase the net negative charge of LDL), small dense LDL, and urinary 8-isoprostanes by 15.5% (P < 0.01), 5.7% (P < 0.05), and 6.6% (P < 0.05), respectively. Hydrogen-rich water intake was also associated with a trend of decreased serum concentrations of oxidized LDL and free fatty acids, and increased plasma levels of adiponectin and extracellular-superoxide dismutase. In 4 of 6 patients with IGT, intake of hydrogen-rich water normalized the oral glucose tolerance test. In conclusion, these results suggest that supplementation with hydrogen-rich water may have a beneficial role in prevention of T2DM and insulin resistance.

Selective stimulation of the growth of anaerobic microflora in the human intestinal tract by electrolyzed reducing water.

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96-99% of the “friendly” or residential microflora of intestinal tract of humans consists of strict anaerobes and only 1-4% of aerobes. Many diseases of the intestine are due to a disturbance in the balance of the microorganisms inhabiting the gut. The treatment of such diseases involves the restoration of the quantity and/or balance of residential microflora in the intestinal tract. It is known that aerobes and anaerobes grow at different oxidation-reduction potentials (ORP). The former require positive E(h) values up to +400 mV. Anaerobes do not grow unless the E(h) value is negative between -300 and -400 mV. In this work, it is suggested that prerequisite for the recovery and maintenance of obligatory anaerobic microflora in the intestinal tract is a negative ORP value of the intestinal milieu. Electrolyzed reducing water with E(h) values between 0 and -300 mV produced in electrolysis devices possesses this property. Drinking such water favors the growth of residential microflora in the gut. A sufficient array of data confirms this idea. However, most researchers explain the mechanism of its action by an
antioxidant properties destined to detox the oxidants in the gut and other host tissues. Evidence is presented in favor of the hypothesis that the primary target for electrolyzed reducing water is the residential microflora in the gut.

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**Pilot study: Effects of drinking hydrogen-rich water on muscle fatigue caused by acute exercise in elite athletes**

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**Abstract**

Background: Muscle contraction during short intervals of intense exercise causes oxidative stress, which can play a role in the development of overtraining symptoms, including increased fatigue, resulting in muscle microinjury or inflammation. Recently it has been said that hydrogen can function as antioxidant, so we investigated the effect of hydrogen-rich water (HW) on oxidative stress and muscle fatigue in response to acute exercise. Methods: Ten male soccer players aged 20.9 ± 1.3 years old were subjected to exercise tests and blood sampling. Each subject was examined twice in a crossover double-blind manner; they were given either HW or placebo water (PW) for one week intervals. Subjects were requested to use a cycle ergometer at a 75% maximal oxygen uptake (VO2) for 30 min, followed by measurement of peak torque and muscle activity throughout 100 repetitions of maximal isokinetic knee extension. Oxidative stress markers and creatine kinase in the peripheral blood were sequentially measured.

Results: Although acute exercise resulted in an increase in blood lactate levels in the subjects given PW, oral intake of HW prevented an elevation of blood lactate during heavy exercise. Peak torque of PW significantly decreased during maximal isokinetic knee extension, suggesting muscle fatigue, but peak torque of HW didn’t decrease at early phase. There was no significant change in blood oxidative injury markers (d-ROMs and BAP) or creatine kinase after exercise.

Conclusion: Adequate hydration with hydrogen-rich water pre-exercise reduced blood lactate levels and improved exercise-induced decline of muscle function. Although further studies to elucidate the exact mechanisms and the benefits are needed to be confirmed in larger series of studies, these preliminary results may suggest that HW may be suitable hydration for athletes.

**Effectiveness of HydrogenRich Water on Antioxidant Status of Subjects with Potential Metabolic Syndrome**

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Summary:

Metabolic syndrome is characterized by cardio metabolic risk factors that include obesity, insulin resistance, hypertension and dyslipidemia. **Oxidative stress is known to play a major role in the pathogenesis of metabolic syndrome.** The objective of this study was to examine the effectiveness of hydrogen rich water (1.5–2 L/day) in an open label, 8-week study on 20 subjects with potential metabolic syndrome. Hydrogen rich water was produced, by placing a metallic magnesium stick into drinking water (hydrogen concentration: 0.55–0.65 mM), by the following chemical reaction: \(\text{Mg} + 2\text{H}_2\text{O} \rightarrow \text{Mg(OH)}_2 + \text{H}_2\). The consumption of hydrogen rich water for 8 weeks resulted in a 39% increase \((p<0.05)\) in antioxidant enzymes superoxide dismutase (SOD) and a 43% decrease \((p<0.05)\) in thiobarbituric acid reactive substances (TBARS) in urine. Further, subjects demonstrated an 8% increase in high density lipoprotein (HDL)-cholesterol and a 13% decrease in total cholesterol/HDL-cholesterol from baseline to week 4. There was no change in fasting glucose levels during the 8 week study. In conclusion, drinking hydrogen rich water represents a potentially novel therapeutic and preventive strategy for metabolic syndrome.
Hydrogen-rich water improves neurological functional recovery in experimental autoimmune encephalomyelitis mice

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10 authors, including:

Xue Jun Sun
Second Military Medical University, Shanghai
249 PUBLICATIONS  4,333 CITATIONS

Some of the authors of this publication are also working on these related projects:

- hydrogen inhalation to stroke
- ischemic neuronal death
Accepted Manuscript

Hydrogen-rich water improves neurological functional recovery in experimental autoimmune encephalomyelitis mice

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Hydrogen-rich water improves neurological functional recovery in experimental autoimmune encephalomyelitis mice

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Abstract

Multiple sclerosis (MS) is a chronic autoimmune demyelinating disease of the central nervous system (CNS). The high costs, inconvenient administration, and side effects of current Food and Drug Administration (FDA)-approved drugs often lead to poor adherence to the long-term treatment of MS. Molecular hydrogen (H₂) has been reported to exhibit anti-oxidant, anti-apoptotic, anti-inflammatory, anti-allergy, and anti-cancer effects. In the present study, we explored the prophylactic and therapeutic effects of hydrogen-rich water (HRW) on the progress of experimental autoimmune encephalomyelitis (EAE), the animal model for MS. We found that prophylactic administration of both 0.36 mM and 0.89 mM HRW was able to delay EAE onset and reduce maximum clinical scores. Moreover, 0.89 mM HRW also reduced disease severity, CNS infiltration, and demyelination when administered after the onset of disease. Furthermore, HRW treatment prevented infiltration of CD4⁺ T lymphocytes into the CNS and inhibited Th17 cell development without affecting Th1 cell populations. Because HRW is non-toxic, inexpensive, easily administered, and can readily cross the blood-brain barrier, our experiments suggest that HRW may have great potential in the treatment of MS.

Key Words:
Multiple sclerosis; Experimental autoimmune encephalomyelitis; Hydrogen-rich water; CD4⁺ T lymphocytes; TH17
1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease characterized by multiple inflammatory demyelinating lesions in the central nervous system (CNS) (Frohman et al. 2006). Most MS patients initially experience relapsing-remitting neurological dysfunction, which develops into a secondary progressive form after 10-15 years (Lassmann et al. 2012). The disease cannot be cured by available treatments thus far, but a reduction of relapse rate and disease progression, as well as other clinical activity markers, e.g., magnetic resonance imaging (MRI) lesions and brain atrophy, can be achieved (Stangel et al. 2015).

Because inflammatory lesions detected by MRI can be up to ten-fold more common than clinically recognized attacks, persistent and long-term administration with disease modifying drugs (DMDs), which can reduce immune attacks and neural damage, has been proven to be an effective way to delay disease progression (Miller et al. 1998). However, current MS medications are not always affordable; common drugs to treat MS can cost as much as $60,000 a year. Some drugs require injecting the patient one or more times per week, and some have side effects that can be very serious. Moreover, the immune-suppressing drugs may cause long-term side effects (Castro-Borrero et al. 2012). The high costs, inconvenient administration, and side effects of these drugs often lead to poor adherence to long-term treatment.

Molecular hydrogen (H₂) is an anti-oxidant gas present within the human body. It is non-toxic, inexpensive, and easily administered by drinking hydrogen-rich water (HRW) (Xue et al. 2014). In recent years, H₂ has been reported to have great potential in preventive and therapeutic applications. Many experiments have been carried out to confirm the properties of H₂, which exhibits anti-oxidant, anti-apoptosis, anti-inflammation, and anti-allergy effects, among others (Zhang et al. 2012). It is reported that administering HRW for 33 days to brain slice cultures derived from mice significantly diminished superoxide formation compared to the control (Sato et al. 2008). In rats with periodontitis, oral intake of HRW for 4 weeks resulted in lowered serum levels of reactive oxygen species (ROS) and oxidized low-density lipoprotein-cholesterol (Ekuni et al. 2012). Inhalation of H₂ gas also protected the brain from ischemia and reperfusion-induced oxidative stress damage (Ohsawa et al. 2007). However, whether H₂ has therapeutic potential for MS has not been reported.

In the present study, we aimed to address the potential role of HRW on an animal model of MS, experimental autoimmune encephalomyelitis (EAE). We found that HRW can significantly reduce EAE severity. It also prevented infiltration of CD4⁺ T lymphocytes into the CNS and inhibited Th17 cell development. Our experiments suggest that HRW may have a novel potential in the treatment of MS.

2. Materials and methods
2.1. Animals and preparation of Hydrogen rich water (HRW)

C57BL/6J mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All experimental mice were maintained under specific pathogen-free conditions and used at 6–8 wk of age. All animal experiments were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and approved by the Second Military Medical University Committee on Animal Care. HRW was purchased from Beijing HuoliQingyuan Co., Ltd. (China) and the core technology of this product is dissolving purified H\textsubscript{2} into pure water for two hours under 0.6 MPa. The saturated hydrogen water was stored under atmospheric pressure at 4°C in an aluminum bag or can with no dead volume. Hydrogen water was freshly prepared every week, which maintained a continuous concentration. The concentration of hydrogen has been confirmed by Li Han et al (Han et al. 2015). In the present study, gas chromatography was also used to further confirm the concentration of hydrogen following the method described by Ohsawa et al (Ohsawa et al. 2007).

2.2. EAE induction and treatment with HRW

The myelin oligodendrocyte glycoprotein (MOG\textsubscript{35–55}) (GLBiochem) used to induce EAE had a purity of 95%. For EAE induction, female C57BL/6 mice 8–10 weeks of age were immunized subcutaneously with 150 µg MOG\textsubscript{35–55} in complete Freund’s adjuvant containing heat-killed Mycobacterium tuberculosis (H37Ra strain; 5 mg/ml; Difco). Pertussis toxin (200 ng per mouse; Calbiochem-EMD Chemicals) in PBS was administered intraperitoneally on day 0 and day 2. Mice were examined daily for signs of disease by researchers ‘blinded’ to the experimental conditions and were assigned scores on a scale of 0–5 as follows: 0, no clinical signs; 1, paralyzed tail; 2, paresis (weakness, incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of both hindlimbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund state or death. Mice were euthanized if they reached a clinical score of 4. 0.36mM HRW (0.8 PPM) for the low-dose group and 0.89mM HRW (2.0 PPM) for the high-dose group were orally administered by gavage for twice a day at the onset of MOG immunization or at the first sign of disease. The volume of HRW administered every time is 20ml/Kg per mouse. The final dosage of molecular hydrogen is 16mg/Kg and 40mg/Kg per mouse for the low- and high-dose group respectively. The same volume of degassed water by gentle stirring was administrated in the same manner for control animals. For paralyzed mice we offer wealth of food and water so that they can easily eat and drink. We did not find mice have a score of 5 during the experiment, no mice suffering because of illness and death, all of the mice were sacrificed before humanely euthanasia.

2.3. Histology

For histopathological studies, animals were humanely euthanatized and spinal cords
were dissected from female mice (n=4), after performing heart perfusion with 4% (w/v) paraformaldehyde. Then, the spinal cords were embedded in paraffin, and 4-μm-thick sections that had been deparaffinized and rehydrated were stained with Hematoxylin and eosin (H&E) or Luxol fast blue (LFB) fast blue for evaluation of inflammation or demyelination, respectively. One of every five successive sections was collected from each animal. One pool of the sections was stained with LFB and counterstained with 0.5% neutral red. Another pool of the sections was stained with H&E solution and mounted in Permount (Fisher Scientific). For H&E analysis, the numbers of lymphocytes and macrophages infiltrated into the spinal cord sections per mm² were counted to estimate the degree of infiltration. For LFB scoring, demyelination was determined by the ratio of shadow white matter areas in LFB stained sections to total white matter area. Scoring for H&E or LFB sections was performed using blind examination by independent readers. At least four animals for each time point in different treated groups were included in one experiment. Three independent experiments were performed.

2.4. Isolation of mononuclear cells from the CNS and draining lymph nodes (DLNs)

The brain and spinal cord of normal or EAE mice were isolated, homogenized, filtrated, and centrifuged and were then suspended in 70% Percoll (GE healthcare) and overlaid with 37% and 30% gradients. After centrifugation, the majority of mononuclear cells, found in the interface of 37% and 70% Percoll, were collected. DLNs were harvested and pooled from EAE mice, and single-cell suspensions were prepared. Cells were cultured at 5x10⁶ cells/well in 24-well U-bottom plates with 10 mg/ml MOG₃₅–₅₅ peptide in complete RPMI 1640 medium (including 10% FBS) (n = 4).

2.5. Intracellular staining and flow cytometry

Surface staining was first performed with FITC Cy5–conjugated anti-mouse CD4 (clone GK1.5, Tianjin Sungene). For intracellular allophycocyanin-conjugated anti-mouse IFN-γ (clone XMG1.2, BD Biosciences) and PE-conjugated anti-mouse IL-17A (clone 17F3, BD Biosciences) staining, cells were isolated from DLNs and CNS infiltrates on day 20 after immunization and stimulated for 4.5 hours with Phorbol 12-myristate 13-acetate (PMA) (50 ng/ml Sigma-Aldrich) and ionomycin (1 mg/ml Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences). Cells were then fixed, permeabilized, and stained, as described previously (Du et al. 2009). For flow cytometry analysis, Moflo XDP (Beckman Coulter, Brea, CA, USA) was used. The software was CellQuest Pro (BD Biosciences) or FlowJo (Tree Star, Ashland, OR).


The data were analyzed using one-way ANOVA with Tukey’s post-hoc test in multiple groups or T-test in two groups. The EAE model was analyzed using the non-parametric Mann-Whitney test to compare two groups. The data are presented as the mean±SEM.
unless otherwise indicated. Values of p<0.05 were considered statistically significant.

3. Results

3.1. HRW delays onset and ameliorates severity of EAE.

We first explored the prophylactic effects of HRW on EAE. EAE was induced using the neurogenic peptide MOG\textsubscript{35–55}. Fresh 0.36mM HRW was initiated at the onset of MOG\textsubscript{35–55} immunization and administered orally twice a day. As shown in Fig 1A, the mean clinical scores of EAE in HRW-treated mice were significantly decreased compared with EAE controls. Therefore, prophylactic administration of 0.36mM HRW is able to delay EAE onset and decrease maximum clinical score.

Meanwhile, we used 0.89mM HRW to explore whether the beneficial effect of HRW was improved at a higher concentration. Fresh 0.89mM HRW, starting on the immunization day, was administered twice daily. The clinical scores of EAE in 0.89mM HRW-treated mice were significantly decreased, which led to a milder EAE (Fig.1A& Table.1). When compared with 0.36mM HRW, prophylactic administration of 0.89mM HRW was more effective in ameliorating EAE severity (Fig. 1A). These data showed a dose-dependent effect of HRW on EAE.

We next evaluated the efficacy when 0.89mM HRW was administered therapeutically by starting twice-daily administrations at the first sign of disease. Treatment with HRW in this mode again led to functional recovery; significant decreases in clinical severity were observed (Fig.1B& Table.2). These results suggest that HRW plays a suppressive role in the progress of EAE.

3.2. HRW altered EAE pathology by inhibit inflammatory infiltration and demyelination of CNS

To determine the effect of HRW on inflammation-induced demyelination in vivo, we evaluated the pathology of CNS inflammation and demyelination. We found that both 0.36mM and 0.89mM can significantly reduce demyelination of LFB staining from CNS of EAE compared with control (Fig. 2A,C). Meanwhile, the H&E staining showed less infiltrating cells around the lesion sites (Fig. 2B,D). The statistical results show that prophylactic HWR treated mice with EAE compared with control mice showed lower infiltration of immune cells, moreover, the proportion of myelin loss was also reduced in the spinal cord (Fig.2C,D). The EAE mice administered 0.89mM HRW therapeutically also had a significant improvement in the extent of demyelination and inflammation in spinal cords (Fig.3). These results indicate that both prophylactic and therapeutic HRW can alleviate the CNS pathology of EAE.

3.3. HRW affects the CD4\textsuperscript{+} population in vivo
To investigate whether inflammatory T cell development was also influenced by HRW during EAE, CD4+ T cells from prophylactic 0.36mM and 0.89mM HRW-treated mice and controls were harvested on day 20 after immunization. We detected infiltrative CD4+ T cells in the CNS of three groups of mice and found fewer CD4+ T cells accumulated in the CNS of prophylactic HRW-treated mice as measured by CD4 surface staining of CNS leukocyte infiltrates (Fig. 4A). Both the frequency and absolute number of CD4+ T cells were lower in prophylactic HRW-treated mice but higher in controls. Moreover, the frequency and absolute number of CD4+ T cells in prophylactic 0.89mM HRW was lower than 0.36mM HRW (Fig. 4B). We next investigate the proportion of CD4+ T cells from therapeutic HWR treated EAE in vivo (Fig.4C), it was found that therapeutic HWR revealed a remarkable inhibition of CD4+ T cells, the frequency and absolute number are quantified as shown in (Fig. 4D). Thus, our data indicate that HRW plays a negative regulatory role in CD4+ T cells development.

3.4. **HRW inhibit the Th17 cell development in vivo**

We identified the proportion of two important pro-inflammatory T helper cells in CD4+ cells. Through intracellular cytokine staining of the proportions of IFN-γ+ and IL-17A+ cells, both in the CNS and lymph nodes(Fig.5A), we found the absolute number of Th17 cells and the proportion of Th17 cells in the CD4+ population were consistently lower in HRW-treated mice compared to controls(Fig.5B,C). Although the absolute numbers of Th1 cells in HRW-treated mice were also decreased, the proportion of Th1 in the CD4+ population was not changed (Fig.5B,C). These results indicated that HRW treatment could largely inhibit Th17 cell development.

4. **Discussion**

H2 has been shown to reduce inflammation in experimental animal models induced by concanavalin A, dextran sodium sulfate, lipopolysaccharide, Zymosan, and polymicrobial sepsis(Das et al. 2007). It can also promote functional recovery of patients with rheumatoid arthritis, a chronic autoimmune inflammatory disease characterized by the destruction of bone and cartilage(Ishibashi et al. 2012). Our finding of the role of HRW in reducing the clinical score of EAE further indicated that HRW might be therapeutic in MS.

Because MS is a chronic disease and not currently curable, the high costs, inconvenient administration, and side effects of current FDA-approved drugs often lead to poor adherence to long-term treatment. H2 is a non-toxic gas present within the human body. It can readily cross the blood-brain barrier and cellular membranes(Itoh et al. 2011). Drinking liquids supplemented with H2 represents a novel method of hydrogen gas delivery that is easily translatable into clinical practice(Ishibashi et al. 2012). Moreover, HRW is commercially available and is much cheaper than current FDA-approved MS drugs. Because HRW is non-toxic, inexpensive, easily administered, and can readily cross the blood-brain barrier, HRW therapy may have
tremendous potential in the long-term treatment of MS patients.

The immunologic mechanisms of MS are very complicated (Kulkarni et al. 2004; Mirshafiey and Jadidi-Niaragh 2010). CD4+ T cells have been shown to play a basic important role in the neuro-inflammatory process of MS and EAE. Th1 and Th17 cells are the main pathogenic CD4+ T cells in disease development (Sonobe et al. 2007; Tzartos et al. 2008). Our present work indicated that HRW prevented infiltration of CD4+ T lymphocytes into the CNS, decreased the absolute number of Th1 and Th17 cells and inhibited Th17 development. In addition to T cells, macrophages and microglia also play important roles in EAE pathogenesis (Goldmann and Prinz 2013). It is reported that paralysis of microglia or ablation of macrophages significantly alleviated EAE severity (Ponomarev et al. 2011). Studies also indicated that treatment with hydrogen inhibits LPS/IFN-γ-induced NO production through modulation of signal transduction in macrophages and ameliorates inflammatory arthritis in mice (Itoh et al. 2011). Thus, the beneficial effects of HRW on EAE may also be mediated by its anti-inflammatory effects on macrophages or microglia.

The molecular mechanism by which HRW modulates severity of EAE may be as follows. Firstly, recent evidences indicate that molecular hydrogen is an effective free radical scavenger (Ohta 2014). Oxidative stress has been implicated as mediators of demyelination and axonal damage in both EAE and MS. Many studies have been showed increased free radical levels in MS patients (Gilgun-Sherki et al. 2004). Treatment of EAE rats with scavenger of ROS markedly suppressed the severity of the disease (Martin and Near 1995). Thus HRW may prevent EAE progress by its antioxidant activity. Secondly, it is reported that HRW treatment decreased the levels of pro-inflammatory molecules, such as TNFα, IL-1β, NF-κB and high-mobility group box 1 protein (HMGB1), whereas increased the levels of an anti-inflammatory cytokine, IL-10 (Tian et al. 2016; Zhang et al. 2014). Among these molecules, TNFα were observed to be increasing in cerebrospinal fluid in patients with MS and correlated with disease activity (Sharief and Hentges 1991). TNFα deficiency, treatment with soluble TNFR1–IgG fusion protein or anti-TNF antibodies prevented the development of EAE (Kassiotis and Kollias 2001; Kollias et al. 1999). Besides TNFα, upregulation of IL-1β was also found in lesion in brain material of MS patients and EAE (Bauer et al. 1993). Reduction of IL-1β action by administration of IL-1ra reduces the neurological defects in animals subjected to EAE (Bettelli et al. 1998; Ruuls et al. 1995). In addition, activation of the downstream signaling molecule of TNFR1 and IL-1β, NF-κB, has also been found in MS brain tissue. Microarray analysis of MS brain tissue identified upregulated expression of genes related to NF-κB (Lock et al. 2002; Mycko et al. 2003). NF-κB inhibition, both in peripheral immune cells and in the CNS, is protective in EAE, suggesting that the therapeutic effect of HRW may be mediated by targeting of the NF-κB pathway (Yan and Greer 2008). HMGB1 is a ubiquitous nuclear protein that released from necrotic cells, such as damaged oligodendrocytes in MS lesions. HMGB1 drives neuroinflammatory responses in EAE, inhibition of HMGB1 signaling ameliorates disease (Robinson et al. 2013). IL-10 also plays an important role in the regulation of autoimmune injury in EAE, as evidenced by increased susceptibility of IL-10−/− mice to EAE (Bettelli et al. 1998), whereas mice over-expressing IL-10 are highly resistant to EAE induction (Cua et al. 1999). Increased IL-10 levels in spinal cord correlate with EAE remission,
and lower production of IL-10 in humans appears to be a risk factor for MS (Croxford et al. 2001; Vandenbark et al. 2001). Thus the EAE suppressor function of HRW may be mediate by it anti-inflammatory functions through up regulating TNFα, IL-1β, NF-κB, HMGB1 and down regulation of IL-10. Thirdly, drinking HRW is also reported to prevent BBB disruption and inhibit the activities of matrix metalloproteinases-9 (MMP-9) (Sun et al. 2012; Takeuchi et al. 2015). The primary functions of MMP-9 in EAE encompass increase of inflammation, disruption of BBB, cleavage of myelin proteins, activation or degradation of disease-modifying cytokines, and direct damage to CNS cells (Opdenakker et al. 2001). Increased MMP-9 levels have been found in the serum, cerebrospinal fluid, and autopsied brains of patients with MS and EAE (Dubois et al. 1999). Thus the beneficial effect of HRW on EAE may also be due to suppression of MMP-9 activity.

The molecular mechanism by which HRW modulates Th17 development is also unclear. Activation of precursor T helper cells in the presence of transforming growth factor (TGF)-β and IL-6 is thought to drive differentiation of Th17 cells in the mouse (Hu et al. 2011). In humans, a combination of TGF-β, IL-1β, and IL-23 induces Th17 differentiation from naive T cells (Valmori et al. 2010). It has been suggested that H2 treatment markedly attenuated IL-1β and IL-6 expression in bronchoalveolar lavage fluid after acute lung injury (Liu et al. 2015). This indicates that HRW may inhibit Th17 differentiation by alleviating these molecules. However, the precise molecular mechanisms of how hydrogen therapy influences Th17 differentiation still require further research.

In summary, the present work determined the prophylactic and therapeutic effects of HRW on the occurrence and development of EAE. To our knowledge, this is the first report demonstrating that HRW significantly reduced the clinical score of EAE by inhibiting Th17 differentiation in the CNS and peripheral immune organs.

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Conflict of interest

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of this paper.

References

Bettelli E, Das MP, Howard ED, Weiner HL, Sobel RA, Kuchroo VK. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. Journal of immunology 161(7):3299-3306.


Ruuls SR, Bauer J, Sontrop K, Huitinga I, t Hart BA, Dijkstra CD. 1995. Reactive oxygen species are


Fig1. Clinical scores of EAE treated with HRW. (A) HRW (prophylactic 0.36 or 0.89 mM) was administrated twice every day from the immunization day; (B) HRW (therapeutic 0.89 mM) was administrated twice every day from the onset of clinical symptoms of EAE. The intake of normal water was set up as control in a similar manner. All data above are representative of three independent experiments, n=5-6 in each group and each experiment. EAE model was analyzed using the non-parametric Mann-Whitney test. *p<0.05, **p<0.01 were considered statistically significant.

Fig2. Histology of spinal cords isolated from HRW-treated and control mice after immunization. (A) EAE mice treated with HRW (0.36 and 0.89 mM) or vehicle in the prophylactic mode and spinal cord sections from mice at 20 days after immunization stained with H&E. Representative LFB staining showed demyelination in the prophylactic mode of HRW or vehicle. The demyelinating lesion in white matter was outlined. (B) H&E-stained section from the spinal cord of EAE mice treated with HRW (0.36 and 0.89 mM) prophylactically at 20 days. Arrows point to regions of inflammatory cells infiltration (H&E). Scale bars represent 100 um. (C) Histogram of demyelinated white matter (WM) in total WM around lesion sites of EAE mice. (D) Histogram of the number of infiltrating cells around lesion sites of EAE mice. Data are representative of three independent experiments (mean and s.e.m.), 5 mice were used in each group and each experiment. *P < 0.05, **P < 0.01, versus control (one-way ANOVA with Tukey’s post-hoc test).

Fig 3. Histology of spinal cords isolated from control mice and HRW-treated mice after immunization. (A) EAE mice treated with HRW (0.89 mM) or vehicle in the therapeutic mode and spinal cord sections from mice at 20 days after immunization stained with H&E. Arrows point to regions of inflammatory cells infiltration (H&E). (B) HRW (0.89 mM) or vehicle in the therapeutic mode and spinal cord sections stained with LFB. Arrows point to regions of demyelination (LFB). Scale bars represent 100 um. (C) Histogram showing quantification of the number of infiltrating cells around lesion sites of EAE mice. (D) Histogram of the percentage of demyelinated white matter (WM) in total WM around lesion sites of EAE mice. Data are representative of three independent experiments (mean and s.e.m.), 5 mice were used in each group and each experiment. *P < 0.05, **P < 0.01, versus control (Student’s t-test).

Fig4. Quantification of CD4+ T cells in mononuclear cell infiltrates isolated from brains and spinal cords of HRW-treated mice after immunization. (A) Mice were treated with 0.36 mM and 0.89 mM HRW prophylactically twice daily
from the onset of EAE after immunized with MOG peptide\textsubscript{35-55}. The intake of degassed water was set up as control in a similar manner. On day 20 after immunization, flow cytometry was performed to assess the infiltrative CD4\textsuperscript{+}T cells in brains and spinal cords. One representative stain is shown. (B) The percentage and absolute number of infiltrative CD4\textsuperscript{+}T cells from (A) are shown. (C) Mice were treated with 0.89mM HRW therapeutically twice daily from the onset of clinical symptoms of EAE after immunization. Flow cytometry was performed to assess the infiltrative CD4\textsuperscript{+}T cells in brains and spinal cords on day 20. One representative stain is shown. (D) The percentage and absolute number of infiltrative CD4\textsuperscript{+}T cells in brains and spinal cords from 0.89mM therapeutic HRW treated mice. The experiments were repeated three times, 7 mice were used in each group and each experiment. **\textit{p}<0.01,***\textit{p}<0.01 were considered statistically significant (one-way ANOVA with Tukey’s post-hoc test).

**Fig 5. Intracellular staining of IL-17A and IFN-\textgamma in the cells in CNS and lymph nodes.** (A) The CD4\textsuperscript{+} cells from CNS and lymph nodes were also stimulated with PMA and ionomycin in the presence of GolgiPlug for 6 h, and then stained with IL-17A and IFN-\textgamma, one representative stain is shown. (B) The statistical analysis of the percentage and absolute number of IL-17A\textsuperscript{+}, IFN-\textgamma\textsuperscript{+} cells from brain and spinal cord. (C) The statistical analysis of the percentage and absolute number of IL-17A\textsuperscript{+}, IFN-\textgamma\textsuperscript{+} cells from lymph nodes. The experiments were repeated three times, 5 mice were used in each group and each experiment. *\textit{P}< 0.05, **\textit{P}< 0.01, versus control (Student’s t-test).
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Table 1. Development of EAE treated with Prophylactic HRW.

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Time of onset</th>
<th>Maximum score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>14 of 15</td>
<td>11.1 (± 0.9)</td>
<td>3.28 (± 0.5)</td>
</tr>
<tr>
<td>Prophylactically (0.36mM)</td>
<td>12 of 15</td>
<td>12.1 (± 0.4)**</td>
<td>1.94 (± 0.16)**</td>
</tr>
<tr>
<td>Prophylactically (0.89mM)</td>
<td>12 of 15</td>
<td>13.7 (± 0.6)**##</td>
<td>1.75 (± 0.27)**##</td>
</tr>
</tbody>
</table>

Incidence indicates number of mice that developed EAE relative to total mice in group; time of onset is presented in days (mean ± s.e.m.); Maximum EAE score is presented as mean (± s.e.m.). All data above are representative of three independent experiments, n=5-6 in each group and each experiment. The data were analyzed using one-way ANOVA with Tukey’s post-hoc test. **p<0.01 versus control (CTRL); #p<0.05, ##p<0.01 versus prophylactically (0.36mM) group.
Table 2. Development of EAE treated with Therapeutic HRW.

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Time of onset (± s.e.m.)</th>
<th>Maximum score (± s.e.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>14 of 15</td>
<td>11.7 (± 0.5)</td>
<td>3.14 (± 0.24)</td>
</tr>
<tr>
<td>Therapeutically (0.89mM)</td>
<td>13 of 15</td>
<td>12.0 (± 0.9)</td>
<td>2.17 (± 0.25)**</td>
</tr>
</tbody>
</table>

Incidence indicates number of mice that developed EAE relative to total mice in group; time of onset is presented in days (mean ± s.e.m.); Maximum EAE score is presented as mean (± s.e.m.). All data above are representative of three independent experiments, n=5-6 in each group and each experiment. The data were analyzed using one-way ANOVA with Tukey’s post-hoc test. **p<0.01 versus control (CTRL);
Graphical abstract

A

B

Mean clinical EAE score vs. time after immunization (days) for CTRL, HRIV (Prophylactic 0.36mM), and HRIV (Prophylactic 0.89mM) groups.

Mean clinical EAE score vs. time after immunization (days) for CTRL and HRIV (Therapeutic 0.85mM) groups.

* indicates statistical significance.
Highlights

- HRW reduced the clinical score of EAE
- HRW altered EAE by inhibit inflammatory infiltration and demyelination of CNS
- HRW functions in EAE development largely by affecting the CD4$^+$ population.
- HRW inhibiting Th17 cells differentiation in CNS and peripheral immune organs.