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ABOUT COVER

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AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

EDITORS FOR THIS ISSUE

ORIGINAL ARTICLE

Basic Study

Effects of alkaline-electrolyzed and hydrogen-rich water, in a high-fat-diet nonalcoholic fatty liver disease mouse model

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Abstract

AIM
To identify the effect of hydrogen-rich water (HRW) and electrolyzed-alkaline water (EAW) on high-fat-induced non-alcoholic fatty acid disease in mice.

METHODS
Mice were divided into four groups: (1) Regular diet (RD)/regular water (RW); (2) high-fat diet (HFD)/RW;

(3) RD/EAW; and (4) HFD/EAW. Weight and body composition were measured. After twelve weeks, animals were sacrificed, and livers were processed for histology and reverse-transcriptase polymerase chain reaction. A similar experiment was performed using HRW to determine the influence and importance of molecular hydrogen (H_2) in EAW. Finally, we compared the response of hepatocytes isolated from mice drinking HRW or RW to palmitate overload.

RESULTS

EAW had several properties important to the study: (1) pH = 11; (2) oxidation-reduction potential of -495 mV; and (3) H_2 = 0.2 mg/L. However, in contrast to other studies, there were no differences between the groups drinking EAW or RW in either the RD or HFD groups. We hypothesized that the null result was due to low H_2 concentrations. Therefore, we evaluated the effects of RW and low and high HRW concentrations (L-HRW = 0.3 mg H_2/L and H-HRW = 0.8 mg H_2/L, respectively) in mice fed an HFD. Compared to RW and L-HRW, H-HRW resulted in a lower increase in fat mass (46% *vs* 61%), an increase in lean body mass (42% *vs* 28%), and a decrease in hepatic lipid accumulation ($P < 0.01$). Lastly, exposure of hepatocytes isolated from mice drinking H-HRW to palmitate overload demonstrated a protective effect from H_2 by reducing hepatocyte lipid accumulation in comparison to mice drinking regular water.

CONCLUSION

H_2 is the therapeutic agent in electrolyzed-alkaline water and attenuates HFD-induced nonalcoholic fatty liver disease in mice.

Core tip: In this work, we compared the effects of two functional waters: Electrolyzed alkaline water and Hydrogen-rich water in a high-fat-diet-induced nonalcoholic fatty liver disease (NAFLD) mouse model. Hydrogen-rich water (HRW) has potential for NAFLD treatment by attenuating hepatic lipid accumulation, inflammation, and CD36 expression. However, neither electrolyzed-alkaline water (EAW) nor HRW with a low H_2 concentration had protective effects on NAFLD. Additionally, we demonstrated that H_2 pretreatment has a protective effect by modifying gene expression. The results demonstrate that H_2 has a surprisingly positive impact in preventing NAFLD in mice and is also the key agent responsible in EAW for these benefits.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is considered the hepatic expression of metabolic syndrome, and in most cases, it is associated with obesity, dyslipidemia, diabetes, and insulin resistance[1]. At present, NAFLD is considered the most common liver disease affecting 20%-30% of the Western World's population[1]. The spectrum of NAFLD ranges from simple, apparently benign, hepatic lipid accumulation (simple steatosis) to nonalcoholic steatohepatitis (NASH)[2]. NASH is characterized by inflammation and collagen deposition (fibrosis)[2] and may progress to hepatic tissue damage (cirrhosis)[3]. Generally, the disease remains in the steatotic stage, characterized by an excessive accumulation of triglycerides in the hepatocytes. However, in about 5% to 10% of cases, the disease will progress to NASH, and from there, 10%-25% will develop cirrhosis with about 1% of those patients progressing to hepatocellular carcinoma[4]. The disease's pathogenesis is complex and multifactorial and involves genetic and environmental factors, including altered gut microbiome as part of the multistep pathogenic NAFLD model[4]. Insulin resistance is a key factor in the disease onset resulting in increased hepatic *de novo* lipogenesis (DNL), adipose tissue lipolysis, and inhibition of free fatty acid (FFA) β-oxidation. The reaction of the liver to intracellular lipid buildup results in a cascade of events, including oxidative stress, mitochondrial and endoplasmic reticulum dysfunction, and inflammation[5].

Lifestyle changes that promote weight loss improve disease status; however, this is often difficult to maintain in the long term[6]. At present, there is no specific pharmacological treatment for this disease as most drugs indicated for NAFLD target secondary features of the disease such as obesity, dyslipidemia, and insulin resistance[7]. Due to the high prevalence and increasing NAFLD incidence coupled with the current treatment's ineffectiveness and health risks, the need for a simple and safe alternative is warranted. Regardless of the factors, NAFLD pathogenesis and progression are linked to excessive oxidative stress and inflammation, the attenuation of which may be a viable approach[8].

Hydrogen-rich and electrolyzed alkaline waters (HRW and EAW, respectively) have been reported as types of functional waters that may ameliorate various disease conditions[9]. EAW is produced *via* electrolysis of water. At the cathode (equation 1), water is reduced to hydrogen gas/molecular hydrogen (H_2) and hydroxide ions (OH^-). The OH^- ions cause an increase in the resulting water's pH making it more alkaline. At the anode (equation 2), water is oxidized to oxygen gas

(O_2) and protons (H^+). The increased hydrogen ion (H^+) concentration makes the water acidic. EAW units have a membrane that separates the cathode and anode compartments, without which the resulting pH would be neutral (equation 3).

Cathode reaction: $4H_2O$ (l) + $4e^-$ → $2H_2$ (g) + $4OH^-$ (aq) Equation 1

Anode reaction: $6H_2O$ (l) + $4e^-$ → + O_2 (g) + $4H_3O^+$ (aq) Equation 2

Overall reaction: $2H_2O$ (l) → $2H_2$ (g) + O_2 (g) Equation 3

EAW has been reported to have anti-obesity, anti-oxidant, anti-diabetic, and hepatoprotective effects[10-13]. However, the properties of EAW associated with these beneficial effects have been debated[11-14]. EAW exhibits a negative oxidation-reduction potential (ORP) due to the dissolved hydrogen gas and high pH[11], which is expected according to the Nernst equation. Nevertheless, H_2 is not recognized by some researchers/commercial companies as the principal therapeutic agent in EAW[15].

H_2 has recently been demonstrated to exert thera-peutic benefits in animal and human clinical studies in ameliorating excessive inflammation and oxidative stress[16]. It was first confirmed to have therapeutic potential in animals for cancer (hyperbaric chamber)[17] and ischemia-reperfusion (inhalation)[18] in studies publi-shed in Science and Nature Medicine, respectively. H_2's potential therapeutic effects have now been confirmed in over 170 different human and animal-disease models and in essentially every organ of the human body[19]. H_2 is nonpolar, hydrophobic, and the smallest molecule, thus allowing it to quickly diffuse through cell membranes and reach the mitochondria, nucleus, endoplasmic reticulum, and other subcellular compartments[20]. These properties make it an attractive molecule for NAFLD treatment[21]. Although more research is needed to elucidate the molecular mechanism(s) and optimal dosing for H_2, preliminary animal and human studies are promising. Clinical studies of drinking hydrogen-rich water (HRW) have demonstrated beneficial effects in several diseases such as Parkinson's disease, type II diabetes, rheumatoid arthritis, mitochondrial myopathies, muscle fatigue, metabolic syndrome, hyperlipidemia, liver inflammation (hepatitis B) and others reviewed previously[19,22-30]. Clinical studies involving metabolic and liver conditions further support the potential benefits of hydrogen on NAFLD.

Several animal and cells studies concerning NAFLD and hydrogen therapy have been reported using different models. For example, one study[31] using hydrogen-rich saline in a NAFLD rat model induced with hyperglycemia and hyperlipidemia, found H_2 significantly lowered levels of oxidative stress and inflammation[31]. In another study using a methionine-choline-deficient diet-induced NASH model, ingestion of HRW significantly attenuated steatohepatitis to a degree comparable to the drug pioglitazone and suppressed hepatic tumorigenesis in a streptozotocin-induced NASH-related hepatocarcinogenic mouse model[32].

Similar positive effects of H_2 have also been reported in other studies[33,34]. In the present study, we used a different NAFLD animal model induced by a high-fat diet (HFD) which is more relevant to human physiology.

We aimed to determine the influence of both types of functional waters, EAW and HRW, on an HFD-induced-NAFLD model, and the importance of H_2 as a therapeutic factor in EAW. Furthermore, we investigated if pretreatment with HRW could protect cells from further exposure to a high-fat environment.

MATERIALS AND METHODS

Animal and animal care
Animal protocols were approved by the Israeli National Committee for Animal Ethics and Welfare. In all experiments, animals had *ad libitum* access to food and water.

Electrolyzed water on NAFLD
Forty-eight C57bl/6J young male mice (three weeks old, approximately 20 g) were obtained from Harlan Laboratories (Israel). The mice were randomly separated into conventional polycarbonate rodent cages (six animals per cage) and fed a regular diet (RD), which was a commercial purified control diet composed of 17% calories from fat, 23% calories from protein, and 60% calories from carbohydrates (TD 120455, Harlan Laboratories, United States). Conventional polycarbonate bottles with a stainless-steel cap were put on the top of each cage. Cages were maintained at 23 ℃ ± 1 ℃ and 40%-60% humidity under a 12:12 h light:dark cycle. During this time, animals were weighed twice a week to check that there were no differences in the initial growth rate.

After a 2-week acclimation period, mice were divided into four groups (*n* = 12) according to the type of diet and water: (1) RD/ Regular Water (RW); (2) RD/EAW; (3) HFD/RW; and (4) HFD/EAW. Mice were individually identified by ear punching. The HFD was a purified commercial diet for inducing obesity (TD 06414, Harlan Laboratories, United States) and was composed of 60.3% calories from fat, 21.3% from carbohydrates, and 18.4% from protein. Formula and specific components of both the RD and HFD are shown in Table 1.

EAW was prepared with a Water Ionizer Batch System (BTM-3000, BionTech, Korea). Commercial mineral water (initial pH 7.8) was electrolyzed conti-nuously for 3 h, and the water at the cathode was collected (pH 11 ± 0.48, ORP of -495 ± 27 mV, H_2 ≈ 0.2 mg/L). The water was transferred to feeding bottles and added to the mice's cages. Fresh electrolyzed water was prepared twice a week.

Body weight was measured weekly at 9 AM before the morning feeding using an Ohaus Scout Pro 200 g scale (Nänikon Switzerland). Body composition: fat

Table 1 Composition of the regular and high-fat diet

Ingredients	Ingredient concentration (g/kg)	
	Regular diet	High fat diet
Casein	210.0	265.0
L-cystine	3.0	4.0
High amylose corn starch	500.0	-
Maltodextrin	100.0	160.0
Sucrose	39.14	90.0
Anhydrous milkfat	20.0	-
Lard	20.0	310.0
Soybean oil	20.0	30.0
Cellulose	35.0	65.5
Mineral mix, AIN-93G-MX (94046) (g/kg)	35.0	48.0
Calcium phosphate, dibasic	-	3.4
Vitamin mix, AIN-93-VX (94047)	15.0	21.0
Choline bitartrate	2.75	3.0
TBHQ, antioxidant	0.01	-
Protein (% by weight)	18.6	23.5
Carbohydrate (% by weight)	50.6	27.3
Fat (% by weight)	6.2	34.3

mass (FM), fat-free mass (FFM), and extracellular fluid were measured using time-domain nuclear magnetic resonance (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany) following a 12 h fast at the end of the acclimation phase and during the experiment at six and 12 wk.

At the end of the experimental period, animals were sacrificed using inhaled isofluorane, and livers were processed for histology and real-time polymerase chain reaction (RT-PCR).

Hydrogen-rich water on NAFLD
Twenty-Four C57bl/6J males (three weeks old, approximately 20 g) were obtained from Harlan Laboratories (Israel). Mice were separated into appropriate cages and fed a RD for two weeks. After this acclimation period, all animals were fed a HFD ad libitum. Mice were then divided into three groups according to the type of water: (1) Group 1 (control group) received regular water; (2) the second group received hydrogen-rich water at low concentration (L-HRW); and (3) the third group received hydrogen-rich water at high concentration (H-HRW). Mice were individually identified by ear punching. Body weight, food consumption, and fluid intake were measured weekly, and at the end of the experiment, livers were processed for histology, RT-PCR and quantification of total fat were done following the protocol by Roopchand *et al*[35].

Preparation and chemical properties of hydrogen-rich water
HRW was prepared using a sachet made of a net cloth containing metallic magnesium (Sigma, St. Louis, United States; 98% turnings) and small natural stones. The sachet was immersed in closed glass bottles containing cold water (4 ℃) that was previously acidified to pH 2.0 with 37% hydrochloride acid (Sigma). The water was vortexed for 1 h, during which time H_2 was produced

according to the reaction: $Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2$. The final pH of the solution reached $\approx$ 11.0 and contained dissolved hydrogen at a concentration of 1.2 mg/L. The solution was then diluted with regular water to make high (0.8 mg/L) and low (0.3 mg/L) concentrations of HRW each at a pH of approximately 8.0.

Each solution was transferred to mice-feeding bottles in which a new magnesium sachet was added. Due to the alkaline pH of the water, the amount of H_2 produced *via* the above reaction was minimal (the rate of escape $\approx$ rate of production) but was enough to maintain the low and high H_2 concentration for 24 h. Fresh HRW was produced every other day. The sachets kept in the feeding bottles were rinsed with 5% acetic acid followed by RW every time the water was changed. The H_2 concentration in water was measured daily.

Determination of hydrogen concentration in water
The hydrogen concentration in water was determined with H_2Blue (H_2Sciences Inc., United States), which is a redox titration reagent composed of methylene blue and a colloidal platinum catalyst[36].

Liver histology
After dissection, the liver was cut into small pieces, immersed in 4% paraformaldehyde at room temperature for 24 h, and then stored at 4 ℃ for 24 h. Samples were dehydrated with ethanol and cleared with xylene and embedded in paraffin. Five-micrometer sections were cut and stained with Hematoxylin and Eosin (HE). Pictures of 4-6 different fields per sample were taken under an Axiovert40-CFL (Zeiss, Germany) microscope equipped with a digital camera (AxioCam MRC, Germany).

Real-time polymerase chain reaction
Total RNA was extracted using Tri-Reagent (T-9424, SIGMA). One microgram of RNA from each sample was

Table 2 Primers used in this study

Gene marker	Full name	Primer sequence (5'-3')
β-*Actin*	Beta-actin	GCCCTGGACTTCGAGCAAGA
		TGCCAGGGTACATGGTGGTG
β-*2m*	Beta-2 microglobulin	TGCTTGTCTCAC TGACCGGCC
		TGGGGGTGAATTCAGTGTGAGCC
SOD2	Super oxide dismutase 2	ACAACAGGCCTTATTCCGCT
		CCCCAGTCATAGTGCTGCAA
SREBP-1c	Sterol regulatory element Binding protein	CACCCTGTAGGTCACCGTTT
		AGAACTCCCTGTCTCCGTCA
ACC1	Acetyl-CoA Carboxylase	GACAGAGGAAGATGGCGTCC
		TACAACTTCTGCTCGCTGGG
TNF-α	Tumor necrosis Factor-alpha	CGTCGTAGCAAACCACCAAG
		AGCAAATCGGCTGACGGTG
CRP	C-Reactive Protein	ACTGTGGGGCCAGATGCAAGC
		GGGGCTGAGTGTCCCACCAAC
CAT	Catalase	TCACTCAGGTGCGGACATTC
		TAGTCAGGGTGGACGTCAGT
IL-6	Interleukin-6	TCTATACCACTTCACAAGTCGGA
		GAATTGCCATTGCACAACTCTTT
Adipo	Adiponectin	GACGACACCAAAAGGGCTCA
		GAGGCCATCTCTGCCATCA
AdipoR2	Adiponectin Receptor 2	CTCTGACAGGATTTGGGGTCAA
		GTGCCCTTTTCTGAGCCGTA

reverse transcribed into cDNA using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative RT-PCR was performed in quadruplicate in 384-well plates using the ABI Prism® 7900HT sequence detection system (Applied Biosystems). Each well contained 2 μL of cDNA template (diluted X 20 after the RT reaction), 2.5 μL SYBR green (Bio-Rad, United States), and 0.25 μL each of both forward and reverse gene-specific primers at 10 μmol/L (primers are listed in Table 2). Beta-2 microglobulin (β2m) and β-Actin were used as endogenous reference genes for normalization. The thermal profile was 95 ℃ for 1 min followed by 40 amplification cycles of 95 ℃ for 15 s and 60 ℃ for 30 s. After that melting curve analysis was done. The resulting Ct values were used to determine the relative gene expression.

In vitro experiments

Hepatocytes from mice that drank H-HRW or RW were isolated by perfusion to test if consumption of H-HRW had a long-term effect.

Twelve C57bl/6J male mice (three weeks old) were obtained from Harlan Laboratories (Israel). Mice were divided into two groups: (1) One that drank HRW (0.8 mg/L) and (2) the second that drank RW; both groups were fed a regular diet. After four weeks, hepatocytes (one mouse from each group) were isolated using perfusion under anesthesia through the portal vein according to Zhang *et al*[37].

The primary hepatocytes were isolated through a two-step collagenase perfusion system as described in a previous report[38]. Isolated hepatocytes from each mouse were seeded in 6-well plates (10^6 cells/wells) for measuring gene expression, and in 12-well plates (200000 cells/well) for the steatosis assay in William's

E medium (Sigma, St. Louis, United States) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, United States). Cells obtained from each mouse were seeded in a different plate. After 3 h, the medium was replaced with fresh medium without FBS, and the plates were prepared for further processing.

Steatosis assay

Cells were exposed overnight to 0.3 mmol/L palmitate-BSA conjugate or BSA as a control (in quadruplicate). Preparation of the palmitate stock solution was carried out as described previously[39]. Briefly, 20-mM palmitate (Sodium Palmitate, SIGMA) stock solution was prepared in 0.01 mol/L NaOH by heating at 80 ℃. A 2 mmol/L FFA-free BSA (Sigma) solution was prepared in ddH2O and maintained at 37 ℃ in a water bath. A 4 mmol/L FFA/1% BSA (5:1) solution was obtained by complexing the appropriate amount of palmitate stock solution to the BSA at 50 ℃ for another 15 min.

After overnight incubation with BSA-palmitate, the medium was removed, and cells were stained with Oil-Red-O to examine fat accumulation (steatosis)[40]. Plates were washed with cold phosphate-buffered saline and fixed in 4% paraformaldehyde for 1 h. After two changes of 70% ethanol, plates were rinsed in distilled water and stained for 30 min by Oil-Red-O and then placed to dry at room temperature. The stain collected by the cells was then extracted with 100% isopropanol. Steatosis was estimated according to the extracted oil red concentration (determined by measuring the absorbance at 520 nm). The steatosis percentage was determined by dividing the average OD value of experimental wells (treated with palmitate) by the average OD value of control cells (treated with BSA only) × 100. In each assay, two plates (HRW or RW)
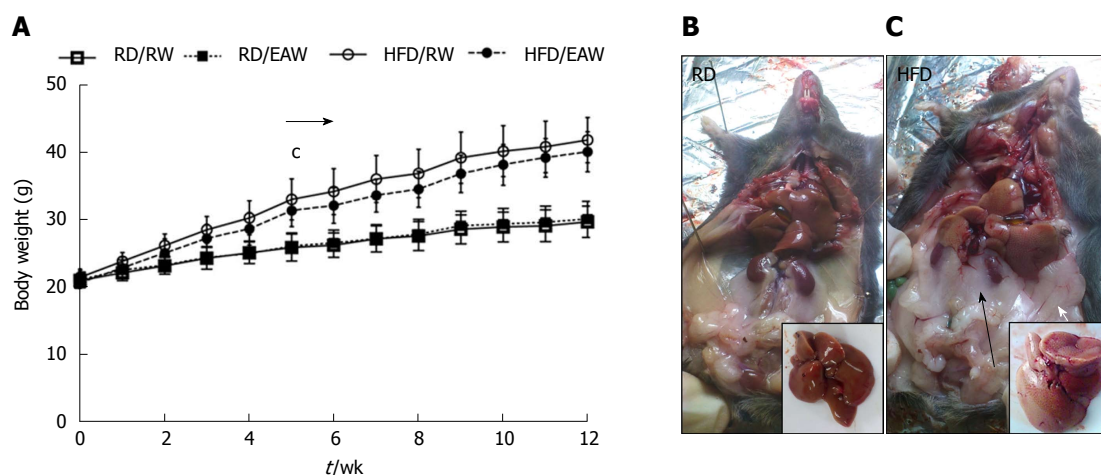
Figure 1 Effect of electrolyzed-alkaline water on body weight and composition. A: Body weight (n = 12, mean ± SD) [c]shows significant diference between high-fat and regular diets. Mann Whitney and Kruskal-Wallis [c]P < 0.001); B and C: Ventral view of dissected mice after 12 wk of the experiment. Black arrow: Retroperitoneal fat pads; white arrow epididymal fat pad. Insert: Fresh dissected liver. RW: Regular water; EAW: Electrolysed alkaline water; RD: Regular diet; HFD: High fat diet.

were processed in parallel.

Gene expression
Six-well plates containing 1×10^6 cells were used. The cells were exposed to 0.3 mmol/L bovine serum albumin-palmitate conjugate for 4 h, after which RNA was extracted from cells as described above.

Statistical analysis
Data are expressed as mean values ± SD. Data were analyzed by Kruskal-Wallis nonparametric test followed by a Mann-Whitney nonparametric test. A *P*-value ≤ 5% is considered statistically significant. The data were analyzed using the SPSS version 24 (SPSS Inc., Chicago, IL, United States). The statistical methods of this study were reviewed by Ms. Adi Sharabi-Nov of Tel-Hai Academic College.

RESULTS

Effect of EAW on body weight and composition
Water and food intake were measured for the entire group (n = 12) and calculated for each mouse once per week. As expected, mice fed an HFD showed a high-calorie intake when compared to mice fed chow (10.65 kcal/mouse/d *vs* 8.30 kcal/mouse/d, P < 0.01). Daily water intake was similar in mice drinking RW and EAW (2.49 mL/ mouse).

Mice fed an HFD had higher body weights compared to RD mice. Significant body weight differences were observed from the fifth week until the end of the experiment (Figure 1). At the end of 12 wk, the weight was 72% greater in HFD mice than in RD mice (P < 0.001). The differences in final body weight were due to an increase in fat mass. Larger retroperitoneal and epidimal fat pads were detected in HFD mice. The HFD also affected liver morphology. Livers from mice maintained on a regular diet were dark red/brown

and showed a clear and homogeneous texture. Livers from mice fed an HFD were light brown and showed a heterogenic flocculent texture. There was no difference in the weight of mice drinking RW or EAW in either group.

Body composition measurements based on NMR are shown in Figure 2. After 12 wk on different diets, the mice fed a HFD contained more fat (21.7 g ± 2.5 g) than RD mice (8.1 g ± 1.7 g. (P < 0.001). However, FFM was similar in both groups (16.5 g ± 0.8 g and 15.1 g ± 1.2 g in RD and HFD, respectively). EAW did not affect body composition of mice in either the RD or the HFD group.

EAW water effects on liver histology
The HE stained sections from livers are shown in Figure 3. Most of the liver parenchyma of HFD mice (Figure 3C and D) was steatotic with many enlarged cells, which were distributed among the remaining normal parenchyma. The enlarged hepatocytes showed micro- and macrovesicular steatosis. RD-fed mice presented liver parenchyma with no signs of steatosis (Figure 3A and B). EAW water did not affect the liver histology in the RD group or the HFD group.

Effect of HRW on NAFLD development
To evaluate the impact of HRW on NAFLD, we used mice fed an HFD only. Mice were separated into three groups according to the type of water. Control group received RW, and experimental groups received HRW at either low (HRW-L, 0.3 mg/L) or high (HRW-H, 0.8 mg/L) concentration. After 12 wk, mice were sacrificed, and liver histology and gene expression were analyzed. Body weight and composition were measured throughout the experiment.

Water and food consumption
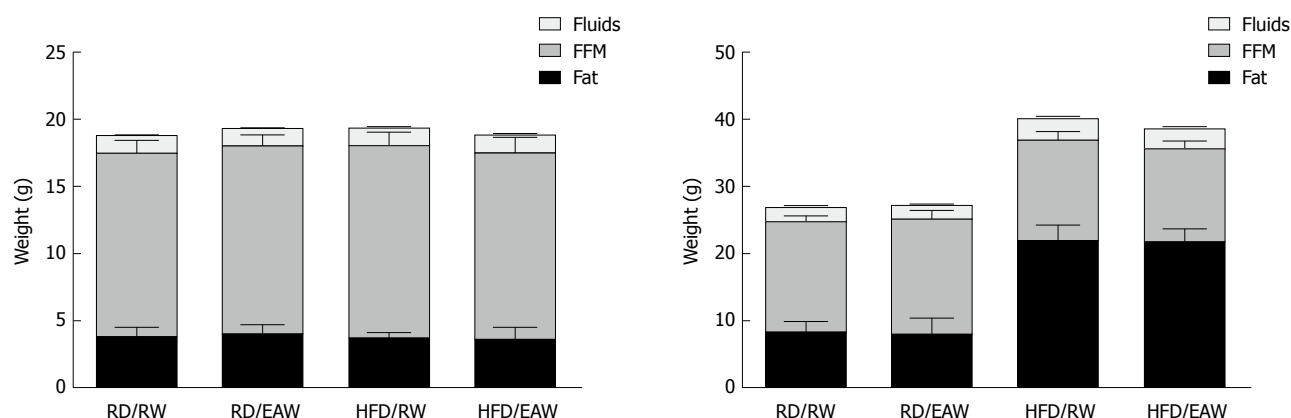Average water consumption was significantly higher

Figure 2 Body mass composition fat, free fatty mass and fluids of mice at different groups (*n* = 12) before the beginning of the experiment (up) and after 12 wk (down). Data are expressed as mean ± SD. Different letters show significant difference in fat content (Mann Whitney and Kruskal-Wallis [a]$P < 0.05$). RD/RW: Regular diet/regular water; RD/EAW: Regular diet/electrolysed alkaline water; HFD/RW: High fat diet/regular water; HFD/EAW: High fat diet/electrolysed alkaline water.

for H-HRW group (8.83 mL/mouse/d) than for L-HRW group (3.66 mL/mouse/d), and control group (3.34 mL/mouse/d, $P = 0.012$). However, the differences in water consumption did not affect appetite and food consumption. All three groups had the same caloric intake consisting of 9.99 kcal/mouse/day for the control group, 9.38 kcal/mouse/day for L-HRW group, and 10.5 kcal/mouse/day H-HRW group ($P = 0.505$).

Body weight and body composition
All three groups gained weight during the experiment; however, the H-HRW gained less weight compared to the two other groups ($P = 0.0567$).

Body composition was significantly affected by HFD. As expected, the total body mass of the three groups increased, mainly due to the addition of fat mass. However, mice in the H-HRW group showed a smaller increase in fat tissue when compared to the other two groups (p = 0.002). At the end of the experiment, mice in the H-HRW group showed a body composition of 46% fat mass and 42% lean body mass, compared to 61% fat mass and 28% lean body mass of the control group. There were also no differences in body water content among the three groups (Figure 4). Since the body mass composition of mice in the L-HRW group was similar to the control group, no further analysis of the L-HRW group was performed.

Liver fat content and histology:
Mice that drank H-HRW water accumulated significantly ($P < 0.01$) less hepatic lipids (30.49 mg ± 4.3 mg/300 mg tissue) in comparison to mice that drank RW (48.24 mg ± 6.0 mg/300 mg tissue). This result is corroborated by the histological sections.

Figure 5 shows liver H&E sections of the control group and H-HRW group (two mice from each group, the heaviest and lightest). Each pair of pictures shows the livers of mice of the two different groups that reached a similar total weight. Figure 5A and B show

livers of mice in the H-HRW group and control group that achieved final weights of 35.5 g and 35.9 g, respectively. The liver in the control group has visible steatosis, mainly at the periphery of the lobule. In contrast, the liver sections of the H-HRW group hardly developed fat inclusions. Figure 5C and D shows mice livers in the H-HRW group and control group weighing 40.0 g and 40.9 g, respectively. Although both livers are steatotic, the steatosis of the control group is visibly more pronounced. The hepatocytes show microvesicular steatosis at acinar zones 2 and 3; macrovesicular steatosis is most concentrated at zone 1. In contrast, the liver of H-HRW group is characterized by only mild steatosis, mainly at zone 1, and the beginning of microvesicular steatosis at zones 2 and 3.

NAFLD-related genes expression
The effect of HRW on the expression of several genes involved in NAFLD development compared to control is shown in Table 3. Acetyl CoA oxidase (ACOX), a gene related to lipid metabolism, was significantly up-regulated ($P < 0.05$), while others (such as carnitine palmitoyltransferase 1 (CPT1) and acetyl-CoA carboxylase), did not change significantly. HRW consumption did not alter the expression of the lipogenic enzymes SREBP1-c, fatty acid synthase (FAS) or acetyl-CoA carboxylase (ACC1). The most significant change was in the expression of CD36, which was markedly reduced in the H-HRW group ($P = 0.028$). Additionally, while the expression of adiponectin receptor 2 (AdipoR2) did not change significantly, a significant increase in adiponectin expression was detected ($P = 0.022$).

Genes related to oxidative stress [such as catalase (CAT) and superoxide dismutase (SOD)] did not change significantly; however, the inflammatory gene tumor necrosis factor (TNF)-α, but not interleukin (IL)-6 was significantly down-regulated. However, when the gene expression was measured after overloading with 0.3 mmol/L BSA-palmitate for 4 h, TNF-α and IL-6 were

Table 3 Expression of genes relevant to nonalcoholic fatty liver disease

Gene	RW		HRW		*P* value
	M	**Sd**	**M**	**Sd**	
IL-6	1.10	0.46	1.17	0.26	0.26
TNF-α	1.19[a]	0.36	0.68[b]	0.22	0.02
SREBP-1c	1.04	0.19	1.02	0.30	0.87
ACOX	1.04[a]	0.21	1.31[b]	0.25	0.04
CD36	1.06[a]	0.28	0.71[b]	0.06	0.02
CPT1	1.08	0.37	1.43	0.40	0.14
ACC2	1.12	0.50	1.05	0.23	0.64
ACC1	1.06	0.14	1.13	0.17	0.13
FAS	1.09	0.42	1.42	0.36	0.29
SOD	1.04	0.17	0.96	0.08	0.86
Cat	1.04	0.21	1.12	0.24	0.80
AdipoR2	1.01	0.22	1.14	0.28	0.23
Adipo	0.92[a]	0.29	1.32[b]	0.38	0.004

Each value is the average of 8 mice after normalization with keeping genes (β2m, β-actin). Statistically significant difference marked by different letters *P* < 0.05 (Kruskal-Wallis, Mann Whitney test). Regular water or hydrogen-rich water.

Figure 3 Histological sections stained by hematoxylin eosin of livers from mice fed regular diet and regular water (A), regular diet and electrolyzed alkaline water (B), high fat diet and regular water (C), high fat diet and electrolyzed alkaline water (D). Note the prominent steatosis in mice fed on high-fat diet. Microvesicular inclusions (big arrow), macrovesicular inclusion (small arrow).

both significantly down-regulated (*P* = 0.036 and *P* = 0.002, respectively) as shown in Table 4.

Long-term influence of HRW on hepatocytes
To investigate the long-term effects of HRW on hepatocytes, we extracted cells from different mice drinking either HRW or regular water and compared the fat accumulation after exposing them overnight to 0.3 mmol/L BSA-palmitate *in vitro*. There was less fat accumulation (*P* = 0.057) in the hepatocytes from the

mice that drank HRW (44.0% ± 18.6%) compared to hepatocytes from mice that drank regular water (52.8% ± 20.9%).

DISCUSSION
The high prevalence of NAFLD and the lack of safe and effective treatments have resulted in the search for natural alternative methods. The claims of purported functional waters for human health have increased in

Table 4 Expression of genes relevant to nonalcoholic fatty liver disease from hepatocytes isolated from mice drinking regular water or hydrogen-rich water after palmitate overloading *in vitro*

| | RW | | HRW | | |
	M	Sd	M	Sd	*P* value
TNF-α	9.16[a]	1.90	5.71[b]	4.59	0.036
IL-6	3.2[a]	0.87	2.24[b]	0.64	0.002
CRP	0.96	0.28	1.20	0.34	0.065
SREBP-1c	0.96	0.12	1.05	0.19	0.196
ACC1	0.84	0.18	0.86	0.14	0.902
SOD	1.07	0.34	1.30	0.56	0.184
AdipoR2	0.80	0.08	0.78	0.18	1.000
Adipo	1.00	0.30	0.79	0.30	0.097

Each value is the average of hepatocytes obtained from 4 different mice after normalization with keeping genes (β2m, β-actin). Statistically significant difference marked by different letters (Kruskal-Wallis, Mann Whitney test).
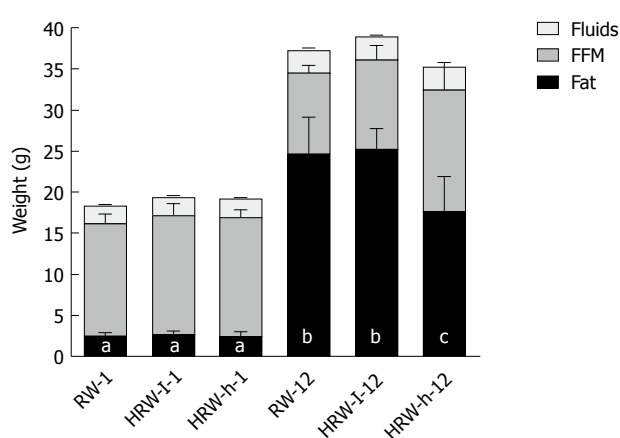

Figure 4 Body mass composition: Fat, fluids and free fatty mass at weeks 1 (first three columns) and 12 (last three columns), (*n* = 8). Data are expressed as mean ± SD. Different letters show a significant difference in fat content (Mann Whitney and Kruskal-Wallis *P* < 0.05). RW: Regular water; HRW-I: Hydrogen-rich water low concentration; HRW-h: Hydrogen-rich water high concentration).

recent years; however, many of these claims are not supported by the scientific literature. Therefore, our study used mice fed a HFD to determine and compare the potential effects of two claimed functional waters (EAW and HRW). We focused on the early stages of NAFLD, a disease associated with obesity and insulin resistance, which has become the leading cause of liver disease affecting adults and children worldwide.

As expected, mice fed a HFD gained significantly more weight (72%) as fat mass with accompanying liver steatosis compared to mice fed a RD. However, contrary to previous findings, using EAW[13], our results found no differences in body weight or liver histology between the regular water group and the EAW group. EAW was prepared by continuous electrolysis (3 h) to optimize its properties (such as pH, ORP, platinum nanoparticles) according to previous protocols[14,41,42]. The resulting water had a high stable pH (11) and a high negative ORP (-495 mV). However, the H_2 concentration in water was only 0.2 mg/L. In this way, mice drinking EAW received high alkaline water with a negative ORP but with only trace amounts of H_2. Due to the non-detectable differences between EAW and RW on body weight or liver parenchyma in either the RD or HFD groups, we did not perform any further analysis (such as gene expression or inflammatory markers).

In contrast to the H_2 concentration in EAW, our method for HRW preparation allowed us to maintain the H_2 concentration. However, L-HRW was also not efficient, but H-HRW was significantly effective at preventing the HFD-induced increase in fat tissue and hepatic lipid accumulation while increasing the amount of lean body mass compared to the control group. We also found that H-HRW significantly abolished the fat inclusions in liver sections, contained fewer triglycerides, and suppressed micro- and macrovesicular steatosis compared to the control. The suppression of fat gain by H-HRW may be attributed to hydrogen's ability to induce the hepatic hormone fibroblast growth factor-21 (FGF21), which causes an increase in energy expenditure[43]. Indeed, administration of FGF-21 can improve obesity and reverse hepatic steatosis[44]. In their study, H_2 water similarly attenuated HFD-induced fatty liver and weight gain in both wildtype and db/db mice. It also reduced hepatic oxidative stress[43].

To understand the mechanism by which H_2 affects NAFLD, we measured the expression of several essential genes related to the disease, including those genes related to oxidative stress, lipid metabolism, and inflammation. From the enzymes related to lipid metabolism, only peroxisomal fatty acyl-CoA oxidase (ACOX) showed significant differences. ACOX is one of the first enzymes in the metabolism of lipids and is a rate-limiting enzyme for beta-oxidation of very-long-chain fatty acids in peroxisomes. The participation of ACOX in suppressing NAFLD is essential. Fan *et al*[45] found that lack of ACOX expression in homozygous (ACOX-/-) mice resulted in liver steatosis, and Kohjima *et al*[46] found that in humans with NAFLD, its expression was increased two-fold, most likely to compensate for the increase in lipid accumulation. We found that mice that drank HRW showed a significant increase in ACOX expression, which would help enhance fatty acid
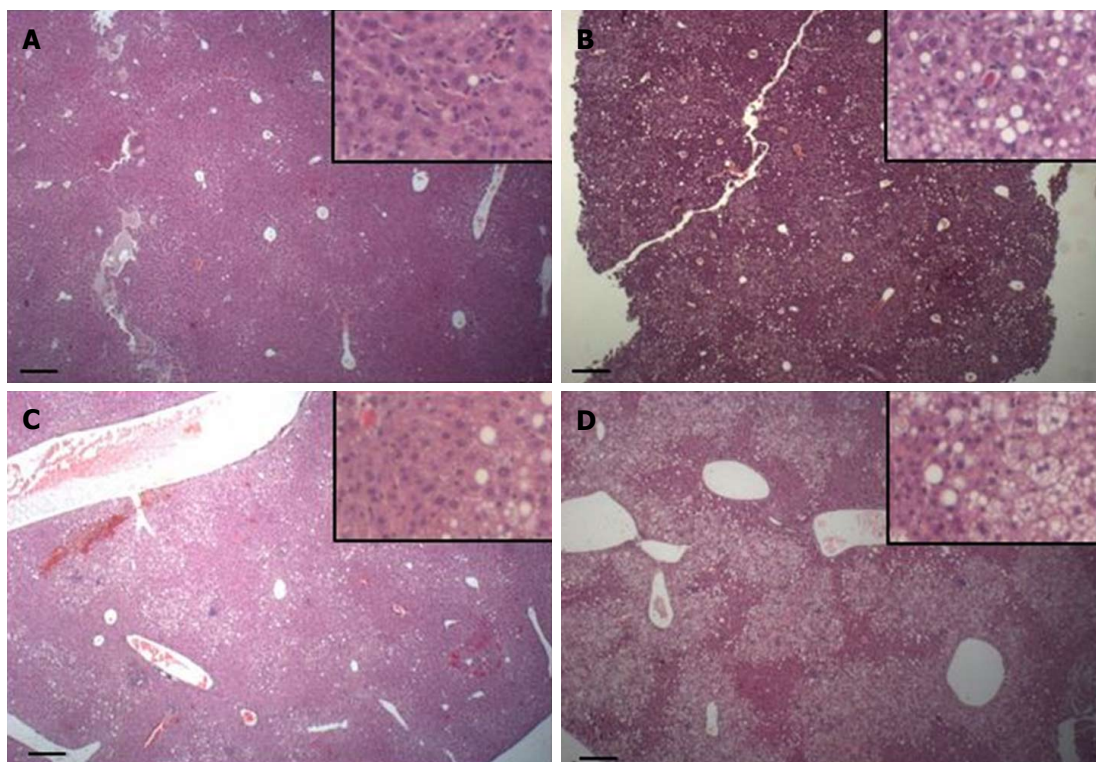
Figure 5 Histological sections of livers (HE) of mice fed a high-fat diet for 12 wk. A and C: Hydrogen-rich water (HRW) group; B and D: Regular water (RW) group. Each row shows sections of different mice that reached a similar weight at the end of the experiment. Note the pronounced steatosis in mice that drank RW in compare to mice of the same weight that drank HRW. Insert detail of each section. Bar = 200 μm HWR: Hydrogen-rich water; RW: Regular water.

oxidation, compared to the control group. The increase in ACOX expression is mediated by the transcription factor peroxisome proliferator-activated receptor[46], which can be upregulated by H$_2$[31,43].

Fatty acid translocase-36 (CD36), also known as FAT (fatty acid translocase), is a member of the scavenger receptor family and is involved in low-density lipoprotein, long chain fatty acid, and phospholipid oxidation[47]. CD36 expression in normal livers is relatively low; however, it was demonstrated that an increase in CD36 expression is related to a HFD, liver steatosis, and NAFLD. Wilson *et al*[48] showed that CD36 deletion in mice fed a HFD reduced their liver lipid contents and induced insulin resistance, while specific induction of CD36 transport in livers led to hepatomegaly and fatty liver[49]. In our study, HRW significantly caused downregulation of CD36 mRNA expression. Suppression of fatty acid uptake and lipid accumulation in Hep G2 cells overloaded with palmitate through CD36 downregulation at the protein level has been reported to have been induced by HRW[33]; however, contrary to our work, the downregulation was only detected at the protein but not the mRNA level[33].

HRW also influenced the expression of other genes related to *de novo* fatty acid synthesis (such as ACC1, FAS), which are upregulated in NAFLD, and the carnitine palmitoyltransferase 1 gene, which is usually downregulated in NAFLD[46]. Another factor affecting NAFLD is adiponectin, which is a peptide mainly released by adipocytes but also can be released by liver cells[50,51]. When adiponectin binds to its receptor (AipoR2) in the liver, it affects intracellular signaling (resulting in a decrease in *de novo* lipogenesis) and FFA influx by causing downregulation of CD36 expression and increase in FFA oxidation[52]. In addition to those metabolic effects, adiponectin also has anti-inflammatory actions *via* inhibition of TNF-α expression through the nuclear factor-κB pathway[53]. In our study, we found that mice that drank HRW showed an increase in the mRNA expression of adiponectin and a decrease in the mRNA expression of TNF-α. The reduction in TNF-α mRNA expression caused by H$_2$ in a similar model has been previously reported[32]. In a double-blinded, human crossover study, drinking HRW for eight weeks also increased adiponectin levels[23].

We did not detect an increase in the mRNA expression of the antioxidant enzymes SOD and CAT in the HFD groups that drank RW or HRW. However, NAFLD mice already have higher levels of SOD and CAT that are required to help neutralize the enhanced HFD-induced reactive oxygen species (ROS) production[46]. Although many H$_2$ studies have reported increased levels of these antioxidants *via* activation of the Nrf2 pathway (reviewed here[19]), in some cases, H$_2$ causes a decrease in their levels because it mitigates their need by attenuating the assault[54-58]. Thus, HRW may have attenuated some ROS by mitigating the HFD-induced damage, which reduced the need for higher antioxidant levels.

In the *in vitro* study, we checked for a residual effect resulting from drinking HRW that could be reflected in hepatocytes overloaded with palmitate *in vitro*. We compared the function of hepatocytes isolated from mice that were fed regular chow and drank HRW to mice that drank regular water. It has previously been demonstrated that hepatocytes respond to palmitate overload by increasing steatosis and cytokine production[59]. Interestingly, hepatocytes from mice that drank HRW showed less fat accumulation and significantly lower mRNA expression of the cytokines TNF-α and IL-6. Therefore, drinking HRW for four weeks before exposure to palmitate appeared to confer hepatocyte protection to palmitate overload *in vitro*, which may partly be explained by HRW's effects on pro-inflammatory cytokine gene expression. This residual effect was sustained for at least 24 h after animals were sacrificed. A similar result was demonstrated in hydrogen preconditioning of *ex vivo* lung grafts, in which it improved post-transplant graft function[60].

Our study demonstrates that the functional water, HRW, may have the potential for the prevention of NAFLD by attenuating HFD-induced increases in fatty tissues, lipid accumulation in the liver, inflammation, and CD36 expression. However, neither EAW nor L-HRW had beneficial protective effects. This result also confirmed the importance of H_2 as the therapeutic agent in these waters. Effectively, all three waters could be considered HRW at different concentrations. EAW still had a negative ORP due to the presence of H_2, whereas L-HRW only contained 0.3 mg/L, and H-HRW contained 0.8 mg/L. However, the mice in the H-HRW group drank nearly three times more water than L-HRW or control. Due to the higher volume of consumed water and higher concentration, the actual dose of H_2 per day in the H-HRW group would have been significantly more (≈ eight times) than what was ingested in the L-HRW group. It is unknown why mice in the H-HRW group ingested nearly 3 times more water. Perhaps the magnesium/water reaction continued to elevate the pH after our initial measurements, which may then result in an increased thirst sensation. Acidic beverages are generally considered more satisfying to quench thirst than those of higher pH. Additionally, we demonstrated that H_2 pretreatment had a residual protective effect by modifying gene expression and conferring future cytoprotection. H_2 is already in use without any reports of known side effects. However, more research is needed to determine the benefits and optimal dosing to be recognized as a conventional therapy for the prevention and treatment for NAFLD.

ARTICLE HIGHLIGHTS

Research background
Nonalcoholic fatty liver disease (NAFLD) is a major growing metabolic health condition. Conventional pharmaceuticals and drugs are not effective in preventing or treating this disease condition. There is a deep interest in searching for novel, safe, and effective methods to prevent and treat NAFLD. For many years there have been many claims about healthy functional waters including alkaline ionized water, also known as electrolyzed reduced water. Some of those claims suggest it is beneficial for obesity and metabolic disturbances. However, there is a paucity of scientific data on this electrolyzed water, but significant noise and claims by commercial companies marketing and selling it. There are untenable claims about the benefits of high pH, alkaline water, or the oft claimed, albeit impossible, microclustering/structuring of the water. Strong claims have also been made regarding the water's negative oxidation-reduction potential. However, electrolysis of water does produce hydrogen gas at the cathode, and H_2 gas does produce a negative oxidation-reduction potential (ORP). Interestingly, biomedical investigations of molecular hydrogen have demonstrated that this small molecule does have therapeutic potential. Perhaps electrolyzed alkaline water does have beneficial effects, but they are not due to its high alkaline pH or other impossible properties, but due to the presence of dissolved H_2 gas produced during electrolysis. Additionally, molecular hydrogen may be an important molecule to combat against NAFLD.

Research motivation
We first wanted to examine if the claimed functional water, electrolyzed alkaline water, could exert therapeutic effects on a mouse model of NAFLD induced by a high-fat diet. We also wanted to compare these results to water with a similar and a greater concentration of molecular hydrogen. This would allow us to know if electrolyzed water has any beneficial effects on NAFLD, and the importance of molecular hydrogen in that water. Additionally, if molecular hydrogen was shown to be therapeutic as previous studies suggest, then it would add to the body of literature lending support for more research.

Research objectives
To determine the effects of H_2 water in preventing NAFLD development under an obesogenic diet. This is the first step to understanding the efficacy of this approach for preventing and treating the disease.

Research methods
In this research mice under an obesogenic high fat diet were used for developing NAFLD. Control mice ingested regular water while the experimental consumed two types of hydrogen-rich water produced in two different ways: *via* electrolysis with an alkaline water ionizer or *via* a chemical reaction between water and metallic magnesium. General parameters as food and water consumption, body weight and composition were measured during the experiment. In the end, livers were sampled for histology and gene expression measuring by means of RT-PCR. In the *in vitro* experiment hepatocytes obtained from mice drinking either regular water or H_2-rich water were exposed to a high-fat environment in order to check the residual protective effects of H_2-rich water.

Research results
This study demonstrated the positives effects of H_2-rich water on NAFLD. Mice fed a high-fat diet that were drinking H_2-rich water showed less weight gain, more lean body tissue, less steatosis, and better liver histology when compared to the control group. It was also demonstrated that electrolyzed water with a high pH, -ORP, but a low H_2 concentration did not result in any improvement. Hepatocytes derived from mice drinking H_2-rich water were more resilient to palmitate overload *in vitro* when compared to hepatocytes obtained from mice drinking regular water. H_2-rich water positively affected the expression of several NAFLD related genes. However, the mechanism of action of H_2-rich water needs further investigation.

Research conclusions
This study demonstrated that the H_2 dissolved in water is the therapeutic agent in functional waters since electrolyzed water with a high pH and a negative ORP did not show any effect on preventing the development of NAFLD. Apparently, H_2 works at a molecular level since it changed the expression of specific genes related to the disease. This study also demonstrates a long-term protective effect of H_2 in an *in vitro* experiment. Functional water rich in H_2 could be a preventive agent in NAFLD. The Therapeutic aspect of H_2 still need to be elucidated and the optimum dosage determined.

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