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Original Article

Treatment with hydrogen molecule attenuates cardiac dysfunction in streptozotocin-induced diabetic mice

Feng Wu [a,1], Yihua Qiu [a,1], Guangming Ye [a,1], Hede Luo [a], Junsong Jiang [b], Feng Yu [a], Wei Zhou [a], Shuai Zhang [a], Jinzhong Feng [a,*]

[a] Department of Cardiology, 98th Military Hospital, Huzhou 313000, China
[b] Department of Stomatology, 98th Military Hospital, Huzhou 313000, China

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ABSTRACT

Introduction: Diabetic cardiomyopathy, a disorder of the heart muscle in diabetic patients, is one of the major causes of heart failure. The aim of present study was to investigate the therapeutic effect of hydrogen molecule on streptozotocin-induced diabetic cardiomyopathy in mice.
Methods: Diabetes was induced in adult male mice by consecutive peritoneal injection of streptozotocin (50 mg/kg/day) for 5 days. Then, they were treated with hydrogen water (1.3±0.2 mg/l) for 8 weeks (four groups, n=83–88 in each group).
Results: Although treatment of diabetic mice with hydrogen water did not significantly affect blood glucose level, it significantly attenuated cardiac hypertrophy and reduced expression of atrial natriuretic factor and β-myosin heavy chain; it alleviated cardiac fibrosis and reduced expression of collagen I and III, transforming growth factor beta, alpha-smooth muscle actin, and osteopontin; it reduced cardiac caspase-3 activity and ratio of bax/bcl-2. Importantly, hydrogen water treatment improved cardiac function in streptozotocin-diabetic mice. Furthermore, it was found that hydrogen water treatment abated oxidative stress, suppressed inflammation, and attenuated endoplasmic reticulum stress in the hearts of streptozotocin-diabetic mice. In addition, hydrogen water treatment suppressed activation of Jun NH2-terminal kinase and p38 mitogen activated protein kinase signaling and nuclear factor κB signaling in the hearts of streptozotocin-diabetic mice.
Conclusion: Treatment with hydrogen molecule attenuated cardiac dysfunction in streptozotocin-induced diabetic mice, which was independent of glycemic control.
Summary: Treatment with hydrogen molecule attenuated cardiac dysfunction in streptozotocin-induced type 1 diabetic mice. Molecular hydrogen could thus be envisaged as a nutritional countermeasure for diabetic cardiomyopathy.

1. Introduction

Cardiomyopathy, a severely disabling complication of diabetes mellitus (DM), is one of the leading causes of increased morbidity and mortality in the diabetic population [1]. Diabetic cardiomyopathy is attributed to myocyte hypertrophy and the changes in the composition of the extracellular matrix with enhanced cardiac fibrosis [2]. This serious and chronic complication has an asymptomatic onset and is characterized by impaired contractility and relaxation of the left ventricle independent of coronary artery disease or hypertension [3,4]. Despite intensive investigations, the molecular mechanisms underlying diabetic cardiomyopathy remain poorly understood.

Accumulating evidence suggests that oxidative stress is considered to be the most important contributor in the development and the progression of diabetic cardiomyopathy [5,6]. Many biochemical pathways associated with hyperglycemia increase the production of free radicals leading to oxidative stress, including glucose auto-oxidation, the polyol pathway, prostanoids synthesis, protein glycation, and the protein kinase C pathway [7]. Given the important role of oxidative stress in the pathogenesis of diabetic cardiomyopathy, there is growing interest in antioxidants used as a compensatory therapeutic approach.

It was reported that several antioxidants such as tempol [6], vitamin E [8], and coenzyme Q10 [9] were able to alleviate cardiomyopathy in diabetic animals. Recently, accumulating reports demonstrated that molecular hydrogen (H_2) is recognized as an emerging therapeutic medical gas for degenerative diseases including Parkinson's disease, metabolic syndrome and osteoporosis [10,11]. In hypertensive rats, injection with hydrogen-rich saline attenuated left ventricular hypertrophy and vascular dysfunction [12,13]. In a streptozotocin (STZ)-induced diabetic rat model,

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* Corresponding author at: Department of Cardiology, 98th Military Hospital, 9 CheZhan Road, Huzhou 313000, China. Tel.: +86 5723269662; fax: +86 5723269651.
E-mail address: fengjz2014@hotmail.com (J. Feng).

[1] The first three authors contributed equally to the work.

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treatment with hydrogen-rich saline attenuated erectile dysfunction [14] and retinopathy [15,16]. H_2 significantly markedly improved glycemic control in STZ-induced type 1 diabetic mice after chronic intraperitoneal and oral administration [17,18]. The aim of this study was to investigate the therapeutic effect of hydrogen molecule in drinking water on STZ-induced diabetic cardiomyopathy in mice.

2. Methods

2.1. Ethical statement

All animals used in this work received humane care in compliance with institutional animal care guidelines and were approved by the Institutional Animal Care and Use Committee of the Institute of Navy. All protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China.

2.2. Materials and animals

All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. C57BL/6 mice (male, 2 months old) were purchased from Vital-Aiver Animal Ltd (Beijing, China) and housed two per cage in a room at controlled temperature (23–25°C), humidity (50%), and lighting (12-h light/dark cycle) with food and water provided ad libitum.

2.3. Animal model

Diabetes was induced in adult male mice (2 months old) by consecutive peritoneal injection of STZ (50 mg/kg/day) for 5 days. Three days after the last injection of STZ, whole blood was obtained from the mouse tail–vein, and random glucose levels were measured using the OneTouch Ultra 2 blood glucose monitoring system (LifeScan, Milpitas, CA, USA). Mice were considered diabetic and used for the study only if they had hyperglycemia (≥15 mmol/l) 3 days after STZ injection. Citrate buffer-treated mice were used as nondiabetic controls (blood glucose <12 mmol/l).

Animals were randomly divided into four groups as follows: control group (n=81), control+hydrogen water (HW) group (n=81), DM group (n=85), and DM+HW group (n=86).

2.4. Hydrogen water

Hydrogen was dissolved in water for 4 h under the pressure of 0.4 MPa, as the method described by Ohsawa et al. [19]. The saturated HW (1.3±0.2 mg/l) was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume for use within 1 week. HW was sterilized by gamma radiation and freshly prepared every week. Each day, HW from the aluminum bag was placed in a closed glass vessel, which ensured that the hydrogen concentration was greater than 0.8 mg/l after 1 day. After induction of diabetes, the control and diabetic groups were fed with HW or normal water for 2 months.

2.5. Measurement of cardiac function by echocardiography

Transthoracic echocardiography was performed noninvasively with a Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada). Mice were lightly anesthetized (0.3 ml of a cocktail containing 100 mg/ml ketamine and 10 mg/ml acepromazine given intraperitoneally) for the duration of the recordings. The heart rate was monitored simultaneously by electrocardiography. Left ventricular (LV) end-diastolic diameter (LVEDD) and end-systolic diameter (LVESD) were used to calculate fractional shortening by the following formula: Fractional shortening (%)=[(LVEDD − LVESD)/LVEDD] ×100%. LV end-diastolic volume, end-systolic volume, and normalized LV mass were calculated as described previously [20].

Ejection fraction was calculated by the following formula:

$$\text{Ejection fraction}(\%) = [(\text{LVEDV}-\text{LVESV})/\text{LVEDV}] \times 100\%.$$

Estimated echocardiographic LV mass was calculated as:

$$\left[(\text{LVEDD} + \text{septal wall thickness} + \text{posterior wall thickness})^3 - \text{LVEDD}^3\right] \times 1.055$$

Here, 1.055 (in mg/mm^3) represents the density of the myocardium.

All echocardiographically derived measures were obtained by averaging the readings of three consecutive beats.

2.6. Blood sample

At the end of the treatment period, the animals were euthanized via an anesthetic overdose (200 mg/kg of ketamine mixed with 40 mg/kg of xylazine delivered by intraperitoneal injection). Then, the blood was collected and the hearts were isolated for further analysis. Serum total cholesterol, triglycerides, and insulin were analyzed using enzymatic methods with an automatic analyzer (JCA-BM8060; JEOL Ltd, Tokyo, Japan).

2.7. Assessment of cardiac function

Hearts were isolated and perfused on a Langendorff system. Myocardial function was then determined as described in previous study [21]. Maximal and minimal first derivatives of force (+dF/dtmax and −dF/dtmin) as the rate of contraction and relaxation were analyzed by PowerLab Chart program (ADInstruments).

2.8. Morphological examination

Hearts were excised, washed with phosphate-buffered saline (PBS), and fixed in 10% formalin. Hearts were then transversely cut close to the apex to visualize the left ventricles. Four-micron sections of the heart were prepared, stained with hematoxylin and eosin, visualized by light microscopy, and photographed. The myocyte outlines were traced and the cell areas measured using “lasso” tool in Adobe Photoshop.

The percentage area of myocardial interstitial fibrosis was calculated in Mallory–Azan-stained sections of each animal [22]. Fibrosis area was measured by the Nikon (Melville, NY, USA) NIS-Elements BR 2.30 program from 10 representative high-power fields of each animal.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was carried out according to the manufacturer's instruction (Cell death detection assay; Promega, Madison, WI, USA). Cardiomyocyte nuclei were quantified by randomly counting 10 fields/section. The apoptotic index (percentage of apoptotic nuclei) was calculated as apoptotic nuclei/total nuclei counted×100.

Random sections were taken and analyzed by two pathologists (Feng Yu and Wei Zhou) blinded to the treatments.

2.9. Real-time quantitative polymerase chain reaction

Total RNA was extracted from left ventricles using the Trizol Reagent (Gibco-BRL) following the manufacturer's instruction. Reactions were performed in a real-time polymerase chain reaction (PCR) thermocycler (iQ5; Bio-Rad, Hercules, CA, USA) using SYBR green as the fluorescence dye. Melting curve analysis was performed to ensure the specificity of the amplicon for each gene. Each sample was run and analyzed in duplicate. Quantification was performed using the samples of known concentrations prepared from amplified DNA fragments extracted and purified from agarose gel for electrophoresis. Using this method, sixth-order linearity was obtained in serial dilutions of the sample. The mRNA expression of the target genes was normalized to the control glyceraldehyde-3-