Hydrogen-Rich Saline Prevents Early Neurovascular Dysfunction Resulting from Inhibition of Oxidative Stress in STZ-Diabetic Rats

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ABSTRACT

Purpose: Diabetic retinopathy (DR) is characterized by increased oxidative and nitrosative stress, both of which lead to neurotoxicity and vascular permeability. Previous studies on a variety of organs indicate that hydrogen-rich saline not only has considerable antioxidant and anti-inflammatory properties, but also suppresses oxidative stress-induced injury. In the present study, we assessed the effects of hydrogen-rich saline on neurovascular dysfunction and oxidative stress in an animal model (rat) of DR.

Materials and Methods: Male Sprague-Dawley rats with streptozotocin (STZ)-induced diabetes mellitus (DM) were injected intraperitoneally with 5 ml/kg hydrogen-saturated (experimental) or plain (control) saline daily for one month. Visual function and blood–retinal barrier (BRB) integrity were evaluated by electroretinography (ERG) and bovine serum albumin (BSA)-fluorescence, respectively. Histological changes in the inner retina were assessed by light microscopy. Biomarkers of oxidative stress, including 4-hydroxynonenal (4-HNE) and 8-hydroxy-2-deoxyguanosine (8-OH-dG), and antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione transferase, were evaluated by ELISA. Synaptophysin and brain-derived neurotrophic factor (BDNF) levels were measured by immunoblotting.

Results: STZ-diabetic rats were marked by clearly reduced b-wave amplitudes and oscillatory potentials, DM-related BRB breakdown and histological changes in the inner retina, all of which were suppressed following treatment with hydrogen-rich saline. Furthermore, hydrogen-rich saline reduced oxidative stress, increased antioxidant enzyme activities and preserved synaptophysin and BDNF levels in the diabetic rat retina.

Conclusions: Based on its inhibition of oxidative stress and up-regulation of anti-oxidative enzymes, we conclude that hydrogen-rich saline is a potentially valuable therapeutic modality for the treatment of DR.

Keywords: antioxidant, diabetic retinopathy, hydrogen, oscillatory potentials, oxidative stress

INTRODUCTION

Diabetic retinopathy (DR), a neurodegenerative disease, causes visual dysfunction in its early stages and is the leading cause of irreversible vision loss in working-age adults.¹² Because many of the complications of diabetes mellitus (DM) are associated with oxidative stress and inflammation,³⁵ reactive oxygen species (ROS) have been suggested as being major factors in the etiogenesis DR.⁶⁻⁷ Considerable evidence indicates that retinal neurons are adversely affected during DM. Many types of...
neuronal deficits have been described for both animal models and patients prior to the onset of vascular compromise. Diabetic blood-retinal barrier (BRB) breakdown, a common feature of DR and a direct result of vision loss, characterizes the early stages of vascular dysfunction in both human and experimental DM. However, whether or not such diabetic neurodegenerative changes can be prevented by suppressing retinal ROS remains to be elucidated. In fact, electroretinography (ERG) oscillatory potentials (OPs), which reflect the functioning of the inner retina, are already abnormal in early DM in humans and animal models. This is partially due to a decrease in levels of synaptophysin, an abundant synaptic membrane protein of the inner plexiform layer (IPL), which plays a critical role in determining OPs. On the other hand, retinal ganglion cells (RGCs) and a subset of inner nuclear layer (INL) amacrine cells are lost to apoptosis in DM, an event that can be attenuated by administration of brain-derived neurotrophic factor (BDNF). Since the relationship between BDNF and oxidative stress in DM is still obscure, evaluating the contribution of ROS in DR may help establish a new therapy.

The underlying mechanisms of DM-associated retinal degeneration are not yet fully elucidated. Furthermore, a definitive therapy for prevention of diabetic retinal degeneration is not available at this time. Most of the ∼4000 antioxidants that have been described to date are electron donors that react with ROS to form harmless end-products. Barriers to utilizing exogenous antioxidants therapeutically include low membrane permeability and high toxicity, both of which would constrain administration of these agents to a narrow window of therapeutic dosage.

Recent studies have identified molecular hydrogen (H₂) as a novel antioxidant with therapeutic effects in a variety of disease models, including ischemia–reperfusion injury, inflammatory disorders and metabolic syndromes. Oharazawa et al. reported that hydrogen-rich saline eye drops reduce retinal ischemia/reperfusion-induced injury caused by transient elevations in intraocular pressure. Wei et al. reported that hydrogen-rich saline protects the retina against glutamate-induced excitotoxic injury in the guinea pig. H₂ selectively neutralizes strong free radicals to reduce oxidative stress with no harmful end-products, easily diffuses into organ and has no known toxic side-effects on the human body.

The goals of the present study were to investigate the efficacy of hydrogen-rich saline for improving neurovascular dysfunction in an animal (rat) model of DR and to determine if its protective effects, if any, occur via inhibition of oxidative stress and up-regulation of anti-oxidative enzyme activities in the diabetic retina.

MATERIALS AND METHODS

Animals and Induction of DM

All animal procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology guidelines and approved by the local ethics committee of the Second Military Medical University, Shanghai, China. Male Sprague-Dawley rats (∼180–200 g, six-weeks old) were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences, China. DM was induced in by a single intraperitoneal injection of streptozotocin (STZ) (60 mg/kg body weight) in sodium citrate buffer, pH 4.5 (Sigma-Aldrich, St. Louis, MO). Only STZ-treated rats with blood glucose levels of >13.9 mmol/l (250 mg/dl) at both 24 h and one week after STZ injection were included in the study.

Administration of Hydrogen-Rich Saline

After one week of STZ treatment, animals were given daily intraperitoneal injections of either hydrogen-rich (5 ml/kg) (experimental) or plain (control) saline for one month. Purified hydrogen was dissolved in normal saline for two hours under an internal pressure of 0.4 MPa. The physiological concentration is 1.73 ml hydrogen per 100 ml saline (average, 0.86 mmol/l). Saturated hydrogen-rich saline was stored at 4°C in an aluminum bag with no dead volume under atmospheric pressure. Hydrogen-rich saline was freshly prepared every week to ensure a uniform concentration of >0.6 mmol/l. Age-matched, non-diabetic rats (controls) were given sodium citrate in place of STZ. Rats were grouped as non-DM (n = 30), i.e., sodium citrate with no treatment; DM (n = 30), i.e., saline only and DM + H₂ (n = 30), i.e., hydrogen-rich saline only. Body weights and blood glucose levels were analyzed at one week and one month after induction of DM. At the end of experiments, eyes were resected from deeply anesthetized animals.

Electroretinography

For ERG, rats were dark-adapted for 30 min and anesthetized by an intraperitoneal injection of ketamine hydrochloride (70 mg/kg). Pupils were dilated with 1% tropicamide. The cornea was anesthetized by topical administration of 0.4% oxybuprocaine hydrochloride. Animals were placed on a heating pad to maintain a body temperature of 37 ± 0.5°C during the procedure, and remained there until they recover completely from anesthesia. A silver electrode
was placed on the cornea, with Vidisic gel as a conducting medium. A reference electrode was placed on the central forehead and a ground electrode on the right ear. A black patch was used to cover the contralatera1 eye during the test. Light stimuli were generated by a Grass PS 22 photic stimulator (UTAS-E 2000) (LKC Technologies, Gaithersburg, MD). Impedance-matched neuroelectric signals were produced by a unity gain preamplifier, and signals were further differentially amplified using appropriate bandpass settings. ERG studies were performed before and after the test. A- and b-waves, and OP amplitudes of scotopic ERG were measured using a series of four bright white flashes applied at 15-s intervals.22

**BRB Function**

Thirty minutes after tail vein injection of bovine serum albumin (BSA)-Alexa-Fluor 488 conjugate (100 mg/kg) (Molecular Probes-Life Technologies, Grand Island, NY), animals were sacrificed and the eyes enucleated. Following enucleation, eyes were imbedded in OCT medium and snap-frozen in liquid nitrogen. Plasma was assayed for BSA fluorescence using a plate reader (excitation 370 nm, emission 460 nm), and a standard curve for BSA fluorescence was established in normal rat serum. Serial sections (10 μm) and imaging (200 μm2) of retinal non-vascular areas allowed detection of extravasation of BSA-fluorescence. Average retinal fluorescence intensity was calculated and normalized to a control retina and to plasma fluorescence intensity for each animal.

**Histopathology**

For histopathology, enucleated eyeballs were fixed in 4% paraformaldehyde and imbedded in paraffin. Tangential sections (6 μm) were cut through the pupil and optic nerve – passing through the temporal ora serrata, optic nerve and nasal ora serrata – and stained with hematoxylin and eosin (H&E). Using IMAGE program, thicknesses of the IPL, INL and outer nuclear layer (ONL) were measured at four points in the posterior retina – two on either side of the optic nerve, each 200 and 500 μm apart. To further standardize for any bias introduced by skewed sectioning angles and to more precisely compare distances between different sections, IPL and INL thicknesses were normalized to that of the ONL at each point. Previous reports indicate that ONL thickness remains unchanged in the diabetic retina, although inner retinal thickness and, consequently, total retinal thickness is reduced in DM.4 Numbers of neurons in the ganglion cell layer (GCL) were determined by counting and averaging nuclei in three sections from each sample.

**ELISA**

Three rat retinas from each group were homogenized in 0.3 ml Tris buffer containing 10 mmol/l Tris-HCl (pH 7.6), 100 mmol/l NaCl, 1 mmol/l EDTA, 1% (w/vol) Triton X-100 and protease inhibitor cocktail. Lysates were then sonicated and centrifuged at 10,000 g for 10 min to remove large particles. Supernatants were used for spectrophotometric measurements of superoxide dismutase (SOD), glutathione peroxidase (GSHPx), glutathione reductase (GSSGRed) and glutathione transferase (GSHTrans) activities by ELISA.

**Western Blot Analysis**

Isolated retinas were placed into lysis buffer containing 10 mmol/l Tris-HCl (pH 7.6), 100 mmol/l NaCl, 1 mmol/l EDTA, 1% (w/vol) Triton X-100 and protease inhibitor cocktail. Lysates were then sonicated and centrifuged for 15 min at 10,000 g at 4°C. Supernatants were collected and protein concentrations determined using the Bradford method. Each sample was separated by SDS-PAGE and electroblotted onto a polyvinylidene fluoride membrane. After blocking, membranes were blotted overnight at 4°C with anti-synaptophysin (1 : 2500), anti-BDNF (1 : 2500) and anti-β-actin (1 : 2500) antibodies (Sigma-Aldrich). After washing, blots were incubated for one hour at room temperature with horseradish peroxidase-conjugated secondary antibody (1 : 2000) (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were re-probed with β-actin to confirm equal loading. Primary antibodies were visualized by enhanced chemiluminescence. Bands were semi-quantified using densitometry.

**Statistical Analysis**

For all analyses, the individual performing the analysis was blinded to study group. All quantitative data are expressed as means ± SD. The significance of differences between means was verified by analysis of variance (ANOVA), followed by Tukey’s test. For analysis of cell count results, a non-parametric Kruskal–Wallis ANOVA was used, followed by Dunn’s test. Statistical significance was set at p < 0.05.

**Hydrogen-Rich Saline and Diabetic Retinopathy**

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RESULTS

Body Weight and Blood Glucose Levels

All diabetic rats, whether treated with hydrogen-rich saline or saline only showed significant decreases in body weight and significant increases in blood glucose compared with age-matched non-diabetic controls (Table 1). Daily treatment with hydrogen-rich saline for one month did not significantly change these metabolic variables in diabetic rats.

Visual Function

ERG was used as an objective method for evaluating the functional status of the inner and outer retina. To determine the effect of daily hydrogen-rich saline treatment on visual function in DM, we used full-field flash ERG (Figure 1a and b). Compared with those in age-matched controls, amplitudes of b-waves and OPs in STZ-induced diabetic rats with no hydrogen-rich saline treatment had significantly decreased by the end of the experimental period. However, amplitudes in diabetic rats administered hydrogen-rich saline were not reduced and were markedly higher than those of untreated STZ-induced diabetic rats (Figure 1c). In other words, reduction of b-wave and OP amplitudes was successfully prevented by hydrogen-rich saline treatment, suggesting that daily hydrogen-rich saline treatment protected the inner and outer retina from the functional deficits typically induced by DM.

BRB Breakdown and Histological Changes

Increased microvascular permeability is an early event in DM, and we observed dramatic leakages in STZ-induced diabetic rats (Figure 2a). Hydrogen-rich saline suppressed DM-related BRB breakdown by 30% in diabetic rats compared with non-DM rats. We also analyzed retinas of one-month DM rats treated with hydrogen-rich saline or saline only. Thicknesses of retinal layers were measured in paraffin sections, and each thickness normalized to ONL thickness (Figure 2b). The normalized thickness of the IPL, where neurites and synapses of inner retinal cells are present, was significantly reduced in the retina of one-month DM rats. However, the change had been clearly circumvented in retinas of hydrogen-rich saline-treated DM rats. The normalized INL thickness was also reduced, suggesting that the number of INL cells had decreased. Moreover, RGC numbers had also declined in the retinas of one-month DM rats treated with saline only (Figure 2c). Cell loss was significantly suppressed by administration of hydrogen-rich saline. These data indicate that DM-induced decrease in inner retinal cells and their neurites is prevented by hydrogen-rich saline.

Levels of 4-HNE and 8-OHdG

To demonstrate retinal ROS, we used ELISA to examine the levels of two oxidative stress markers – 4-HNE

TABLE 1. Body weights and blood glucose concentrations of four-week STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Blood glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-DM</td>
<td>30</td>
<td>289.9 ± 5.2</td>
<td>5.2 ± 1.3</td>
</tr>
<tr>
<td>DM</td>
<td>30</td>
<td>171.9 ± 14.5**</td>
<td>21.7 ± 3.1**</td>
</tr>
<tr>
<td>DM + H2</td>
<td>30</td>
<td>175.2 ± 16.2**</td>
<td>19.8 ± 4.1**</td>
</tr>
</tbody>
</table>

Values are means ± SD. **p < 0.01 versus non-DM.

FIGURE 1. (a and b) Representative ERG tracings at one month in STZ-induced diabetic rats and (c) amplitudes of b-wave and OP. At one month after onset of DM, b-wave amplitudes and OPs of the DM group were clearly lower than those of the non-DM group. These reductions were successfully suppressed by hydrogen-rich saline treatment. Values are means ± SD, n = 6. *p < 0.05, **p < 0.01.
and 8-OHdG (Figure 3). As expected, retinal concentrations of 4-HNE and 8-OHdG in the DM group were increased dramatically compared with the non-DM group. However, increases in 4-HNE and 8-OHdG (1.12 ± 0.11-fold and 1.25 ± 0.13-fold, respectively) were suppressed in the DM + H₂ group compared with those (1.56 ± 0.21-fold and 2.12 ± 0.27-fold, respectively) of the DM group. Therefore, DM-induced oxidative stress in the retina is effectively reduced by hydrogen-rich saline.

Antioxidant Enzyme Activities

To elucidate the effect of hydrogen-rich saline on antioxidant enzyme activation, we performed ELISA analyses of SOD, GSGrRed, GSHPx and GSHTrans activities, all of which were significantly decreased (57.1%, 66%, 88.9% and 54.5%, respectively) in DM rats compared with controls (Figure 4). However, reduction of these antioxidant enzyme activities was significantly inhibited by hydrogen-rich saline-treated retinas compared with vehicle-treated retinas. Thus, DM-induced reduction of antioxidant enzyme activities is suppressed by hydrogen-rich saline.

Levels of Synaptophysin and BDNF

We analyzed synaptophysin, a major synaptic vesicle protein, which plays a critical role in the cellular source of OPs. Immunoblot analysis showed that synaptophysin levels decreased to 0.71 ± 0.06-fold in diabetic retinas compared with controls (Figure 5a and c). Administration of hydrogen-rich saline from the very onset of DM preserved synaptophysin levels in diabetic retinas compared with controls (0.94 ± 0.09-fold in DM + H₂ group compared with non-DM group; p > 0.05). Since DM causes decreases in BDNF, which is one of the major trophic factors in...
retinal neurons, we also measured BDNF by immunoblot analysis in one-month DM retinas (Figure 5b and d). We found that DM-associated decreases in BDNF were attenuated by administration of hydrogen-rich saline (0.52 ± 0.1-fold in DM group versus 0.816 ± 0.141-fold in DM + H2 group; *p < 0.05).

DISCUSSION

Our results demonstrate that regular administration of hydrogen-rich saline not only improves retinal electrophysiological dysfunction in early stage STZ-diabetic rats, but also inhibits increased BRB permeability of the inner retina. Previous studies have indicated that ERG response amplitudes of STZ-induced diabetic rats are lower than those of control rats as early as two weeks after onset of DM. The ERG b-wave is known to be related to Müller cell, bipolar cell, amacrine cell and ganglion cell function.23,24 Amacrine cell-generated OPs consist of four-to-six wavelets that are present on the rising phase of the b-wave.25 OPs arise in the IPL, and are reflective of inner retinal functioning. Therefore, reduced amplitudes in OPs and b-waves are commonly noted in the early stage of DM, even before the onset of the vascular compromise of DR. ERG specifically demonstrates retinal functions, providing an early warning of retinal abnormalities in DM, even before visible alterations are detectable.26,27 In our study, as expected, amplitudes of b-waves and OPs in STZ-induced DM rats were significantly reduced compared with those of non-diabetic controls. In addition, we found that hydrogen-rich saline inhibited the reduced amplitudes in OPs and b-waves. DR is frequently complicated by macular edema, resulting from BRB breakdown. The BRB plays a critical role in partitioning retinal neural elements from the circulation, consequently protecting them from circulating inflammatory cells and their cytotoxic products. In our evaluation of BRB integrity, we found BRB breakdown to be significantly suppressed by administration of hydrogen-rich saline. This suggests that hydrogen-rich saline has a protective effect on tight junctions, thereby ameliorating BRB function in diabetic rats. Moreover, structural changes in the retina of STZ-induced diabetic rat were prevented by the constant administration of hydrogen-rich saline.

Chronic hyperglycemia leads to sorbitol pathway hyperactivity, non-enzymatic glycation/glycoxidation and protein kinase-C activation, all of which processes have been implicated in the pathogenesis of diabetic complications, including DM.28-30 One of the most dangerous consequences of DM-associated “glucose toxicity” is oxidative stress resulting from increased production of ROS. Thus, we evaluated two oxidative stress-associated biomarkers – 4-HNE and 8-OH – and found that hydrogen-rich saline dramatically decreased their levels in the diabetic retina, with no associated alterations in body weight or blood pressure.
glucose levels. It appears, therefore, that administra-
tion of hydrogen-rich saline in DR protects lipids from
peroxidation and DNA from oxidation. Previous
studies on diabetic rats have indicated that there is
increased retinal oxidative stress, as demonstrated by
the accumulation of lipid peroxidation end-products.
Therefore, adequate levels of key antioxidant
enzymes, responsible for scavenging free radical, are
essential for redox homeostasis. 31–33 Our results sug-
gest that up-regulation of anti-oxidative enzymes,
including SOD, GSHPx, GSSGRed and GSHTrans,
successfully builds an antioxidant defense under
conditions of high glucose-induced free radical gen-
eration in diabetic rats treated with hydrogen-rich
saline.

Lack of synaptophysin causes decreased synaptic
vesicles, thus disturbing neurotransmitter release and
synaptic network activity. 34 Furthermore, preserved
synaptic activity promotes cell survival and protection
of vision via increased neuronal levels of the
intracellular calcium ions triggered by neuronal
electric stimuli. 35,36 Our results show that
synaptophysin levels are preserved even when ROS
levels are decreased by hydrogen-rich saline. Hence,
visual function is preserved when normal synaptophysin
levels are maintained by inhibition of oxidative stress.

We also found that reduction of BDNF was atten-
uated by hydrogen-rich saline, partially due to exces-
sive oxidative stress. Maintenance of BDNF levels by
hydrogen-rich saline may also have been due to:
preparation of synaptophysin levels and subsequent
neuronal synaptic activity. 37 BDNF not only regulates
neurotransmitter release and neuronal activity, but
also promotes survival of inner retinal cells. 38
Reduction of BDNF in the diabetic retina may be
responsible for the visual impairment indicated by
ERG. Oxidative stress-mediated BDNF reduction may,
to some extent, have contributed to the subsequent
and significant histological changes of the inner
retina. Structural changes in the inner retina were
clearly observed in two-month diabetic rats. This
long-term effect may be caused by DM-activated
multiple signals. However, the ability of hydrogen-
rich saline to inhibit (i) generation ROS, (ii) up-
regulation of anti-oxidative enzyme activities, (iii)
maintenance of synaptic activity and (iv) preserva-
tion of BDNF production may be responsible for
survival of neurons in the inner retina, by interrupting
the positive feedback loop for progressive
neurodegeneration.

It is well known that the retina has high levels of
polyunsaturated fatty acids as well as the highest
oxygen consumption per gram of tissue in the body.
The retina is also exposed to light, thus rendering it
highly susceptible to oxidative stress and lipid
peroxidation. 39 DM disturbs retinal homeostasis by
preserving glial cell activation, reducing neurotrophic
support and increasing inflammation. All of
these processes accelerate apoptosis and BRB
dbreakdown, resulting in macular edema and
neovascularization. 40

Clinically, people with DR do not often notice
changes in their vision during the early stages of
disease. However, with progression, DR often leads to
irreversibly impaired vision. Treatment for DR is
presently limited to laser photocoagulation, an inva-
sive procedure with serious side-effects. The lack of
approved pharmacological treatment for DR makes it
essential to identify effective therapeutic pharma-
logic approaches. Diatonic hydrogen, a novel antiox-
idant, has several potential advantages compared
with more commonly used pharmaceutical drugs. 15
High concentrations of hydrogen are well tolerated
and have few systemic side-effects. 19 Accordingly,
hydrogen-rich saline is suitable for the treatment of
prolonged diseases, such as DR. Hydrogen diffuses
very rapidly across the BRB to reach subcellular
compartments. While it selectively scavenges the
most aggressive hydroxyl radical (•OH), it is far less
effective against the superoxide anion and hydrogen
peroxide, both of which play physiological roles in
stimulating generation of endogenous antioxidant
enzymes. Consequently, the therapeutic potential of
hydrogen as a non-toxic, convenient, safe and
effective antioxidant holds enormous promise for
treatment of oxidative stress-related diseases such as DR.

It is clear that the visual impairment and BRB
dbreakdown occurring in early DM was obviated by
administration of hydrogen-rich saline. We dem-
strated that hydrogen-rich saline ameliorates experi-
mental DR via (i) inhibition of oxidative stress, (ii)
up-regulation of anti-oxidative enzyme activities and (iii)
preservation of neuroprotective factors and, thus, has
promise as a highly useful therapeutic agent. DR, as a
chronic metabolic disease, requires long-term treat-
ment. Therefore, the advantages of fewer systemic
side-effects, easy diffusion and low cost, render
hydrogen-rich saline a potentially useful, new therape-
utic pharmacologic strategy for reducing the retinal
damage caused by DM.

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REFERENCES


36. Ikegami K, Koike T. Membrane depolarization-mediated survival of sympathetic neurons occurs through both