Protective Effects of Hydrogen Saline on Diabetic Retinopathy in a Streptozotocin-Induced Diabetic Rat Model

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Abstract

Purpose: Diabetic retinopathy is the leading cause of blindness in the working population of the developed countries and also a significant cause of blindness in the elderly. This study aimed at examining the protective effect of H2 saline on diabetic retinopathy in a streptozotocin-induced diabetic rat model.

Methods: Sprague–Dawley male rats were divided into 3 groups as follows: (1) nondiabetic control group (non-DM control); (2) diabetic control group (DM control); and (3) diabetic rats receiving H2 saline therapy (DM H2 saline). Rats in DM H2 saline group were intraperitoneally injected with H2 saturated saline (5 mL/kg) every day for 4 weeks. Retinal vascular permeability was assessed by measuring Evans blue leakage into the retina. Retinal apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining and measuring caspase-3 activity. Retinal thickness was observed by hematoxylin and eosin staining.

Results: Our results showed that H2 saline treatment could depress the caspase activity, reduce the retinal apoptosis, and vascular permeability. The H2 saline could also prominently attenuate the retinal parenchyma thickening that resulted from diabetic retinopathy.

Conclusions: Our preliminary studies indicated that H2 saline may have potentials in the clinical treatment of diabetic retinopathy.

Introduction

Diabetic retinopathy is the leading cause of blindness in the working population of the developed countries and also a significant cause of blindness in the elderly. The overall prevalence of retinopathy among patients with diabetes is about 26%. It has been known that diabetic retinopathy is the result of microangiopathy caused by diabetes, but the pathogenesis is still poorly understood.

Diabetic blood–retinal barrier (BRB) breakdown has been known to be a direct result of vision loss, and it could characterize early stages of vascular dysfunction in both human and experimental diabetes. Some researches considered that retinopathy was related with oxidative stress. Researches on samples of diabetic retinopathy from eye banks found that apoptosis of retinal vessel perithelial cells and endothelial cells obviously increased. In induced diabetic rats, gene expression associated with apoptosis in retinal cells up-regulated 3 days after being induced. More and more researches verified that function of retinal neurons and gliacyte changed earlier than that of vascellum in patients with diabetes. Hydrogen gas has been found to have protective effects in the brain, heart, and liver after ischemia-reperfusion damage. Hydrogen gas can neutralize free radicals and reduce oxidative stress. Our laboratory has found that hydrogen saline has neuroprotective effects in the neonatal hypoxia-ischemia rat model. However, a few researchers investigated its effects on diabetic retinopathy. Since hydrogen gas is not safe and convenient for clinical use, in this study, we tested the effects of intraperitoneal application of saturated hydrogen saline (H2 saline) on diabetic retinopathy in a streptozotocin-induced diabetic rat model.

Methods

Experimental groups

All experimental procedures were performed in adherence to the Association for Research in Vision and Ophthalmology...
statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines on Animal Care approved by Second Military Medical University Animal Protocol Management and Review Committee. Male Sprague–Dawley rats, weighing ~200 g, were used in this study. Rats were randomly divided into 3 groups as follows: (1) non-diabetic control group (non-DM control, n=40); (2) diabetic control group (DM control, n=80); and (3) diabetic rats receiving H2 saline (DM H2 saline, n=80). The Animal and Ethics Review Committee at the Second Military Medical University evaluated and approved the protocol used in this study.

**Induction and maintenance of diabetes mellitus with streptozotocin**

After being anesthetized by an intraperitoneal injection of ketamine (50 mg/kg), rats in DM control and DM H2 saline groups were induced with a single 65 mg/kg intraperitoneal injection of streptozotocin dissolved in citrate buffer, pH 4.5. Rats in non-DM group received an equivalent amount of citrate buffer alone. Twenty-four hours later, rats with blood glucose levels higher than 250 mg/dL were declared diabetic. Blood glucose levels were measured every week until they were killed. The general state of all the rats was monitored during the study.

**H2 saline therapy protocols**

Purified H2 was dissolved into normal saline for 2 h under 0.6 MPa. There is 1.73 mL hydrogen in per 100 mL H2 saturated saline media, and the average concentration of hydrogen in this H2 is 0.86 mM. H2 saturated saline was then administered by an intraperitoneal injection (5 mL/kg) immediately after induction in DM H2 saline rats within 2 h. Then, from the second day on, rats received H2 saline in the same manner till they were killed.

**Hematoxylin and eosin staining and observation**

After sacrifice, eyeballs were removed and processed for histopathology, including fixation in 4% paraformaldehyde, embedding in paraffin, and hematoxylin and eosin (HE) staining by standard techniques. For HE staining, the 4-μm sections were hydrated in 1% toluidine blue at 50°C for 20 min. After rinsing with double distilled water, they were dehydrated and mounted with permount. The retinas from each animal were captured, and Imaging-Pro-Plus was used to perform quantitative analysis of cell numbers. The integral cells in the retinal ganglial cells (RGCs) layer of each sample were counted in 10 highpowered fields (HPF, 40×); 3 sections per eye were averaged, and there were 6 rats in each group.

**Measurement of BRB breakdown using Evans blue**

After being anesthetized by an intraperitoneal injection of 2% pentobarbital (50 mg/kg), the left vena iliaca and left iliac artery of rats were separated, then the left iliac artery was cannulated with polyethylene tubing, and filled with heparinized saline (250 units heparin/mL saline). Evans blue (30 mg/mL) was injected through the vena iliaca at a dosage of 45 mg/kg within 10 s, and then 1 mL normal saline was injected to wash the colorant in both pinhead and vein. Two minutes later, 0.2 mL blood was drawn from the iliaca to obtain the initial Evans blue plasma concentration. At 15 min intervals, 0.1 mL blood was drawn from the right iliac artery up to 2 h after injection of Evans blue to obtain the time-averaged Evans blue plasma concentration. Two hours later, the chest cavity was opened, and 0.2 mL blood was drawn from the vena cava to obtain the final Evans blue plasma concentration. Subsequently, the rat heart was perfused for 2 min at 37°C with citrate buffer (0.05 M, pH 3.5). After perfusion, both eyes were enucleated, and retinas were carefully dissected. After measurement of the retinal wet weight, retinas were dried in a Speed-Vac (37°C) for about 2 h. The Evans blue was extracted by incubating every single retina in 150 μL formamide for 18 h at 70°C. The extract was centrifuged at a speed of 70,000 r.p.m. for 1 h at 4°C. A total of 60 μL of supernatant was used for triplicate spectrophotometric measurements. A background-subtracted absorbance was determined by measuring each sample at 620 nm, the absorbance maximum for Evans blue, and 740 nm, the absorbance minimum. The concentration of Evans blue in each extract was determined from a standard curve of dye in formamide. The BRB breakdown was calculated and expressed as μL plasma/g retinal dry weight/h.

**TUNEL staining**

TUNEL staining was performed on paraffin-embedded sections by using the in situ cell death detection kit (Roche). According to standard protocols, the sections were dewaxed and rehydrated by heating the slides at 60°C. Then, these sections were incubated in a 20 μg/mL proteinase K working solution for 15 min at room temperature. The slides were rinsed thrice with phosphate-buffered saline (PBS) before they were incubated in TUNEL reaction mixture for 1 h at 37°C. The area around the sample was dried, and Converter-AP was added to the samples for 1 h at 37°C. After rinsing with PBS (5 min, 3 times), sections were colored in the dark with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. To compare the TUNEL-positive cells in each group, the TUNEL-positive cells in the RGC layer of each sample were counted in 10 HPFs (40×); 3 sections per eye were averaged, and there were 6 rats in each group.

**Cell counts of RGCs**

The number of RGCs in the retina was counted with a fluorescent microscope. More than 3 retinal preparations were analyzed for each sample. The data were represented as the number of cells per high-power field. Each group contained at least 8 rats for obtaining the mean density.

**Caspase-3 activity assay**

The activities of caspase-3 were measured with caspase-3/CPP32 Fluorometric Assay Kit (BIOVISION Research Products). Briefly, retinal samples were homogenized in ice-cold cell lysis buffer and kept at 4°C for 1 h. Retinal homogenate was centrifuged at 12,000 g for 15 min at 4°C. The treated samples were liquated and stored at –80°C until use. Protein content was measured by Enhanced BCA Protein Assay Kit. Equal amounts of the protein samples were incubated in a 96-well plate with 50 μL of 2× Reaction Buffer. Reactions were initiated by adding 5 μL of the 1 mM Ac-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethyl coumarin) (DEVD-AFC) substrate. After incubation in the dark at 37°C, the plate was read in a flurometer equipped with a 400 nm excitation filter and 505 nm emission filter.
Data analysis

All quantitative data were expressed as mean ± standard deviation. The significance of differences between means was verified by analysis of variance (ANOVA) followed by Tukey test. For analyzing the results of cell counting, a non-parametric Kruskal–Wallis ANOVA was used followed by Dunn’s test. Differences were considered statistically significant if $P < 0.05$.

Results

Induction of diabetes

The blood glucose levels heightened from the day after being streptozotocin induced, and retained 400–700 mg/dL during the next 4 weeks in both DM control and DM H$_2$ saline rats (Fig. 1).

Body weight

Body weight was decreased by 9.5% after 1 week in DM control rats compared with non-DM control rats, and it was 59.7% of non-DM control rats after 4 weeks. The body weight in DM H$_2$ saline rats was almost the same as DM control rats (Fig. 2).

Histopathologic examination using HE staining

Light-microscopic examination using HE-stained retinal sections revealed the number of RGCs reduced prominently in DM control rats compared with non-DM control rats and DM H$_2$ saline rats ($P < 0.01$) (Fig. 3).

Quantification of BRB breakdown using Evans blue

After 4 weeks, the retinal vascular leakage (permeability) of Evans blue (mean ± standard error) in non-DM control rats, DM control rats, and DM H$_2$ saline rats was 7.8 ± 2.9, 16.3 ± 3.8, and 11.1 ± 3.3 µL plasma/g retinal dry weight/h, respectively, demonstrating significant differences between the values of DM control and DM H$_2$ saline rats, and between the values of non-DM control and DM control rats ($P < 0.01$) (Fig. 4).

TUNEL staining

The representative graphs with different magnifications of TUNEL staining in samples were collected after 4 weeks. At higher magnification, the nuclei of cells were clearly stained in retina. The results indicated that TUNEL-positive cells were markedly increased in DM control rats’ retina, whereas the administration of DM H$_2$ saline dramatically reduced the number of TUNEL-positive cells ($P < 0.01$). A few TUNEL-positive cells were identified in samples from non-DM control rats (Fig. 5).

Caspase-3 activity

The activity of caspase-3 was measured after 4 weeks. The ratio relative to non-DM control rats was 1.64 ± 0.16 and 3.21 ± 0.18 in DM control and DM H$_2$ saline rats. The H$_2$ saline could significantly depress the activity of caspase-3 in retina ($P < 0.01$) (Fig. 6).

Discussion

This study investigated the effect of H$_2$ saline on diabetic retinopathy in a streptozotocin-induced diabetic rat model. We examined the histopathologic changes of retinal sections and quantitatively assessed the effect of H$_2$ saline on BRB breakdown by direct measurement of BRB permeability using Evans blue. Markedly increased retinal vascular permeability was observed in DM control rats compared with non-DM control rats and DM H$_2$ saline rats. The H$_2$ saline could significantly depress the activity of caspase-3 in retina ($P < 0.01$) (Fig. 6).
neovascularization of iris, loss of retinal capillary endothelial and mural cells, focal basement membrane thickening, and variations in capillary diameter had been found in streptozotocin-induced diabetic rats. Ultimately, fusiform microaneurysms appeared. Some other researchers have described the breakdown of the BRB occurring in the streptozotocin-treated rats, and it is widely known that apoptosis of retinal cells is also an evident phenomenon in diabetic retinopathy. Studies on the relationship between diabetes and oxidative stress showed that diabetes could increase oxidative stress, and the elevated oxidative stress played an important role in the pathogenesis of diabetic complications. It is supposed that increased oxidative stress in diabetes promotes the development of neuropathy, nephropathy, myocardial injury, and retinopathy. Autooxidation of glucose, breakdown in redox balances, decreased tissue concentrations of low-molecular-weight antioxidants such as reduced glutathione and vitamin E, and impaired activities of antioxidant defense enzymes such as catalase and superoxide dismutase may all be the possible resources of oxidative stress. Oxidative stress-induced biochemical changes facilitate both structural and functional changes in retinal microvasculature. Structural changes range from basement membrane thickening, and microvascular cell loss.

Retina has a high content of polyunsaturated fatty acids and the highest oxygen uptake and glucose oxidation compared with other tissue, which make retina more susceptible to oxidative stress. Reactive oxygen species (ROS), generated through high glucose metabolism, are considered an important mediator between elevated glucose and any other metabolic abnormalities in the development of diabetic complications.

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FIG. 3. Hematoxylin and eosin (HE) staining and the retinal ganglial cells (RGCs) counts. Twelve rats in each group were killed 4 weeks after streptozotocin induction, and the representative sections were shown here. (A) Light-microscopic examination of HE-stained retinal sections. Arrowhead showed the RGCs. (B) The counts of RGCs. The number of RGCs reduced prominently in DM control rats compared with non-DM control rats and DM H₂ saline rats (**P < 0.01) (n = 10 in non-DM; n = 20 in DM; n = 20 in DM H₂ saline).

FIG. 4. Evans blue-albumin permeation. After 4 weeks, the retinal vascular leakage of Evans blue in non-DM control rats, DM control rats, and DM H₂ saline rats were 7.8 ± 2.9, 16.3 ± 3.8, and 11.1 ± 3.3 μL plasma/g retinal dry weight/h, respectively, demonstrating significant differences between the values of DM control and DM H₂ saline rats, and between the values of non-DM control and DM control rats (**P < 0.01) (n = 10 in non-DM; n = 20 in DM; n = 20 in DM H₂ saline).
Neutralizing free radicals, especially the hydroxyl radical (•OH) and peroxynitrite anion (ONOO−), is the key pathway through which hydrogen can exhibit neuroprotective effect. Hyperglycemia increases oxidative stress, and ROS and reactive nitrogen species, such as •OH and ONOO−, appear to play a key role in cell death. In recent years, Oh-sawa et al. found that molecular hydrogen could selectively reduce (scavenge) •OH and ONOO− in cells and exhibit therapeutic antioxidant activity in the rat middle cerebral artery occlusion model in which the ability of reducing or eliminating •OH and ONOO− of hydrogen may be responsible for the protective effect. Besides, some other Japanese researchers reported that hydrogen gas provides protective effects on ischemic insult in both brain and liver by selectively scavenging •OH and ONOO−. It is also reported that oral administration of H2 saline could prevent the stress-induced decline in both learning and memory,
which were caused by chronic physical restraint. In the previous studies of our laboratory, we found that inhalation of 2% hydrogen also provided the protective effects in the cerebral hypothia-ischemia neonatal model.

In this study, we have proved that H2 saline could significantly reduce both retinal vascular leakage and retinal cells apoptosis in a streptozotocin-induced diabetic rat model. H2 saline may have potentials as a safe and effective treatment for the prevention of vision loss in diabetes, which also has an important impact on public health.

Author Disclosure Statement

No competing financial interests exist.

References
