Protective effect of hydrogen-rich medium against high glucose-induced apoptosis of Schwann cells in vitro

YANG YU¹,², XIAOYE MA¹,², TAO YANG¹,², BO LI²,³, KELIANG XIE¹,², DAAQUAN LIU⁴, GUOLIN WANG¹,² and YONGHAO YU¹

¹Department of Anesthesiology, General Hospital of Tianjin Medical University; ²Anesthesiology Research Institute of Tianjin, Tianjin 300052; ³Department of Anesthesiology, The Second Hospital of Tianjin Medical University, Tianjin 300210; ⁴Institute of Acute Abdominal Disease Research Center of Tianjin Integrated Chinese and Western Medicine, Tianjin 300102, P.R. China

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Abstract. Diabetic peripheral neuropathy (DPN) is considered to be one of the most prevalent and life threatening microvascular diabetic complications. DPN affects up to 50% of patients with diabetes mellitus and there are currently no efficacious therapeutic strategies available for its treatment. Previous studies have reported that oxidative stress and poly(ADP-ribose) polymerase-1 (PARP-1) may be unifying factors for hyperglycemic injury. The aim of the present study was to investigate the protective effects of hydrogen-rich medium (HM) on high glucose (HG)-mediated oxidative stress, PARP-1 activation and the apoptosis of Schwann cells (SCs) in vitro. The cells were divided into different groups, and were treated for 48 h. Cell viability and apoptosis were evaluated using Cell Counting kit-8 and annexin V/propidium iodide assays, respectively. The concentrations of 8-hydroxy-2-deoxyguanosine (8-OHdG) and peroxynitrite (ONOO⁻) were detected using an enzyme-linked immunosorbent assay. The presence of intracellular oxygen free radicals was confirmed using flow cytometric analysis. Colorimetric assays were performed to determine the activity of caspase-3, and western blotting was performed to detect the protein expression levels of PARP-1, cleaved PARP-1, PAR, apoptosis-inducing factor (AIF), B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein. HG was found to induce severe oxidative stress and promote the caspase-dependent and caspase-independent apoptosis of SCs. Treatment with HM inhibited HG-induced oxidative stress by suppressing hydroxyl and ONOO⁻ production, levels of 8-OHdG, caspase-3 activity and apoptosis in the SCs. Furthermore, treatment with HM downregulated the HG-induced release of PAR, the activation of PARP-1 and nuclear translocation of AIF, and upregulated the expression of Bcl-2 in the SCs. These results indicated that HM inhibited the HG-induced-oxidative stress-associated caspase-dependent and caspase-independent apoptotic pathways in SCs. Therefore, HM may have potential as a treatment for DPN.

Introduction

Diabetic peripheral neuropathy (DPN) affects up to 50% of patients with diabetes mellitus (1) and is considered to be one of the most prevalent and life threatening microvascular diabetic complications. There are currently no efficacious therapeutic strategies available for the treatment of DPN (2,3). DPN is a complex disorder and its exact pathogenesis remains to be fully elucidated; however, various hypotheses have been suggested (4,5). The four predominant pathways associated with DPN are the advanced glycation end products (AGEs) pathway, polyl pathway, protein kinase C (PKC) pathway and hexosamine pathway (6). Based on previous evidence, oxidative stress, which is induced by the excessive production of reactive oxygen species (ROS), is a key factor in all the above-mentioned pathways (7). Therefore, inhibition of the production of ROS, or alleviation of its detrimental effects, may offer potential for the treatment of DPN.

Molecular hydrogen (H₂) is the smallest natural gas molecule (8) and it has been suggested that H₂ may be promising for extensive use in medical applications, without side effects (9), as a potential oxidation inhibitor. Compared with traditional antioxidants such as vitamin E, H₂ possesses two unique properties. Firstly, it has the ability to readily penetrate cell membranes and rapidly translocate to the nucleus and mitochondria (10). Secondly, it is able to selectively neutralize the more cytotoxic hydroxyl (OH⁻) and peroxynitrite (ONOO⁻), without reacting with less potent ROS (11). It is widely accepted that H₂ inhalation or the administration of hydrogen-rich saline (HS) may protect against numerous diseases, including type 2 diabetes (12), neurodegenerative disease (13), multiple organ dysfunction syndrome (14), sepsis (15) and atherosclerosis (16). However, whether H₂ benefits patients with DPN remains to be elucidated.
There is increasing evidence suggesting that ongoing hyperglycemia, which invokes oxidative stress events and impairs neurons and peripheral nerve Schwann cells (SCs) (17), is a risk factor in the progression of DPN (18). Excessive ROS-induced DNA breakage can rapidly activate poly(ADP-ribose) polymerase-1 (PARP-1), an enzyme associated with DNA repair, resulting in energy depletion and cell apoptosis (3,7). Notably, overactivation of PARP-1 can cause the substantial release of PAR fragments from the nucleus into the cytoplasm, triggering the caspase-independent apoptotic pathway via translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus (19). However, excessive expression of PARP-1 can also activate caspase-3, initiating the caspase-dependent apoptotic pathway (20). PARP-1 itself is degraded by caspase-3 from 116 kDa, into two cleavage products (89 kDa and 24 kDa), thus partially losing its activity (21).

In the present study, SCs were treated with high glucose (HG), in order to produce a cellular research model of DPN. The aim of the present study was to clarify the role of HG in the process of SC apoptosis, and to examine whether treatment with hydrogen-rich medium (HM) alleviates HG-induced injury in vitro.

**Materials and methods**

*Preparation of HM.* HM was prepared, according to a method previously described by Ohsawa et al (11), with minor modifications. Briefly, H$_2$ (1 l/min) mixed with air (1 l/min) was dissolved in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) for 4 h to reach supersaturation (~0.6 mM), under 0.4 MPa pressure. The saturated HM was then stored in a sealed aluminum bag under atmospheric conditions at 4°C. HM was freshly prepared every week and was sterilized using γ-radiation (Co-60γ irradiation facility; Tianjin Institute of Technical Physics, Tianjin, China), in order to maintain a continuous concentration.

*Cell culture and treatment.* Primary rat SCs (cat. no. R1700; ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in low glucose DMEM (5.6 mM), supplemented with 5% fetal bovine serum, 1% SC growth supplement and 1% penicillin/streptomycin solution (ScienCell Research Laboratories) in a humidified atmosphere containing 5% CO$_2$ at 37°C. Once the cells had reached ~80% confluence, 0.05% trypsin-EDTA (Gibco) was used to detach the cells. The cells were resuspended at 2x10$^5$ cells/ml and labeled with DCFH-DA (10 µM) at room temperature for 20 min at 37°C. Following incubation, the cells were washed twice with PBS and the labeled cells were collected. A flow cytometer (Versamax; Molecular Devices, LLC, Sunnyvale, CA, USA), with the cell viability expressed as the percentage of cytoprotection, was used to analyze the data.

*Cell viability assay.* Cell viability was detected using a Cell Counting kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc., Kuamamoto, Japan). Briefly, the cells were seeded at a density of 2x10$^3$ per well in 96-well plates in the different treatment conditions for 48 h, with five parallel wells for each treatment group. Subsequently, 10 µl CCK-8 reagent was added to each well and incubated at 37°C for another 3 h. Cell density was determined by measuring the absorbance at 450 nm using a microplate reader (VERSAmax; Molecular Devices, LLC, Sunnyvale, CA, USA), with the cell viability expressed as the percentage of cytoprotection, vs. control group set at 100%.

*Cellular apoptosis assay.* Apoptosis was determined using an Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) kit (Nanjing Keygen Biotech. Co., Ltd., Nanjing, China), according to the manufacturer's instructions. After 48 h treatment, the SCs in each group were washed twice with pre-cooled phosphate-buffered saline (PBS) and were resuspended at 2x10$^5$/ml in binding buffer (Nanjing Keygen Biotech. Co., Ltd.). A total of 5 µl annexin V-FITC and 10 µl PI was then added to each well, and the cells were incubated in the dark at room temperature for 15 min. Flow cytometry (FACSCalibur; BD Biosciences, San Diego, CA, USA) was performed to analyze the cells immediately following incubation. CellQuest Pro software v.5.2.1 (BD Biosciences) was used to analyze the data.

*Caspase-3 activity assay.* An EnzChek Caspase-3 Assay kit (Molecular Probes Life Technologies, Carlsbad, CA, USA) with Z-DEVD-AMC substrate was used to measure caspase-3 activity, according to the manufacturer's instructions. Briefly, after 48 h treatment of the cells (2x10$^5$ cells/well) in the different treatment conditions, the cells were collected, lyzed with 1X cell lysis buffer (Molecular Probes Life Technologies) and assayed in a standard black 96-well plate. Once the reactions had been performed with 2X reaction buffer (Molecular Probes Life Technologies), a fluorescence microplate reader (Fluoroskan Ascent; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to measure the fluorescence at 360 nm excitation and 460 nm emission wavelengths.

*DCFH-DA assay.* Intracellular OH$^·$ levels were detected using a DCFH-DA assay (Beyotime Institute of Biotechnology, Haimen, China). Briefly, following treatment for 48 h, the cells in each group were harvested. The cells were seeded into a 6-well plate at 2x10$^4$/ml and labeled with DCFH-DA (10 µM) in a humidified atmosphere containing 5% CO$_2$, in the dark for 20 min at 37°C. Following incubation, the cells were washed with PBS and the labeled cells were collected. A flow cytometer (BD Biosciences) was used to detect the fluorescence intensity.

*Enzyme-linked immunosorbent assay (ELISA).* The concentrations of 8-hydroxy-deoxyguanosine (8-OHdG) and ONOO$^·$, were measured using a highly sensitive 8-OHdG Check ELISA kit (Japanese Institute for the Control of Ageing, Shizouka, Japan) and ONOO$^·$ ELISA kit (Yueyan Bio, Shanghai, China), respectively. The cellular supernatants were obtained following centrifugation at 3,000 x g for 15 min at
4°C, and the levels of 8-OHdG and ONOO· in the supernatants were determined, according to the manufacturer's instructions, with minor modifications, such as to the centrifugation speed. The absorbance was read using the VERSAMax microplate reader.

Western blot analysis. Western blotting was used to detect the relative expression levels of the target proteins. Following treatment of the cells in each group, the cells were lysed with radioimmunoprecipitation buffer (Beijing Solarbio Science & Technology, Co., Ltd., Beijing, China). The lysates (50 µg/lane) were separated by 10% SDS-PAGE and were transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free milk for 2 h and were then incubated with the following antibodies: Rabbit polyclonal anti-AIF (1:1,000; cat. no. ab1998; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-β-actin (1:1,000; cat. no. A5060; Sigma-Aldrich, St. Louis, MO, USA), rabbit polyclonal anti-cleaved PARP-1 (1:100; cat. no. sc-25780; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit polyclonal anti-Bcl-2 (Bcl-2; 1:100; cat. no. sc-492; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-Bcl-2-associated X protein (Bax; 1:100; sc-493; Santa Cruz Biotechnology, Inc.) and anti-β-actin (1:2,000; cat. no. A5060; Sigma-Aldrich, Shanghai, China), at 4°C overnight with mildly consistent agitation. The immunoblots were washed three times with Tris-buffered saline containing 0.05% Tween (10 min/wash), followed by incubation with goat anti-rabbit immunoglobulin G (1:5,000; cat. no. 05557; Sigma-Aldrich) for 2 h at room temperature. The blots were washed again, as mentioned above, and were treated with a prepared chemiluminescent horseradish peroxidase substrate (EMD Millipore). The blots were visualized using Quantity One software, version 4.5.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the integrated optical density was analyzed using a Gel-Pro analyzer (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. The data are presented as the mean ± standard deviation. One-way analysis of variance with least significant difference comparison were used to analyze differences between the groups. SPSS 21.0 software (IBM SPSS, Armonk, NY, USA) was used to perform the statistical analyses. *P<0.05 was considered to indicate a statistically significant difference.

Results

HM increases the viability of SCs exposed to HG. In order to investigate the effects of HM on cellular viability following treatment with HG, the viability of the SCs was examined using a CCK-8 assay (Fig 1). The viability of the SCs exposed to HG was significantly reduced, compared with the control group (P<0.05), following treatment for 48 h. Furthermore, treatment with HM improved cell viability under HG conditions (P<0.05). However, no significant difference were observed between the mannitol and control groups (P>0.05).

HM prevents apoptosis of SCs under HG conditions. It is well known that HG-induced oxidative stress promotes apoptosis (18,23). To further examine the curative effects of HM on HG-induced apoptosis, the apoptotic rate and caspase-3 activity of the SCs were determined using annexin V-FITC/PI and caspase-3 activity assays, respectively. The proportion of apoptotic cells in the HG group was markedly increased, compared with the control cells cultured in primary DMEM (P<0.05; Fig. 2A and B). Treatment with HM significantly reduced the percentage of apoptotic cells in the HG condition (P<0.05). Concordantly, caspase-3 activity was significantly increased in the HG-treated SCs (P<0.05), which was alleviated by treatment with HM (P<0.05; Fig. 2C), whereas treatment with mannitol demonstrated no arresting effects (P>0.05) on either the apoptotic rate or caspase-3 activity. These results indicated that treatment with HM significantly suppressed HG-induced apoptosis of SCs in vitro.

HM inhibits the production of OH· and ONOO· under HG conditions. The levels of intracellular ROS, ONOO· and OH· were detected in the treatment groups using ELISA and DCFH-DA assays, respectively. The intracellular levels of OH· (Fig. 3A and B) and ONOO· (Fig. 3C) were higher in the HG group, compared with the control group (P<0.05). Treatment with HM suppressed the intracellular concentrations of OH· and ONOO· under HG conditions (P<0.05), whereas no significant effects of mannitol on levels of ROS were observed (P>0.05).

HM mitigates HG-induced 8-OHdG levels in SCs. Treatment with HG significantly increased the levels of 8-OHdG, a sensitive oxidative stress-induced DNA damage biomarker, compared with the control group (Fig. 4; P<0.05). However, treatment with HM markedly reduced the generation of 8-OHdG (P<0.05), whereas treatment with mannitol had no effect (P>0.05). These results suggested that HM inhibited oxidative stress-induced DNA damage in the SCs under HG conditions.

Effects of HM on the PARP-1/AIF pathway. To clarify whether HM exhibits a protective effect against HG-induced caspase-independent PARP-1/AIF apoptosis, the acti-
Figure 2. Apoptotic rate of the Schwann cells was determined using annexin V-FITC/PI and caspase-3 activity assays. (A) Representation of the apoptotic cells following flow cytometry, >5,000 cells were assessed from each group. The percentage of gated cells (those in the circle) in the lower-right and upper-right quadrants of the plots indicate the proportion of early and late apoptotic cells. Results of the (B) annexin V-FITC/PI and (C) caspase-3 activity assays are expressed as the mean ± standard deviation (n=3/group). *P<0.05, compared with the control group; #P<0.05, compared with the HG group. FITC, fluorescein isothiocyanate; PI, propidium iodide; H2, hydrogen; HG, high glucose.

Figure 3. Determination of intracellular OH· and ONOO− levels. Intracellular levels of OH· and ONOO− were detected using flow cytometry and enzyme-linked immunosorbent assays, respectively. (A) Flow cytometric results of the DCFH-DA assay, 20,000 cells were assessed from each group. Quantitative analysis of (B) OH· and (C) ONOO− are expressed as the mean ± standard deviation (n=3/group). *P<0.05, compared with the control group; #P<0.05, compared with the HG group. H2, hydrogen; HG, high glucose; OH·, hydroxyl; ONOO−, peroxynitrite; M1, DCFH-DA labelled cells.
viation of PARP-1 (ratio of cleaved-PARP-1/PARP-1), expression of PAR and nuclear translocation of AIF (ratio nuclear AIF/AIF) were detected in each treatment groups using western blot analysis (Fig. 5A). The relative expression levels of cleaved-PARP-1/PARP-1 (Fig. 5B), PAR (Fig. 5C) and nuclear AIF/AIF (Fig. 5D) were markedly increased in the HG group, compared with the control group (P<0.05). Treatment with HM significantly reduced the activation of PARP-1, expression of PAR and nuclear translocation of AIF (P<0.05). However, no significant differences in the these proteins were observed between the mannitol and control groups (P>0.05).

**Discussion**

It is widely recognized that hyperglycemia, which exists in all patients with diabetes mellitus, is the predominant cause of diabetic complications (6). SCs are a unique type of glial and myelin-forming cell in the peripheral nervous system, which have a critical role in maintaining normal function and morphology and also in the pathogenesis and development of peripheral nerves (24). SCs can rapidly respond to hyperglycemia, generating high levels of antioxidative enzymes, and enhancing antioxidant defense mechanisms to relieve hyperglycemia-induced oxidative stress (25). As injury to the DPN in SCs is reversible (26), it is possible that SCs is a promising target for the treatment of DPN. HG-induced injury has also been widely investigated as a model of chronic disease in vitro (27). Therefore, the present study used SCs cultured under HG conditions as an in vitro cellular model of DPN.

The present study aimed to investigate the protective effects of H₂-rich medium on oxidative stress-mediated SC apoptosis under HG conditions in vitro. The results of the present study indicated that (i) in normal SCs, treatment with H₂-rich medium exhibited little effect on cell trauma and viability; (ii) treatment with HG induced severe oxidative stress in SCs with significant injury to the peripheral neural system, and this was significantly improved following treatment with 0.6 mM HM for 48 h following HG administration; (iii) HM inhibited oxidative stress-associated DNA damage under hyperglycemic conditions in SCs; and (iv) treatment with HM inhibited HG-induced caspase-dependent and caspase-independent apoptosis in SCs.

Oxidative stress is considered to be an imbalance between the production of ROS and activated anti-oxidative mechanisms in cells (28). Hyperglycemia-induced overproduction of free radicals is considered to be the source of DPN complication through increased glycolysis (29). The production of ROS, including superoxide anions (O₂⁻) and OH•, is unavoidable in all mammalian cells under normal circumstances. In healthy cells, the generation of ROS is closely monitored, whereas during metabolic disturbance, the overproduction of ROS may lead to damage, dysfunction and deletion of nerve fibers in the PNS (30). In DPN, the hyperglycemia-induced overproduction of O₂ can result in the combination of NO and O₂, resulting in the formation of the ONOO⁻, a potent antioxidant that can cause cell death (31). In addition to the formation of surplus ROS, hyperglycemia can also induce the dysfunction of organelles and the nucleus, which may lead to the activation of caspase-3, translocation of AIF and cytochrome c, and mitochondrial biogenesis and fission (32). Accordingly, the four predominant pathways associated with DPN include the AGE pathway, polyol pathway, PKC pathway and hexosamine pathway (6), all of which lead to programmed cell death. Our previous study demonstrated that, under hyperglycemic conditions, SCs were a target of oxidative damage, and that inhibition of ROS may inhibit the progression of neuropathy (33).

PARP-1 is the most abundant protein in the PARP family, and is a ubiquituous nuclear enzyme, the activation of which in neurons and SCs of the PNS indicates the pathogenesis of diabetic complications (34). Hyperglycemia-associated oxidative stress can initiate excessive DNA single or double strand breaks; however, PARP-1 can respond to this damage by promoting DNA repair procedures via nicotinamide adenine dinucleotide (NAD⁺)-dependent poly(ADP-ribose)ylation on histones, or by itself, altering the mitochondrial membrane potential and thus releasing apoptosis-inducing factor from the mitochondria (35,36). Furthermore, excessive PARP-1 activation can result in the generation of increased PAR fragments in the nucleus, which are released into the cytoplasm, promoting AIF nuclear translocation. Therefore, it is possible to directly trigger apoptosis via a caspase-independent pathway (19). Superfluous activation of PARP-1 can markedly consume NAD⁺ stores, deplete levels of adenosine triphosphate and destroy the integrity of the oxidative respiratory chain, which can lead to molecular death from necrosis or caspase-dependent apoptosis (37). PARP-1 itself is a substrate of caspase-3, can be decomposed from a 116 kDa protein into two cleavage products (89 and 24 kDa), resulting in partial loss of its activity (21). The present study induced apoptosis of SCs using 50 mM glucose, and demonstrated that HG significantly upregulated the levels of 8-OHdG and increased the expression of cleaved PARP-1, release of PAR, nuclear...
tanslocation of AIF and activation of caspase-3. These results indicated that HG induced severe oxidative stress and promoted caspase-independent and caspase-dependent apoptosis of the SCs, both of which are associated with PARP-1.

Since oxidative stress is involved in all DPN pathways, a logical therapeutic strategy may be to prevent oxidative stress by increasing antioxidant defences. Numerous clinical studies have demonstrated that H₂ or HS exhibit antioxidative (9,11,12,38), anti-apoptotic (10) and anti-inflammatory (14,15) effects in several disease models. The protective effects of H₂ or HS on zymosan-induced organ impairment (14), lipopolysaccharide (LPS)-associated lung injuries (39), LPS-associated sepsis (15,38,40) and ouabain-induced auditory neuropathy (41) have also been observed in vivo. The results of the present study indicated that HM significantly inhibited oxidative damage in the SCs by selectively suppressing the production of ONOO⁻ and OH⁻, formation of 8-OHdG and activation of PARP-1, leading to a reduction in SC apoptosis via the caspase-independent and caspase-dependent pathways. In addition, treatment with HM markedly upregulated the expression of anti-apoptotic Bcl-2 and downregulated the expression of pro-apoptotic Bax, indicating that HM protected the SCs from HG-induced apoptosis.

The results of the present study indicated that HG selectively induced the production of ROS and activation of PARP-1, and promoted caspase-dependent and caspase-independent apoptosis of SCs. Furthermore, the findings demonstrated that treatment with HM inhibited HG-induced oxidative stress and the PARP-1 activation-associated apoptosis of SCs by down-regulating the caspase-independent and caspase-dependent apoptotic pathways.

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References


