Hydrogen Relieves Neuropathic Pain in Diabetic Rats by Inhibiting MCP1 and CCR2 Expressions

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Abstract: We aimed to explore the mechanism for hydrogen in the treatment of neuropathic pain in diabetic rats. Eight-week-old male SD rats were randomly divided into control, model and hydrogen treatment groups. The hydrogen treatment group was intraperitoneally injected with hydrogen-rich saline daily in 7th and 8th weeks after modeling. Before and 2, 4, 6 and 8 weeks after modeling, the neurological function, behavioral changes and levels of inflammatory factors TNF-α and IL-6 in the sciatic nerve were detected, and MCP1 and CCR2 protein expressions were measured by Western blotting. Compared with the model group, the hydrogen treatment group had significantly increased motor nerve conduction velocity, thermal withdrawal latency and mechanical withdrawal threshold (P<0.05). The significantly higher levels of TNF-α and IL-6 in the sciatic nerve of the model group than those of the control group decreased in the hydrogen treatment group (P<0.05). The protein expressions of MCP1 and CCR2 in the sciatic nerve of the model group significantly exceeded those of the control group. Such expressions of the hydrogen treatment group were lower than those of the model group. Hydrogen alleviated the inflammatory response of peripheral nerves in diabetic rats by inhibiting the MCP1-CCR2 signaling pathway, thus mitigating neuropathic pain.

Keywords: Diabetic neuropathy, Hydrogen, MCP1, CCR2, TNF-α, IL-6

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INTRODUCTION

Diabetes is one of the most influential and harmful diseases in the world, which leads to metabolic damage, cardiovascular disorders and obesity, accompanied by vascular complications. Damage to vascular endothelial cells caused by hyperglycemia results in microvascular complications such as diabetic peripheral neuropathy (DPN), diabetic nephropathy and retinopathy. DPN is the most common yet intractable complication of diabetes, usually manifested as distal symmetrical sensorimotor polyneuropathy and autonomic neuropathy [1]. Over 50% of diabetic patients have peripheral neuropathy as the chronic disease develops, of whom 40-50% suffer from pain symptoms such as hyperalgesia, numbness and paraesthesia. Some of them may even suffer from gangrene in the lower limbs with the aggravation of disease and have to receive amputation [2]. Diabetes is the main cause for lower limb amputation. There are approximately 80,000 diabetic patients undergoing amputation annually in the USA only. The pain and risk of amputation caused by diabetes have severely affected the quality of life as well as physical and psychological health, and hugely burdened the society and families. However, due to the unknown pathogenesis of diabetes, patients’ suffering can be relieved only by controlling blood glucose level and alleviating pain symptoms. Hence, it is urgent to find new drugs based on multiple pathogenic mechanisms.

Many factors are involved in the pathogenesis of DPN, including oxidative stress, pre-inflammatory changes and formation of advanced glycation end products. These processes lead to many types of cell damage, such as neuronal damage and vascular endothelial impairment. Among all the factors, oxidative stress and inflammatory mechanism play central roles in the onset of diabetic neuropathy. It is well-documented that monocyte chemoattractant protein-1 (MCP-1) and its specific receptor C-C chemokine receptor 2 (CCR2) play important roles as inflammatory mediators in the pathophysiological processes of many diseases [3-5]. They participate in all stages of inflammatory response, covering adhesion, migration, removal of inflammatory substances and repair, and also essentially regulate inflammation, immunity, infection and other diseases.

Hydrogen is a novel antioxidant found in recent years. In the past, most biologists considered hydrogen physiologically inert. Cytological and molecular biological studies have proven that hydrogen can selectively eliminate hydroxyl radical and peroxynitrite anion [6]. In 2009, Mao et al. [7] dissolved hydrogen into normal saline at high pressure to prepare 0.6 mmol/L hydrogen-rich saline (HRS) or hydrogen-saturated medium for in vitro and in vivo experiments. Since then, HRS has been widely applied to study different disease models, but the specific mechanism remains largely unknown. Compared with other antioxidants, hydrogen is typified by selectivity, no toxicity, strong permeability, no residue and low cost, with well-established protective and therapeutic effects on 63 disease models. Nevertheless, its influence on DPN has never been reported hitherto. Since DPN is closely related to oxidative stress damage and inflammatory response, we postulated that HRS may be clinically effective for treating DPN.

MATERIAL AND METHODS

Ethical Approval

The study has received ethical approval of Affiliated Jiangning Hospital of Nanjing Medical University, China, (AJHNMU-201903006).

Experimental Animals

SPF 8-week-old healthy male SD rats weighing 200-220 g were purchased from Shanghai SIPPR-Bk Lab Animal Co., Ltd. [China, license: SCXK (Shanghai) 2018-0007]. All rats were fed in a well-ventilated environment with free access to food and water on a 12 h/12 h light/dark cycle.

Establishment of Diabetic Model

After fasting for 12 h, healthy male SD rats were intraperitoneally injected with streptozotocin (STZ) at a concentration of 1% (65 mg/kg) dissolved in sodium citrate buffer (pH 4.2-4.5). In control group, an equal volume of sodium citrate buffer was injected only. All rats had free access to food and water. After 48 h, the blood was drawn via the caudal vein to measure the fasting blood glucose using Accu-Chek blood glucose meter and test paper (Roche, Germany). Fasting blood glucose ≥16.67 mmol/L indicated successful modeling.

Preparation of HRS

Hydrogen was dissolved in normal saline using a hydrogen generator under the pressure of 0.4 MPa till the saturated condition, such a condition was maintained for 6 h, and it was stored in a refrigerator at 4°C prior to use. HRS had never been reported hitherto. Since DPN is closely related to oxidative stress damage and inflammatory response, we postulated that HRS may be clinically effective for treating DPN.

Preparation Grouping and Treatment

Before modeling, 6 rats of the same age were randomly
selected as control group. After successful modeling, 12 rats were selected and randomly divided into model group and hydrogen treatment group. The optimal therapeutic regimen determined in the preliminary experiment was used for the hydrogen treatment group, i.e. HRS was intraperitoneally injected (5 mg/kg) daily in 7th and 8th weeks after modeling. For control and model groups, an equal volume of normal saline was injected.

**Behavioral Test**

Thermal withdrawal latency (TWL) and mechanical withdrawal threshold (MWT) were detected using the tail-flick test, hot plate test and Von Frey filaments, respectively. 1) TWL was detected before STZ injection (baseline state) and 2, 4, 6 and 8 weeks after injection. In the hot-water tail-flick test, the tail was immersed in hot water at 52.5±0.5°C, and the duration (s) from tail immersion to tail flick or struggle was recorded for 3 times at an interval of 15 min. The average was taken as the tail-flick latency (TFL). 2) In hot plate test, the heating temperature of YLS-6B hot-plate analgesia meter was set at 52±1°C, and the duration from placement of rats on the hot plate to heating response (paw licking, screaming and jumping) was recorded for 3 times at an interval of 10 min. The average was taken as TWL. 3) MWT was detected before STZ injection (baseline state) and 2, 4, 6 and 8 weeks after injection. After the rats were placed on a mesh plane for 30 min, MWT (g) of the right hind paw was measured using an electronic Von Frey device 3 times at an interval of 15 min. The average was taken as MWT.

**Neurological Function Test**

Before STZ injection (baseline state) and 2, 4, 6 and 8 weeks after injection, the motor nerve conduction velocity (MNCV) of the sciatic nerve was determined. After anesthesia, the rats were fixed in a prone position, and the body temperature was kept at 35-37°C to resist the cooling effect of anesthesia. The sciatic nerve trunk at the ischial tuberosity and hip joint was exposed and separated. The proximal stimulating electrode was placed at the sciatic notch, the distal stimulating electrode at the medial ankle, the recording electrode at the first femoralis of ipsilateral toes, and the reference electrode between the stimulating electrode and the recording electrode. Upon the stimulation of 3 V single-pulse square wave, the latency of action potential of the proximal and distal sciatic nerves was recorded, and the distance between the two stimulating electrodes and the recording electrode was measured. Finally, MNCV was calculated: MNCV (m/s) = distance between two stimulating electrodes and recording electrode - action potential latency of two stimulating electrodes.

**Sample Collection**

After the last behavioral test, the rats were deeply anesthetized with 10% chloral hydrate (300-350 mg/kg), and the thoracic cavity was quickly cut open to expose the heart. The heart was held using a tweezer in the left hand, and the perfusion needle was inserted into the cardiac apex upwards till the ascending aorta. At the same time, a small incision was made using scissors at the right auricle, from which the normal saline was perfused quickly until the outflow liquid was colorless (about 120 mL). In a prone position, the limbs were fixed, the superficial skin was cut open along the femur, and the muscle was bluntly separated to expose the femur. The intermuscular fascia was separated to find the sciatic nerve trunk. The bilateral sciatic nerves were taken, frozen in liquid nitrogen and thawed repeatedly, and its weight was measured.

Then the nerves were added with pre-cooled phosphate buffer containing PMSF and protease inhibitor, and homogenized, followed by centrifugation at 12,000 xg and 4°C for 20 min. Finally, the supernatant was stored at -80°C.

**Measurement of Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) Levels by ELISA**

ABC-ELISA was performed. Anti-rat TNF-α (Cat. No. ab208348, Abcam, USA) and IL-6 monoclonal antibodies (Cat. No. ab100772, Abcam, USA) were coated on an ELISA plate, and bound to TNF-α and IL-6 in the standards and samples. Then biotinylated TNF-α and IL-6 antibodies were added, forming an immune complex on the plate. Afterwards, HRP-labeled streptavidin was added to bind biotin, and the substrate solution was used for color development. Finally, sulfuric acid was added to terminate the reaction. The optical density (OD) at 450 nm was measured, which was directly proportional to the concentration of TNF-α or IL-6. The concentrations of inflammatory factors in samples were detected according to the standard curve.

**Detection of MCP1 and CCR2 Protein Expressions in the Sciatic Nerve by Western Blotting**

Tissue blocks stored in liquid nitrogen were cut into pieces, added with RIPA lysis buffer and PMSF, homogenized on ice and lysed for 30 min, followed by centrifugation at 14,000 rpm and 4°C for 10 min. The supernatant was collected into a centrifuge tube, and the protein concentration was measured using the BCA method. The samples were mixed with loading buffer (1:3), heated at 100°C and cooled to room temperature, followed by centrifugation at 14,000 rpm for 5 min. The supernatant was loaded (10 μL/well), and 5 μL of marker protein was used for labeling. Then the protein sample was subjected to electrophoresis. Subsequently, the product was transferred onto a PVDF membrane which was blocked with skimmed milk on a shaker for 1 h, and incubated with MCP1 (1:500 diluted; Cat. No. ab9858, Abcam, USA), CCR2 (1:500 diluted; Cat. No. ab203128, Abcam, USA) and β-actin (1:500 diluted; Cat. No. ab8226, Abcam, USA) primary antibodies at 4°C.
overnight. After the membrane was washed with TBST at room temperature for 5 min (5 times in total), the protein was incubated again with goat anti-rabbit secondary antibody (1:5000) at room temperature on the shaker for 1 h. After the membrane was washed again with TBST for 5 min (5 times in total), ECL solution (solutions A and B mixed at 1:1; Cat. No. ab65623, Abcam, USA) was added (0.125 mL/cm²) for 2-3 min of reaction, followed by exposure in a darkroom. Finally, OD of protein band was analyzed using Quantity One, and the relative expression level of target protein was expressed as the ratio of its gray value to that of β-actin. The experiments were performed in triplicate independently.

**Statistical Analysis**

All data were statistically analyzed by SPSS 19.0 software. The quantitative data were represented as mean ± standard deviation. Repeated-measures analysis of variance was performed for intergroup comparisons at different time points. First, the differences between two groups and the time differences of measured values were compared. In the case of intergroup difference, further comparison at each time point was carried out by the independent-sample t test. The SNK-q test was employed to compare the time differences of each group. P<0.05 was considered statistically significant.

**RESULTS**

**Changes of Blood Glucose Level and Body Weight**

Compared with the control group, the model group suffered from polyphagia, polydipsia, polyuria, no weight gain and obvious elevation of blood glucose level (P<0.05), gradually became thin and had lusterless hair. The body weight and blood glucose level of diabetic rats were not significantly improved by hydrogen treatment (Fig. 1), indicating that hydrogen cannot inhibit the progression of diabetes by lowering blood sugar.

**Behavioral and Neurological Function Test Results**

Compared with the baseline values (W0), MNCV, TFL, TWL and MWT of the control group were similar (P>0.05). In the model group, MNCV, TFL, TWL and MWT significantly decreased (P<0.05), and neurological impairment and hyper-algesia occurred from the end of the 2nd week till the 8th week, which were aggravated progressively.

Compared with the model group, the hydrogen treatment group had significantly increased MNCV, TWL and MWT (P<0.05) (Table 1), suggesting that hydrogen effectively elevated MNCV and relieved hyperalgesia in diabetic rats.

**TNF-α and IL-6 Levels in Sciatic Nerve**

Eight weeks after STZ injection, the levels of TNF-α and IL-6 in the sciatic nerve of the model group were significantly higher than those of the control group (P<0.05), which were significantly lower in the hydrogen treatment group (P<0.05) (Fig. 2). Therefore, hydrogen treatment prevented diabetes-induced elevation of levels of inflammatory factors TNF-α and IL-6 in the sciatic nerve. In other words, inflammatory response was involved in the generation of peripheral neuroinflammatory responses to diabetes, and hydrogen treated neuropathic pain by mitigating inflammation damage.

**Effects of Hydrogen on MCP1 and CCR2 Protein Expressions in Sciatic Nerve**

Western blotting showed that the protein expressions of MCP1 and CCR2 in the sciatic nerve of the model group were significantly up-regulated compared with those of the control group 8 weeks after STZ injection, suggesting that the MCP1-CCR2 pathway was activated in diabetic...
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rats. The protein expressions of MCP1 and CCR2 in the hydrogen treatment group were lower than those of the model group (Fig. 3). Based on the results of behavioral experiments, it was found that the activation of the MCP1-CCR2 pathway is consistent with the production of pain sensitivity. Accordingly, hydrogen may alleviate the symptoms of DPN by inhibiting the activation of the MCP1-CCR2 pathway.

Table 1. Behavioral and neurological function test results

<table>
<thead>
<tr>
<th>Time</th>
<th>MNCV (m/s)</th>
<th>TFL (s)</th>
<th>TWL (s)</th>
<th>MWT (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Model</td>
<td>Hydrogen Treatment</td>
<td>Control</td>
</tr>
<tr>
<td>W0</td>
<td>57.4±2.0</td>
<td>56.3±2.9</td>
<td>-</td>
<td>11.7±0.9</td>
</tr>
<tr>
<td>W2</td>
<td>55.2±3.0</td>
<td>38.3±2.0*</td>
<td>-</td>
<td>10.8±0.9</td>
</tr>
<tr>
<td>W4</td>
<td>55.3±2.5</td>
<td>31.7±2.0*</td>
<td>-</td>
<td>11.2±0.9</td>
</tr>
<tr>
<td>W6</td>
<td>56.1±2.4</td>
<td>27.9±2.6*</td>
<td>-</td>
<td>10.5±0.3</td>
</tr>
<tr>
<td>W8</td>
<td>55.8±3.1</td>
<td>29.8±2.3*</td>
<td>48.9±2.3#</td>
<td>11.0±0.8</td>
</tr>
</tbody>
</table>

* Compared with control group, P<0.05; #compared with model group, P<0.05

Fig 2. TNF-α (A) and IL-6 (B) levels in sciatic nerve

Fig 3. Effects of hydrogen on MCP1 and CCR2 protein expressions in sciatic nerve. * Compared with control group, P<0.05; #compared with model group, P<0.05
Discussion

The incidence of diabetes mellitus, a worldwide epidemic, has increased by 50% in the past decade. The complications of vascular damage caused by chronic hyperglycemia can be divided into two types: microvascular and macrovascular. The microvascular complications include diabetic neuropathy, retinopathy and nephropathy. Diabetic neuropathy is the most common and refractory complication of diabetes, affecting more than half of diabetic patients, of which nearly 15% are at risk of lower limb amputation, or even threatening lives. The early manifestations of pain symptoms are spontaneous pain, hyperalgesia and paralgesia. In the later stages, the symptoms are manifested as hypoalgesia decrease, which seriously affects the quality of life of patients. Due to the complex pathological mechanism of DPN, there is still no effective treatment. STZ is a toxin that specifically destroys islet cells. A single injection can lead to sudden hyperglycemia. The model of diabetes induced by STZ is the most classic model for DPN research. STZ models usually show the changes of pain behavior, nerve function and nerve morphology that are basically consistent with those of diabetic patients. Early neurological symptoms are usually characterized by time-dependent aggravation of hyperalgesia, paralgesia, and decreased nerve conduction velocity, while late neurologic symptoms are manifested as hypoalgesia, neuro-degeneration, demyelination and loss of epidermal nerve fibers. Obvious hyperalgesia and paralgesia usually occur two weeks after STZ injection, and can continue to aggravate to the eighth week. After eight weeks, the symptoms such as hypoalgesia appear, which may be related to the irreversible degeneration of nerve fibers. Nerve function indicators include motor nerve conduction velocity and sensory nerve conduction velocity, which are currently considered as the gold standard for evaluating the degree of nerve damage. Their decline is the manifestation of nerve hypoxia and ischemia, which is more sensitive and objective than the change of pain threshold in the study of nerve injury [8,9]. In this study, a model was established with reference to literature methods, and the results were consistent with the above theory. Compared with the control group, the rats appeared to have diabetic symptoms such as polyphagia, polydipsia and polyuria 48 h after STZ injection, and their blood glucose increased to above 16.67 mmol/L. The results of the behavioral and neurological experiments showed that the mechanical pain threshold and thermal pain threshold decreased significantly, and the sciatic nerve conduction velocity reduced significantly 14 days after STZ injection in a time-dependent manner until the eighth week after injection, indicating that DPN was successfully induced and continued to develop.

The long course of diabetes and poor glycemic control are the main risk factors for neuropathy. Due to the lack of understanding of its pathogenesis, there is still no effective treatment except for the control of blood glucose and pain treatment. Some studies have found that even the strict control of blood glucose cannot prevent the development of DPN, indicating that hyperglycemia is only a pathogenic inducer, and DPN can also play a damaging role through a variety of mechanisms at the downstream [10,11]. In addition, because the development of microvascular complications is a process from reversibility to irreversibility, it is of great significance to effectively control and reverse the disease by finding the early markers of its onset or pathogenic causes and dealing with them.

Neuropathic pain refers to the type of pain in which a nociceptive response still exists after the noxious stimulus to nerves and surrounding tissues is removed. It is mainly manifested in overreaction to harmful stimulus or abnormal response to mild stimulus. Postherpetic neuralgia trigeminal neuralgia and DPN are the three most common types in clinical practice. Neuropathic pain is still regarded as one of the most difficult types of chronic pain to deal with, which seriously affects the quality of life of patients and brings a huge burden to society. A variety of neural injury models have confirmed that the production of pain symptoms is closely related to the inflammatory response. It has been found in clinical studies that the levels of inflammatory factors in the blood of patients with type 1 or type 2 diabetes were higher than those of healthy people, and this increase often indicated the occurrence and progression of neuropathy [12]. Increased levels of inflammatory factors also play an important role in the production and maintenance of DPN. Drug and gene intervention studies have also proven that the production of pain symptoms is closely related to the inflammatory response. It has been found in clinical studies that the levels of inflammatory factors in the sciatic nerve of rats in the DPN group were both higher than those in healthy rats, which indicate that the inflammatory response may be involved in the occurrence of DPN. In this study, TNF-α and IL-6 levels in the sciatic nerve of rats in the DPN group were both higher than those in healthy rats, which also verified this hypothesis. Molecular hydrogen has been successfully used in a variety of acute oxidative stress environments due to its effective antioxidant and free radical scavenging effects. Its main molecular targets are not yet clear. At present, the most important mechanism is the selective scavenging of free radicals and peroxynitrite. Moreover, it also has the role of regulating gene expression [14]. Hydrogen inhalation can significantly reduce the levels of serum and tissue oxidation products, improve the activity of
antioxidant enzymes, increase the survival rate of mice with moderate or severe sepsis, reduce multiple organ damage caused by sepsis, and effectively prevent the occurrence of multiple organ failure. Meanwhile, no obvious side effects were found by detecting various physiological indices of animals in the process of hydrogen treatment. In addition to direct anti-oxidative stress and scavenging free radicals, the main mechanism of its action can also play a further therapeutic role by down-regulating the expression of inflammatory protein and up-regulating the expression of antioxidant protein in vivo. Herein, HRS inhibited the aggravating symptoms and functional impairment of neuropathic hyperalgesia in diabetic rats, and the effect was correlated with the decrease of proinflammatory cytokine levels. Therefore, we postulated that molecular hydrogen may play a protective role in the chronic oxidative stress environment of the nervous system and the inflammatory damage caused by it, so as to treat DPN.

Hyperglycemia causes damage through a variety of mechanisms, involving multiple damages to the metabolic environment, nerves and blood vessels: it can lead to polyol bypass activation, excessive production of terminal glycosylation products, excessive activation of the MCP1-CCR2 pathway, inflammatory response, and mitochondrial damage. These mechanisms, through their own effects or mutual effects, can eventually lead to the excessive production of ROS and the breakdown of the body's redox balance, resulting in oxidative stress. Thus, oxidative stress is the common result of these pathways and plays an important role in the formation of DPN. Since neuropathic pain in diabetic rats is closely related to neuroinflammatory response, and the MCP1-CCR2 pathway is an important regulator of DPN inflammatory response, this study aimed to assess the effect of HRS on the MCP1-CCR2 pathway. Six weeks after STZ injection, the levels of MCP1 and CCR2 in the sciatic nerve of the DPN group were significantly down-regulated by continuous injection of HRS for 14 days, the content of proinflammatory cytokines regulated by STZ was also decreased, the thermal and mechanical pain thresholds were significantly increased, and the nerve conduction velocity was improved.

In summary, hydrogen may reduce the inflammatory response of peripheral nerves in diabetic rats by inhibiting the activation of the MCP1-CCR2 signaling pathway, thereby improving neuralgia. Due to the complexity of the pathogenesis of DPN and the limited therapeutic strength for one mechanism alone, it is necessary to find a comprehensive treatment that can inhibit the etiology, control the inflammation and strengthen the self-defense mechanism so as to prevent and recover the nerve damage and resolve pain.

Availability of Data and Materials

All data and materials are available from the corresponding authors upon reasonable request.

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Competing Interests

There is no conflict of interest.

Authors’ Contributions

PW, LY and QL designed this study and prepared this manuscript; HW, YL and WC performed this study and analyzed experimental data. All authors read and approved the final version of the manuscript.

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