Int J Clin Exp Med 2016;9(2):2831-2839
www.ijcem.com /ISSN:1940-5901/IJCEM0017105

Original Article

Rosalaevigata Michx extract inhibits oxidative stress in diabetic nephropathy by activating Nrf2/ARE signaling

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Received September 29, 2015; Accepted December 19, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: Objective: Oxidative stress is a key factor in the development of diabetic nephropathy (DN), and transcription factor NF-E2 related factor (Nrf2) is a central regulator of cellular anti-oxidative stress. Therefore, this study investigated the Nrf2/ARE (anti-oxidative response element) signaling mechanisms in Rosalaevigata Michx extract, which inhibits DN oxidative stress. Methods: The streptozotocin (STZ)-induced DN rats were randomly divided into DN model group (DN group) and Rosalaevigata Michx treatment group (DN+RLM group), with normal control group (NC group) and Rosalaevigata Michx control group (NC+RLM group) as controls. The levels of reactive oxygen species (ROS), malondialdehyde (MDA), IL-6, IL-1β and monocyte chemoattractant protein-1 (MCP-1), and the activity of superoxide dismutase (SOD) and total anti-oxidant capacity (T-AOC) in serum and kidney tissues were measured. The expression of molecules in the Nrf2/ARE signal pathway affected by the plant extract was analyzed by immunohistochemistry, Western blot and qRT-PCR. Results: In serum and kidney tissues of DN rats, the levels of ROS, MDA, IL-6, IL-1β and MCP-1 significantly increased, and T-AOC and SOD activity decreased significantly. Rosalaevigata Michx extract significantly reduced the levels of IL-6, IL-1 and MCP-1 as well as the levels of ROS and MDA, and increased the activity of SOD and T-AOC in serum and kidney tissue of the diabetic nephropathy rats. Rosalaevigata Michx extract significantly up-regulated DN rat renal Nrf2 protein expression and up-regulated antioxidant genes hemeoxygenase-1 (HO-1), γ-glutamate synthase (γ-GCS) expression. Conclusion: Rosalaevigata Michx extract improves DN oxidative stress and inflammation, mediated by the activation of Nrf2 /ARE signal pathway and up-regulation of HO-1 and γ-GCS expression.

Keywords: *Rosalaevigata* Michx, diabetic nephropathy, oxidative stress, ROS, SOD, inflammation, Nrf2/ARE signal pathway, hemeoxygenase-1, γ-glutamate synthase

Introduction

Diabetic nephropathy (DN) is the most common complication and the main cause of death in diabetes as well as the main cause of end-stage renal failure [1]. Worldwide, the incidence of diabetic patients is estimated at 550 million in 2030, 30%-50% of whom will be afflicted with diabetic nephropathy [2]. However, the pathogenesis of diabetic nephropathy is complex, mediated by multiple factors and pathways. The related molecular mechanism has yet to be elucidated. Currently, a large number of studies suggest that oxidative stress (OS) plays a key role in the origin and development of diabetic nephropathy [3, 4]. Kidney is highly sensitive to oxidative stress, and is damaged by reactive oxygen species (ROS). In diabetes, multiple factors such as glucose metabolic disorders lead to a large number of highly active molecules in renal tissues, generating ROS. The activity of antioxidant enzymes such as superoxide dismutase (SOD) is decreased via base oxidation, attenuating the free radical-scavenging ability, resulting in the formation of oxidative stress and activation of a series of signal transduction pathways resulting in abnormal renal tissue morphology and function, and the development of DN [5]. In addition, ROS also activate redox signaling similar to second messenger molecules, and increase cellular oxidative damage. Therefore, diabetic nephropathy is mediated by increased free radical levels and decreased antioxidant capacity, resulting in oxi-

dative stress [7]. Inhibition of oxidative stress significantly delays the initiation and development of diabetic nephropathy [8]. The underlying mechanism has yet to be elucidated.

Transcription factor NF-E2 related factor (Nrf2) is a key component of the anti-oxidative response element (ARE), which mediates the cellular oxidative stress response [9]. Nrf2 interacts with ARE to regulate the downstream target genes, including SOD, hemeoxygenase (HO-1), γ-glutamate synthase chain (γ-GCSh) and light chain (γ-GCSL) [10]. Nrf2/ARE signal transduction is the most important endogenous antioxidant stress pathway [11]. Recent studies suggest thatNrf2 is the central regulator of cellular anti-oxidative response, and also the treatment target in oxidative stress [12, 13]. Nrf2/ARE signaling plays an important role in combating environmental stress, neural protection, anti-apoptosis and anti-tumor. The functional mechanism of this pathway in diabetes has yet to be established fully [7, 14].

Discovery of an effective preventative and therapeutic intervention against DN is a hot-button issue. The active constituents of natural Chinese herbal medicine include flavonoids, saponins and lipids, which are widely found in the stems, leaves and fruits of plants [15]. Chinese herbal medicine and its extracts have been widely used in the treatment of various diseases, including the treatment of diabetic nephropathy [16]. *Rosalaevigata* Michx (RLM), also known as Cherokee rose fruit, belongs to family rosaceae. It has often been used as a traditional medicine called Xiaoke for diabetes. In addition to significantly decreasing the levels of serum cholesterol and β2 lipoprotein, reducing the fat deposits in the liver and heart, inhibiting urinary incontinence and aging, RLM also displays significant antioxidant and anti-inflammatory effect [17]. Our previous study found that the fruit of RLM significantly improved disorders of blood glucose and lipids in experimental diabetic SD rats, with a renal protective effect in diabetic rats [18, 19]. The RLM extract has strong antioxidant effect *in vitro* [20], and reduces oxidative stress injury in diabetic rat kidneys [21, 22]. In this study, we used an experimental DN model to investigate the molecular mechanism of RLM extract inactivating and improving the oxidative stress and inflammatory response via Nrf2/ARE signaling. We provide experimental evidence supporting RLM in the prevention and treatment of DN.

Materials and methods

Experimental animals

Animal experiments were conducted in accordance with the animal welfare legislation and after approval by the University of South China Ethics Committee. Sixty Sprague-Dawley (SD) healthy male rats, were purchased from the experimental animal Department of University of South China (license number: SYXK (Xiang) 2009-0011), with an average weight of 150±20 g. The animals were maintained at a room temperature of around 25°C and a relative humidity of about 70%, with adaptive feeding for 1 week.

Drugs

The RLM aqueous extract containing the crude drug 2.5 g/mL was purchased from Hengyang City Chinese herbal medicine company (Hengyang, China). Briefly, the RLM crude drug was de-seeded and powdered, added to Chinese herbal medicine infusion (ZG-280 Zhong Cheng Pharmaceutical Machinery Factory Hunan, Jishou, China). After soaking in water for about 30 minutes, the aqueous fruit extract of RLM was obtained at a final concentration of 2.5 g/mL. Bacterial streptozotocin (STZ) (Sigma, St. Louis, MO, USA) (0.1 mol/L) prepared in sodium citrate buffer (pH 4.2) was stored in an ice bath for immediateuse within 10 min, to reduce the STZ titer.

Diabetic rat model

After 1 week of daily 8 h fasting (with water), SD rats with no abnormal initial blood glucose levels were used for the follow-up experiments. Forty SD rats were fed high-glucose and high-fat diets (provided by the Animal Department of the University of South China, containing sucrose 37%, lard 10% and cholesterol 2%). After 8 weeks, following 8 h fasting with water, blood glucose was detected and the diabetic rat model was produced by intraperitoneal injection of STZ (65 mg/kg). After intraperitoneal injection for 3 days, fasting blood glucose (FBG) levels greater than 14 mmol/L as indicated by glucose test strips were representative of diabetic rat model [23, 24]. The remainder of the rats were randomly divided into two groups: a DN model group (DN group) comprising 17 rats and an RLM treatment group (DN+RLM group) consisting of 16 rats. In addi-

tion, 20 age-matched SD rats were randomly assigned to normal control (NC) and RLM control (NC+RLM) groups, with 10 rats each. Rats in the DN+RLM and NC+RLM groups were, respectively, fed the fluid RLM extract (5 g/kg/D) by gavage. DN and NC groups were treated with equal volume of saline gavage. After successful model development, all the rats were fed the usual feed, normal drinking water, and the experiments ended after 16 weeks of continuous administration. During the experiment, erythromycin ointment was applied to the rat tail to prevent infection.

At the end of the experiment, under chloral hydrate anesthesia abdominal aortic blood was drawn, bilateral kidneys were extracted, weighed and the renal index (kidney/body weight) was calculated. Using the physiological saline to flush the blood, a portion of the kidney tissues was fixed in 10% formalin, and the remainder preserved at -80°C.

Serum and renal tissue oxidative stress and antioxidant capacity

At the end of the experiment, blood was collected from the abdominal aorta and serum was isolated. A portion of the renal cortex was mixed with physiological saline to obtain a 10% tissue homogenate. The supernatant was removed after low temperature centrifugation. The rat serum and renal tissue homogenate levels of malondialdehyde (MDA), SOD, total anti-oxidant capacity (T-AOC), and ROS were detected using commercial MDA, SOD, T-AOC detection kit (Nanjing Jiancheng Biomedical Engineering Co. Ltd., Nanjing, China). The ROS levels in rat serum and kidney tissues were detected using the ROS detection kit (Shanghai Yueyan Biological Technology Co., Ltd., Shanghai, China). All procedures were performed in strict accordance with the kit instructions.

ELISA of IL-6, IL-1β and MCP-1 levels in serum and kidney tissues of rats

At the end of the experiment, blood was collected and serum was isolated from the abdominal aorta. A portion of the renal cortex was mixed with 10% tissue homogenate, and the supernatant was removed at low temperature. The levels of IL-6, IL-1β and MCP-1 in rat serum and kidney were detected using ELISA (Wuhan Bo Shi Dei, Wuhan, China). All procedures were performed in strict accordance with the kit instructions.

Immunohistochemistry

Nrf2 antibody was purchased from Santa Cruz Biotechnology in USA (Delaware Ave Santa Cruz, USA). The SP immunohistochemistry detection kit, DAB chromogenic agent, 0.1 M PBS, and 0.01 M citric acid salt for antigen repair were purchased from Fuzhou Maixin Biotechnology Company (Fuzhou, China). All procedures were conducted strictly in accordance with kit instructions.

Positive cells were identified by brown particles in the cytoplasm/cell nucleus. Semi-quantitative analysis was carried out according to the degree of positive staining and percentage of colored cells. Negative cells were not stained. The weakly-staining positive cells were light brown with no background color. The number of positive cells was less than 25%. Moderately positive cells were stained dark brown with a background light brown. The number of positive cells was more than 25% and less than 50%. The strongly positive cells were stained dark brown with no background color, and the number of positive cells was more than 50%.

Western blot

About 0.2 g of kidney tissue was removed from the -80°C refrigerator, washed with pre-cooled PBS 3 times and the tissue was cut into EP tubes using ophthalmic scissors. Appropriate volume of liquid (100 mmol/L NaCl, 10 mmol/L Tris-HCl pH 7.6, 1 mmol/L EDTA PH 8.0, 1 μg/mL Aprotinin, and 100 μg/mL PMSF) was added to the specimen. The total and nuclear protein was extracted using the BCA protein detection kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions, followed by Western blotting. ImagJ2 software (Madison, WI, USA) was used to detect the optical density of the imprinted region.

Real-time fluorescence quantitative PCR

Total RNA was extracted using the Total RNA Kit (Invitrogen, Carlsbad, CA, USA) according to the kit instructions and reverse-transcribed to cDNA with AMV enzyme (Promega, Madison, WI, USA). Expression of target gene was detected with qRT-PCR using SYBR-green-containing

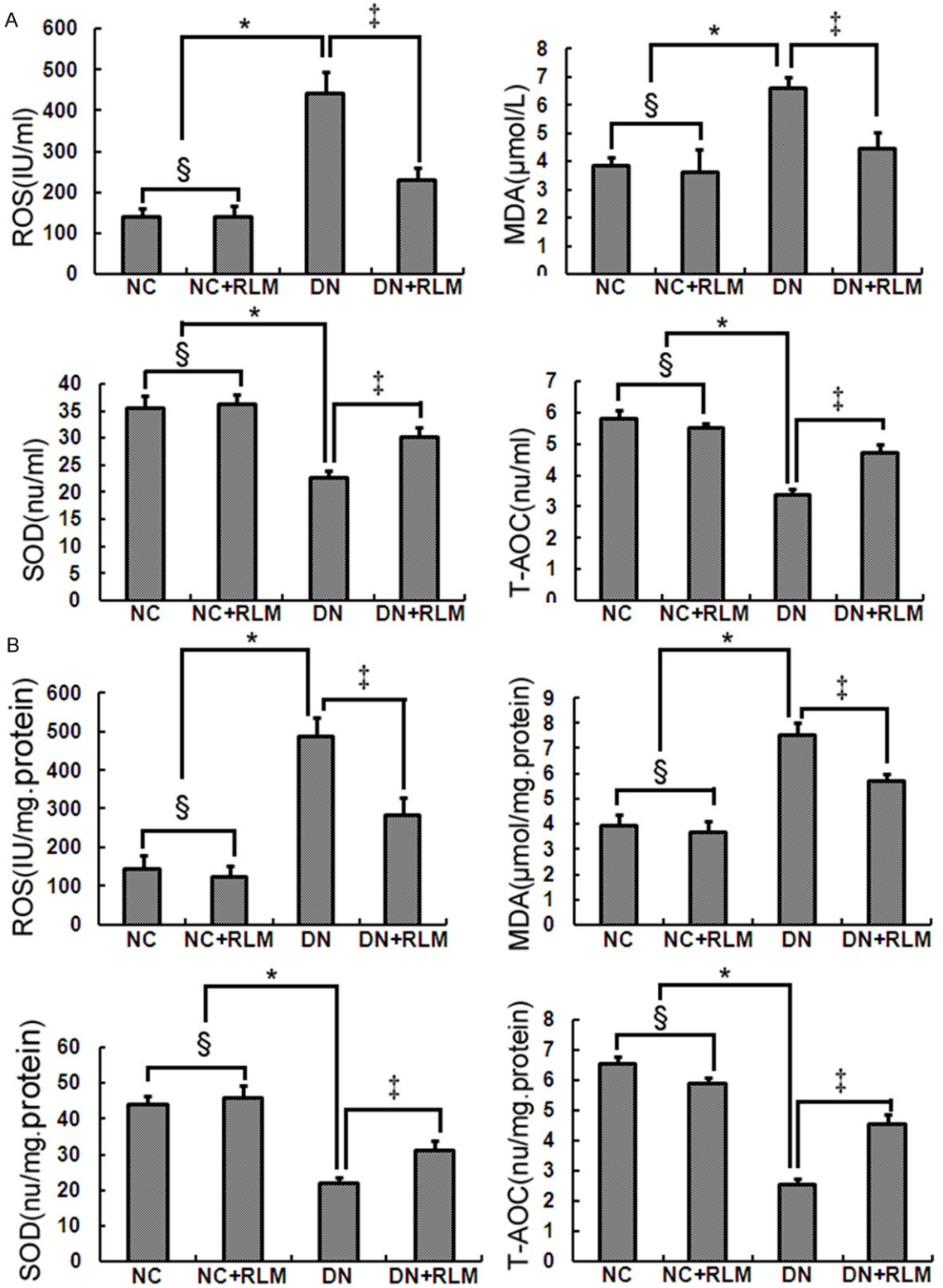

Figure 1. The effect of RLM extract on ROS, MDA, T-AOC and SOD levels in rat serum and kidney tissue. The levels of ROS, MDA, T-AOC and SOD in rat serum (A) and renal tissue homogenate (B) of each group were tested. NC: normal control group, NC+RLM: RLM control group, DN: model group, DN+RLM: RLM treatment group; and compared with NC group: *: $P < 0.05$, §: $P > 0.05$; compared with DN group: ‡: $P < 0.05$. ROS, reactive oxygen species; MDA, malondialdehyde; T-AOC, total anti-oxidant capacity; SOD, superoxide dismutase.

Table 1. Changes in rat serum IL-6, IL-1β and MCP-1 ($\bar{x}$±s)

Groups	Cases	IL-6 (ng/L)	IL-1β (ng/ml)	MCP-1 (pg/ml)
NC	10	16.8±1.89	0.07±0.04	129.5±6.28
NC+RLM	10	15.5±2.69§	0.08±0.01§	123.8±7.13§
DN	15	81.9±5.33*	0.47±0.18*	238.4±9.38*
DN+RLM	15	48.7±3.53*,‡	0.16±0.11*,‡	162.6±8.24*,‡

Compared with NC group: *, $P < 0.001$, §, $P > 0.05$; Compared with DN group: ‡: $P < 0.01$. vs. NC group: *, $P < 0.001$, §, $P > 0.05$; vs. DN group: ‡: $P < 0.01$.

Table 2. Changes in IL-6, IL-1β and MCP-1 in rat renal cortex homogenate ($\bar{x}$±S)

Groups	Cases	IL-6 (ng/mg protein)	IL-1β (ng/mg protein)	MCP-1 (pg/mg protein)
NC	10	12.3±1.31	0.08±0.02	108.7±8.31
NC+RLM	10	12.5±1.64§	0.09±0.01§	107.2±6.28§
DN	15	58.3±6.62*	0.33±0.05*	201.4±10.36*
DN+RLM	15	32.9±5.37*,‡	0.12±0.08*,‡	151.5±9.26*,‡

Compared with NC group: *, $P < 0.001$, §, $P > 0.05$; Compared with DN group: ‡: $P < 0.01$. vs. NC group: *, $P < 0.001$, §: $P > 0.05$; vs. DN group: ‡: $P < 0.01$.

PCR kit (GenePharma, Shanghai, China). Primers were as follows: HO-1 (NM_012580.2): upstream primer 5'-CGCCTCCAACCAGCGAGTGG-3'; downstream primer: 5'-ATGCTGTCGAGCTGTGGGCG-3' (127 bp); γ-GCS (NM_012815.2): upstream primer 5'-GGAAACGCCGGAAGGAGGCT-3'; downstream primer 5'-ACCAAAGCGGGGGTGCTTGT-3' (188 bp); GAPDH (NM-017008): upstream primer 5'-TCAAGAAGGTGGTGAAGCAG-3'; downstream primer 5'-AGGTGGAAGAATGGGAGTTG-3' (111 bp). Using GAPDH as the standard reference gene, the relative expression of each target gene was determined using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 (SPSS, Chicago, IL, USA). The numerical value was expressed by $\bar{x}$±s. Pairwise comparisons were analyzed using one-way ANOVA and Student's t-test. $P < 0.05$ (two-sided) suggested statistical significance.

Results

The RLM extract significantly reduced the levels of oxidative stress in serum and renal tissue of DN rat, and increased the antioxidant capacity

In the course of this experiment, 7 SD rat models were not successful and therefore, were excluded. In the successful group, the DN group showed 2 deaths, 1 in DN+RLM, while the NC group and NC+RLM showed no death. We have successfully established the DN model. RLM extract improved the DN rat blood glucose and lipid levels as well as renal dysfunction and pathology [19, 22].

We evaluated the effect of RLM extract in DN rat, by measuring the levels of oxidative stress and antioxidant capacity of DN rat serum and renal tissue in each group. As shown in **Figure 1**, compared with NC group rats, ROS and MDA levels in rat serum and renal tissue of the DN group increased significantly, T-AOC and SOD activity decreased significantly ($P < 0.05$). After treatment of DN rats (DN+RLM group) with RLM extract, the ROS and MDA levels in rat serum and renal tissues decreased significantly ($P < 0.05$), T-AOC and SOD activity increased significantly ($P < 0.05$), without reaching the level of NC group ($P < 0.05$). The serum and renal ROS and MDA levels as well as T-AOC and SOD in the NC and NC+RLM groups were not significantly different ($P > 0.05$). The results showed that RLM exhibited a strong antioxidant effect, increased total antioxidant capacity in DN rat serum and renal tissue, enhanced the activity of antioxidant enzymes and reduced ROS and lipid oxidation resulting in amelioration of DN oxidative stress injury.

RLM extract significantly lowers renal inflammation in DN rats

Oxidative stress is usually associated with inflammation, which in turn aggravates oxidative stress damage. IL-6, IL-1β, and MCP-1 are inflammatory factors closely related to DN. Therefore, we determined their levels in rat serum and kidney using ELISA. The results (**Tables 1** and **2**) show that compared with the NC group of rats, the levels of MCP-1, IL-1β and IL-6 in rat serum and kidney in DN group increased significantly ($P < 0.001$), while the

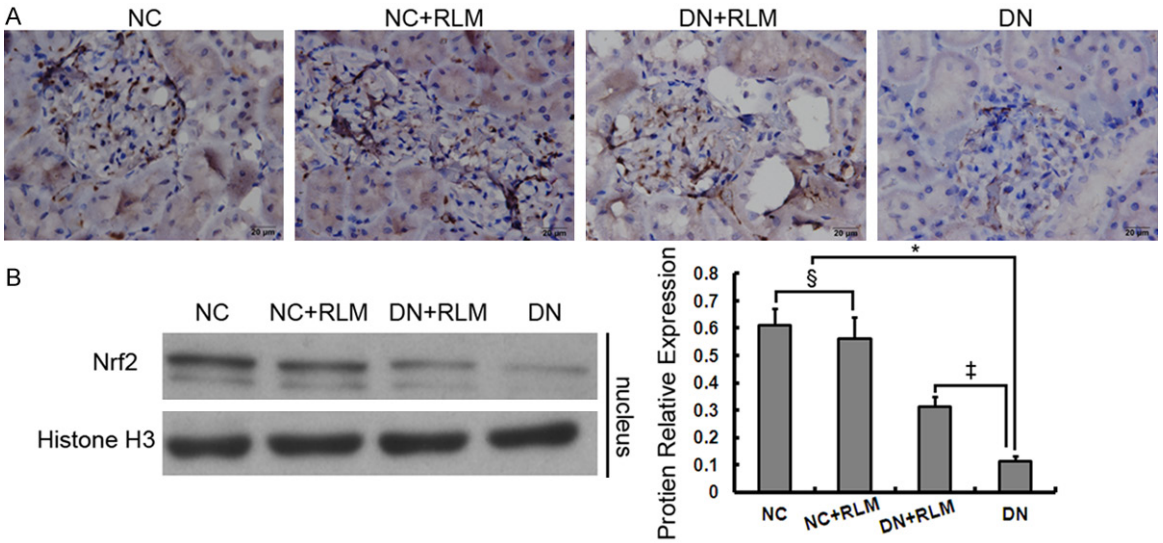

Figure 2. Effect of RLM on the expression of Nrf2 protein in DN rats. Expression of Nrf2 protein in renal tissues of rats was detected by immunohistochemistry and Western blot. A: Immunohistochemistry of Nrf2 protein ×400 Bar = 2040; B: Expression of Nrf2 protein and gray scale scan analysis in renal tissue was detected by Western blot. NC: normal control group, NC+RLM: RLM control group, DN: model group, DN+RLM: RLM treatment group. Compared with group NC: *: P: < 0.01, §: P > 0.05; compared with group DN: ‡: P < 0.05.

levels of IL-6, IL-1β, and MCP-1 in rat serum and renal tissues in DN+RLM group decreased significantly (P < 0.01), but were still higher than in the NC group (P < 0.01). The levels of IL-6, IL-1β and MCP-1 between NC and NC+RLM groups showed no significant changes (P > 0.05) suggesting that RLM exhibited a strong antioxidant effect. RLM improved total antioxidant capacity in DN rat serum and renal tissues, enhanced the body's antioxidant enzyme activity, reduced ROS and lipid peroxidation, and attenuated oxidative stress injury.

RLM extract significantly promotes the expression and activation of Nrf2 in renal tissue of DN rats

The Nrf2/ARE signal transduction pathway is the most important endogenous antioxidant stress mechanism. Nrf2 is the central regulator of cellular oxidative stress. Therefore, we examined the effects of RLM extract on the expression of Nrf2 protein in DN rats. Immunohistochemistry showed that Nrf2-positive expression manifested light yellow, yellow, brown, and dark brown color, mainly in the glomerular cytoplasm and nucleus, with a small amount of expression in renal tubular epithelial cells. Nrf2 expression in NC and NC+RLM groups in rat renal tissues was strongly positive. Nrf2 expression in renal tissue of DN group rats was weakly positive. However, after the treatment of DN rats with RLM extract, Nrf2 protein expression increased significantly (**Figure 2A**). Subsequently, Western blot analysis also revealed that compared with NC group rats, the expression of Nrf2 protein in the renal tissue of DN rats was reduced significantly (P < 0.01), while after treatment with RLM extract (DN+RLM group), renal Nrf2 protein expression of DN rat increased significantly (P < 0.05) (**Figure 2B**). The results showed that DN may significantly down-regulate Nrf2 protein expression leading to abrogation of the antioxidant capacity of the renal tissue of diabetic rats. The RLM extract significantly up-regulated Nrf2 expression and activation, and augmented renal tissue antioxidant capacity.

Effect of RLM extract on the expression of HO-1 and γ-GCS genes downstream of Nrf2 in renal tissue of DN rats

Studies had shown that Nrf2 protein regulated the expression of downstream antioxidant genes after translocation from cytoplasm to the nucleus, leading to enhanced resistance to oxidative stress. Therefore, we detected the expression of HO-1 and γ-GCS downstream of Nrf2 antioxidant enzymes. In DN and NC groups, the expression of HO-1 in renal tissue and γ-GCS was significantly reduced (P < 0.01).

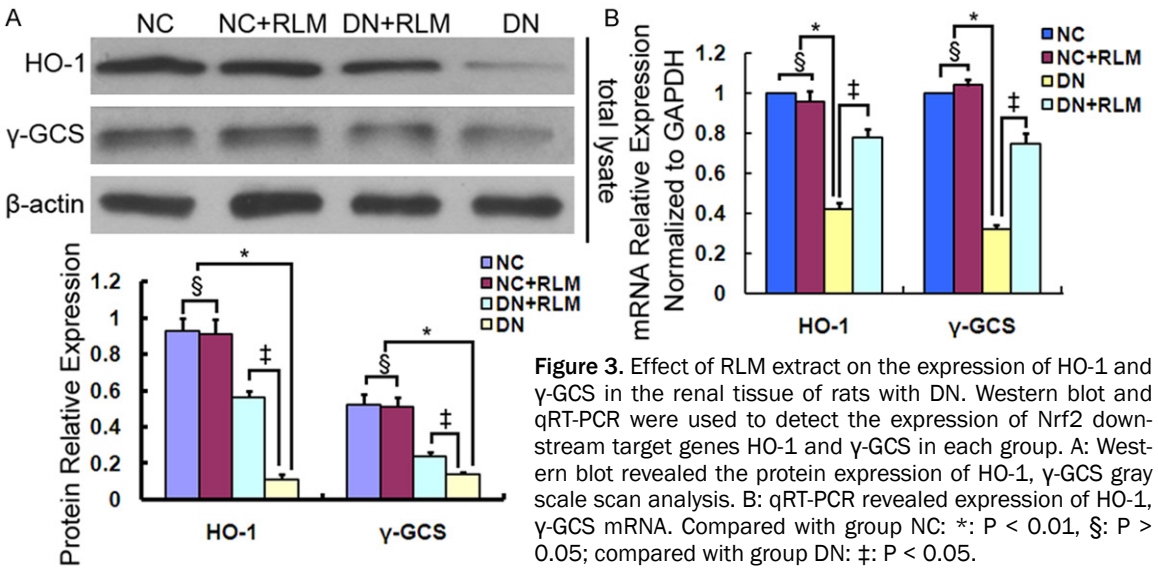

Figure 3. Effect of RLM extract on the expression of HO-1 and γ-GCS in the renal tissue of rats with DN. Western blot and qRT-PCR were used to detect the expression of Nrf2 downstream target genes HO-1 and γ-GCS in each group. A: Western blot revealed the protein expression of HO-1, γ-GCS gray scale scan analysis. B: qRT-PCR revealed expression of HO-1, γ-GCS mRNA. Compared with group NC: *: $P < 0.01$, §: $P > 0.05$; compared with group DN: ‡: $P < 0.05$.

In DN rats, the expression of HO-1 and γ-GCS in renal tissue was significantly increased ($P < 0.05$) after treatment with RLM extract (DN+RLM group). The expression of HO-1 and γ-GCS in renal tissue of rats in the two groups of NC and NC+RLM showed no significant change ($P > 0.05$) (**Figure 3**). These results suggested that the RLM extract up-regulated the gene expression of antioxidant enzymes via activation of the Nrf2/ARE signaling and enhanced renal tissue antioxidant capacity, resulting in resistance to oxidative stress in DN.

Discussion

In vivo and *in vitro* experiments showed that oxidative stress was significantly enhanced in diabetes mellitus, and was one of the most fundamental causes of chronic complications in diabetes mellitus [4, 24]. Oxidative stress is usually associated with inflammatory reactions, and increased oxidative stress injury. Oxidative stress refers to the imbalance between oxidation and antioxidation *in vivo*, which is mainly skewed toward oxidation to generate a large number of intermediate products. When the body is exposed to a variety of harmful stimuli, excessive ROS and reactive nitrogen free radicals (RNS) overwhelm the antioxidant capacity, resulting in tissue damage. ROS is the main cause of oxidative stress [25], and the primary marker of the level of oxidative stress in the organism [26]. MDA is the end-product of lipid peroxidation. It is commonly used to evaluate the level of oxidative stress. The MDA levels in renal tissue represent the severity of diabetic kidney damage [28]. SOD is the first line of defense against free radicals [29]. The strength of SOD activity reflects the ability of the body to remove free radicals, which is reflected in the antioxidant capacity. T-AOC directly reflects the body's antioxidant capacity. SerumIL-6, IL-1β and MCP-1 levels are used to evaluate DN patients' inflammatory response clinically [30]. In this study, we found that the levels of MCP-1, IL-1β and IL-6 and the level of ROS and MDA in the serum and kidney tissues of rats with DN were significantly increased compared with that of normal rats, while the activities of T-AOC and SOD were significantly lower than in normal rats. The results indicate that oxidative stress and inflammatory reaction mediated the development of DN, which suggests a new therapeutic target.

The use of RLM has a long history. Studies showed that RLM not only exhibited strong hypolipidemic, anti-diuretic and anti-aging properties, but also had significant antioxidant and anti-inflammatory effects [17, 31], with no obvious side effects [32]. We modified the traditional Chinese medicine to obtain the RLM extract, with stronger antioxidant effects *in vitro* [20]. In this study, we assessed the effects of RLM extract on oxidative stress and inflammatory reaction in rats with diabetic nephropathy *in vitro*. The results showed that RLM extract significantly reduced the rat serum and renal tissue IL-6, IL-1β, MCP-1 and ROS, the MDA level of DN group, and significantly increased

the activity of T-AOC and SOD, consistent with previous reports [21, 22]. The results showed that the RLM improved rat serum and renal total antioxidant capacity in DN, enhanced the activity of antioxidant enzymes in the body, reduced ROS and lipid peroxidation, inhibited the release of inflammatory factors, ameliorated the oxidative stress and inflammatory reaction in DN rats.

In response to oxidative stress injury, mediated by ROS, the body induces a series of protective proteins, to counter the cellular damage, which is mediated by ARE. Recent studies show that the transcription factor Nrf2 is an important activating molecule of ARE, which is the central regulator of cellular antioxidant response [14], which mediates cell resistance to various environmental stresses and endogenous defense response. Nrf2 interacts with ARE to trigger the expression of downstream target genes, including that of antioxidant proteins and phase II detoxification enzymes such as SOD, HO-1, γ-GCSh and γ-GCSL [12]. Nrf2 significantly increased the expression of antioxidant enzymes such as SOD, HO-1 and γ-GCS, and enhanced the antioxidant capacity to inhibit the oxidative stress [34]. In this study, we found that treatment of DN rats with RLM extract significantly up-regulated and activated renal Nrf2 expression accompanied with the up-regulation of HO-1 and γ-GCS expression. Results indicated that Nrf2 down-regulation in DN attenuated the antioxidant capacity. Treatment with RLM extract improved DN oxidative stress and inflammation, which may be mediated via activation of Nrf2/ARE signaling, and up-regulation of HO-1 and γ-GCS expression. The related molecular mechanisms need to be explored further.

In summary, significant oxidative stress and inflammation in the kidney induce the development of DN. The Nrf2/ARE pathway may play an important role in the process. RLM inhibits DN oxidative stress and inflammatory response, which may be related to the activation of Nrf2/ARE signaling that further delays or prevents the development of DN.

Acknowledgements

Grants: Natural Science Foundation of Hunan Province (13JJ4077); The Project Foundation of the Science and Technology program of the Hunan Province (No: 2010FJ6025).

Disclosure of conflict of interest

None.

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