

Protective effect of resveratrol on kidney in rats with diabetic nephropathy and its effect on endoplasmic reticulum stress

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Abstract. – OBJECTIVE: Diabetic nephropathy (DN) can cause chronic renal insufficiency and significantly reduce the life quality of patients with diabetes mellitus, and may eventually lead to death. The study investigated the expression of endoplasmic reticulum stress-related factors, which have important roles in the progress of DN and to explore effects of resveratrol on DN and the possible mechanism.

MATERIALS AND METHODS: Specific pathogen free (SPF) grade healthy male Sprague Dawley (SD) rats were divided into different groups for different treatments. The diabetic rat model was established by intraperitoneal injection of low-dose streptozotocin (STZ) (40 mg/kg). The normal rats and diabetes model rats were divided into four groups including normal control group (N), normal control + resveratrol (N+R), model group (M), and model + resveratrol group (M+R) for different treatments. The changes of renal histology were observed by immunohistochemistry. Glucose oxidase/peroxidase method was used to measure FPG, UP 24 h content was measured by bicinchoninic acid (BCA) assay, BUN, Scr and Cys C content were measured by automatic biochemical analyzer. The expressions of 78 kDa glucose-regulated protein (GRP78), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP) were analyzed by Western blot.

RESULTS: Resveratrol treatment significantly reduced the fasting blood glucose level, urinary protein level and renal pathological damage. The phosphorylation of PERK in the kidney of rats with diabetes was up-regulated, while resveratrol treatment reduced this change. The expression of p-PERK, GRP78, ATF4, and CHOP was significantly increased in rats with diabetes, while resveratrol treatment can reduce the increased level of those endoplasmic reticulum stress related factors.

CONCLUSIONS: Resveratrol has a good therapeutic effect on DN in rats without side effect. The mechanism may be related to the regulation of endoplasmic reticulum stress response.

Key Words:

Diabetic nephropathy, Endoplasmic reticulum stress, Resveratrol.

Abbreviations

DN: Diabetic nephropathy; ER: Endoplasmic reticulum; SPF: specific pathogen free; SD rats: Sprague Dawley rats; NPD: normal pellet diet group; HFD: high-fat diet group; BCA: bicinchoninic acid; STZ: streptozotocin; HE: Hematoxylin and Eosin; ECL: enhanced chemiluminescence; GRP78: glucose-regulated protein; PERK: protein kinase RNA-like endoplasmic reticulum kinase; ATF4: activating transcription factor 4; CHOP: C/EBP-homologous protein; SD: standard deviation.

Introduction

Diabetes mellitus, which usually can lead to chronic renal failure, causes the increasing unacceptable high mortality and morbidity rates all over the world^{1,2}. The life quality of the patients with diabetes can be significantly reduced not only due to the abnormal physiological conditions caused by diabetes itself but also due to the pathological changes caused by the complications of diabetes^{3,4}. Diabetic nephropathy (DN), affecting about 30% of type 1 diabetes patients and 25% type 2 diabetes patients, is the main cause of cardiovascular and end-stage renal failure mortality of patients with diabetes mellitus^{5,6}. Many treatment methods have been developed to treat DN in the medical care of patients with diabetic nephropathy including glycemic control, management of hypertension, reducing dietary salt intake, phosphorus and potassium restriction in advanced cases⁷. Diabetic nephropathy is the most hazardous of the complications of diabetes, and is responsible for

excess morbidity and mortality in patients with type 1 and type 2 diabetes⁸. So it is of great clinical value to develop new drugs or new treatment methods to improve the life quality and survival rate of the patients with DN. A previous research has reported other natural compounds such as thymoquinone and proanthocyanidin, which might attenuate renal damage in diabetic nephropathy in rats⁹. Resveratrol, a natural phenolic compound, can function as antioxidant to protect the body from the damage caused by internal factors and external factors^{10,11}. Previous studies have shown that resveratrol had good curative effects in the treatment of various human diseases including cardiovascular disease¹², Parkinson's disease¹³, cancer¹⁴ and so on. It has been shown that resveratrol treatment can reduce the oxidative stress to alleviate DN through AMPK/SIRT1-independent pathway¹⁵. Resveratrol can also modulate angiogenesis to deduce the damage to kidney caused by diabetes¹⁶. Endoplasmic reticulum (ER) stress, which can affect transcriptional regulation and metabolism, is the key player in the pathogenesis of DN¹⁷. In cancer cells, resveratrol was found to be able to induce apoptosis by regulating the expression of ER stress-related factors through the interaction with PERK pathway¹⁸. So the interaction between resveratrol and ER stress-related factors may also play a role in the pathogenesis of DN, and the identification of those interactions may facilitate the development of new treatment method to treat DN. In our work, the effects of resveratrol on DN were explored by detecting the expression of ER stress-related factors in normal and DN rats with and without resveratrol treatment. The effects of resveratrol renal function were also observed. We found that resveratrol had a good therapeutic effect on DN in rats and no side effect was found. The possible mechanism is that resveratrol can reduce ER stress by downregulating ER stress-related factors in rats with DN.

Materials and Methods

Experimental Animals

Specific pathogen free (SPF) grade adult male Sprague Dawley (SD) rats with body weight of 200 ± 20 g were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). All the rats were bred in SPF environmental ($22 \pm 3^\circ\text{C}$, 50% humidity)

with a 12-h-light/12-h-dark cycle. The animal research protocol was approved by the institutional guidelines of the Animal Care and Ethics Committee at Yantaishan Hospital (Yantai, Shandong Province, China). Unless otherwise specified, all other reagents were purchased from Sinopharm Group Chemical Co., Ltd., (Shanghai, China).

Rat Diabetes Model Establishment

The rats were randomly divided into normal pellet diet group (NPD) as normal control group and high-fat diet group (HFD), 24 rats in each group. After 4 weeks' breeding, high-dose of STZ (Sigma-Aldrich, St. Louis, MO, USA) (50 mg/kg) was injected intraperitoneally into the rats of HFD group, while rats in the NPD group were injected intraperitoneally with an equal volume of citrate-sodium citrate buffer. One week after the operation, fasting blood was extracted from the rats for 3 consecutive days to measure the blood glucose. Blood glucose ≥ 16.7 mmol/L indicates the successful establishment of diabetes model.

Animal Groups and Treatments

The normal rats and diabetes model rats were divided into four groups including normal control group (group N), normal control + resveratrol (group N+R), model group (group M), and model + resveratrol group (group M+R) for different treatments. The rats in N+R and M+R groups were orally administered with resveratrol (Sigma-Aldrich, St. Louis, MO, USA) (50 mg/kg/day) and the rats in N and M groups were treated with the same volume of 0.5% carboxymethyl cellulose (CMC) sodium solution (Sigma-Aldrich, St. Louis, MO, USA).

Specimen Collection

After drug treatment, the rats were only fed with water for 12 h. Blood samples were extracted from tail vein and centrifuged to collect serum for the detection of blood glucose, TC, TG, and other biochemical indicators. 24 h urine was collected, centrifuged and stored at 4°C for the detection of urine protein (UP 24 h). Next, the animals were sacrificed, the heart was treated with pre-cooling saline intubation to wash the kidney blood. After taking the kidneys, a part of the kidneys was fixed with 4% paraformaldehyde for immunohistochemistry. The rest of the kidneys were preserved for Western blot.

Blood Glucose, Blood Lipids, Renal Function and Urinary Protein Determination

Glucose oxidase/oxidase method (Shanghai Meilian Biotechnology Co. Ltd., Shanghai, China) was used to measure the fasting plasma glucose (FPG). Levels of blood urea nitrogen (BUN), serum creatinine (Scr) and cystatin C (Cys C) were measured by automatic biochemical analyzer (Siemens, München, Germany). The concentrations of total cholesterol (TC) and triglycerides (TG) were analyzed by *CHOD-PAP* method (Shanghai Meilian Biotechnology Co. Ltd., Shanghai, China). The 24 h urinary protein (UP24h) content was measured by bicinchoninic acid assay (BCA assay) (Beyotime Biotechnology, Shanghai, China).

Histological Examination of the Renal Tissue

The renal tissue was fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 24-48 h. Then, the tissue was embedded in paraffin (Sigma-Aldrich, St. Louis, MO, USA) and cut in slices with a thickness of 4 μ m. Hematoxylin and eosin (HE) staining (Solarbio, Beijing, China) was performed. After staining, the tissue was observed under a microscope (Olympus, Tokyo, Japan) for pathological examination.

Western Blot

Total protein was extracted by conventional method and bicinchoninic acid (BCA) method was used to determine the protein content. The extracted protein was first denatured at 95°C for 5 min. After that, 30 μ g protein from each sample was subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transmembrane to the polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific, Waltham, MA, USA). The membrane was blocked with 5% skimmed milk followed by incubation with the primary an-

tibodies including rabbit anti-GRP78 polyclonal antibody (1:2000, ab21685, Abcam, Cambridge, MA, USA), rabbit anti-ATF4 polyclonal antibody (ab23760, Abcam, Cambridge, MA, USA), and anti-CHOP monoclonal antibody (1:1000, ab11419, Abcam, Cambridge, MA, USA) were added and incubated overnight at 4°C. After washing, secondary antibody- HRP-goat anti-rabbit IgG antibody (1:2000, HangZhou HuaAn Biotechnology Co., Ltd., Hangzhou, China) was added and incubated with the membrane at 37°C for 2 h. After washing, electrochemiluminescence (ECL) solution (Roche, Basel, Switzerland) was added. Bio-Rad gel imaging system (Bio-Rad, Hercules, CA, USA) was used to detect the signal. β -actin was used as the endogenous control.

Statistical Analysis

All data were expressed as mean $\pm$ standard deviation (Mean $\pm$ SD) and processed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The comparisons between more than two groups were performed by one-way ANOVA analysis. Least significant difference (LSD) test was used for the comparison between multiple groups. $p < 0.05$ was considered to be statistically significant.

Results

Effects of Resveratrol on Body Weight (BW), Kidney Index, Kidney Weight to Body Weight (KW/BW), FPG, TC and TG in Rats with DN

The levels of all the indexes including BW, KW/BW, FPG, TC, and TG in rats of M group were all increased significantly compared with those in the rats of N group ($p < 0.01$) (Table I, Figures 1-2). For example, the average body weight of the rats in N group was 285.3 ± 24.2 g, while the average body weight of the rats in M group was 348.9 ± 49.2 g. It was found that resveratrol treatment could significantly reduce BW,

Table I. Effects of resveratrol on BW, KW/BW, FPG, TC and TG (mean $\pm$ SD).

Group	BW (g)	KW/BW (g/kg)	FPG (mM)	TC (mM)	TG (mM)
N	285.3 $\pm$ 24.2	3.78 $\pm$ 0.73	4.96 $\pm$ 0.87	1.63 $\pm$ 0.38	0.43 $\pm$ 0.06
N+R	273.7 $\pm$ 31.6	3.94 $\pm$ 0.64	4.59 $\pm$ 0.79	1.48 $\pm$ 0.33	0.41 $\pm$ 0.09
M	348.9 $\pm$ 49.2 ^a	7.02 $\pm$ 0.77 ^a	18.54 $\pm$ 2.65 ^a	2.86 $\pm$ 0.29 ^a	0.89 $\pm$ 0.05 ^a
M+R	288.4 $\pm$ 51.6 ^b	4.35 $\pm$ 0.52 ^{a,b}	13.04 $\pm$ 2.96 ^{a,b}	1.98 $\pm$ 0.43 ^{a,b}	0.65 $\pm$ 0.09 ^{a,b}

^a $p < 0.01$ vs. group N; ^b $p < 0.01$ vs. group M.

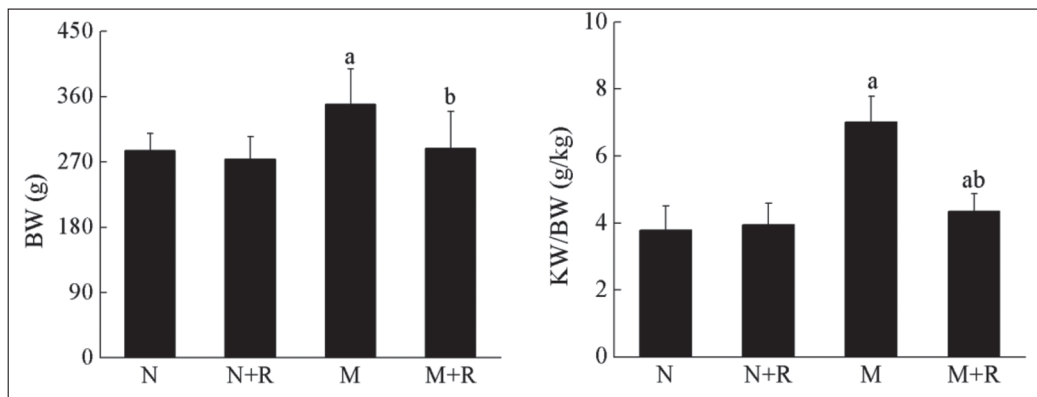


Figure 1. The changes of the body weight (BW), kidney index in rats of each group. ^a $p < 0.01$ vs. group N; ^b $p < 0.01$ vs. group M.

KW/BW, FPG, TC, and TG levels in rats with DN ($p < 0.01$). The average body weight of the rats in M group was 288.4 ± 51.6 g, which is similar to that of the rats in N group, and significantly lower than that of the rats in M group ($p < 0.01$). It was found that resveratrol treatment could slightly reduce BW, KW/BW, FPG, TC, and TG levels in normal rats, but no significant difference was found when compared with the normal rats without resveratrol treatment. Our data indicate that resveratrol can improve the physiological conditions of the rats with DN, but its effects on normal rats were not significant.

Effects of Resveratrol on Renal Function of Rats with DN

In this work, BUN, SCr, Cys C, and UP 24 h levels were used to reflect the renal function of the rats. The average levels of BUN, SCr, Cys C, and UP 24 h of the rats in group M were 13.08 ± 2.64 mM, 83.46 ± 12.34 μ M, 0.75 ± 0.13 mg/L and 80.32 ± 5.83 mg/L, respectively, which were all significantly higher than those in group N (6.85 ± 1.64 mM, 2.63 ± 10.44 μ M, 0.43 ± 0.06

mg/L and 23.54 ± 1.84 mg/L) ($p < 0.01$) (Table II and Figure 3). After resveratrol treatment, the average levels of BUN, SCr, Cys C, and UP 24 h of the rats with DN were significantly reduced to 9.13 ± 1.97 mM, 64.62 ± 16.98 μ M, 0.50 ± 0.18 mg/L and 49.35 ± 7.75 mg/L, respectively ($p < 0.01$). However, no significant difference was found in those indexes between N group and N+R group ($p > 0.05$). Our data indicate that resveratrol can improve the renal function of rats with DN but its effects on the renal function of normal rats were not significant.

Pathological Examination of the Rats in Different Groups

As we can see from Figure 4, no difference was found in the pathological examination between N and N+R group. In the renal tissue of the rats in M group, the glomerular was enlarged, the amount of glomerular mesangial matrix was increased, no significant increase in the number of mesangial cells was found, renal cysts and capillaries narrowed, although renal tubular morphology is still clear, tubule cell hypertrophy, swelling

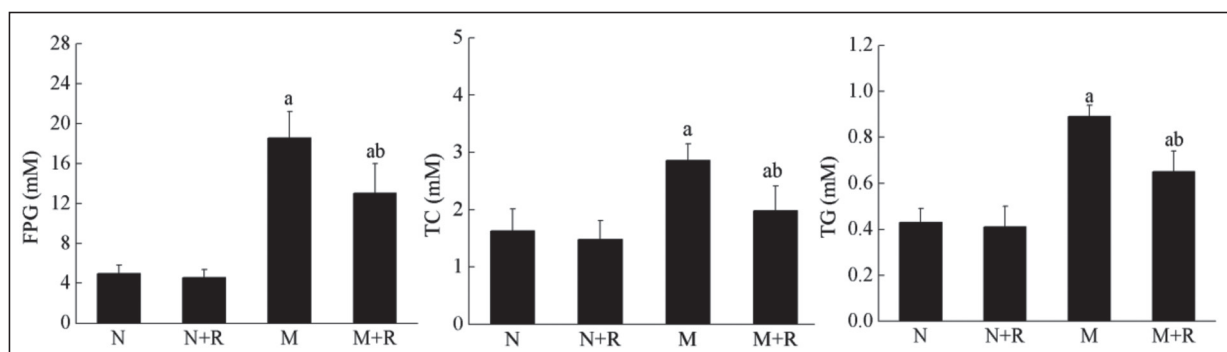


Figure 2. The changes of the content of FPG, TC and TG in rats of each group. ^a $p < 0.01$ vs. group N; ^b $p < 0.01$ vs. group M.

Table II. Effects of resveratrol on renal function of rats with DN (mean $\pm$ SD).

Group	BUN (mM)	Scr (μ M)	Cyst C (mg/L)	UP 24 h (mg/L)
N	6.85 $\pm$ 1.64	42.63 $\pm$ 10.44	0.43 $\pm$ 0.06	23.54 $\pm$ 1.84
N+R	6.94 $\pm$ 1.19	45.47 $\pm$ 9.63	0.47 $\pm$ 0.12	25.03 $\pm$ 2.75
M	13.08 $\pm$ 2.64 ^a	83.46 $\pm$ 12.34 ^a	0.7 $\pm$ 0.13 ^a	80.32 $\pm$ 5.83 ^a
M+R	9.13 $\pm$ 1.97 ^b	64.62 $\pm$ 16.98 ^b	0.50 $\pm$ 0.18 ^b	49.35 $\pm$ 7.75 ^b

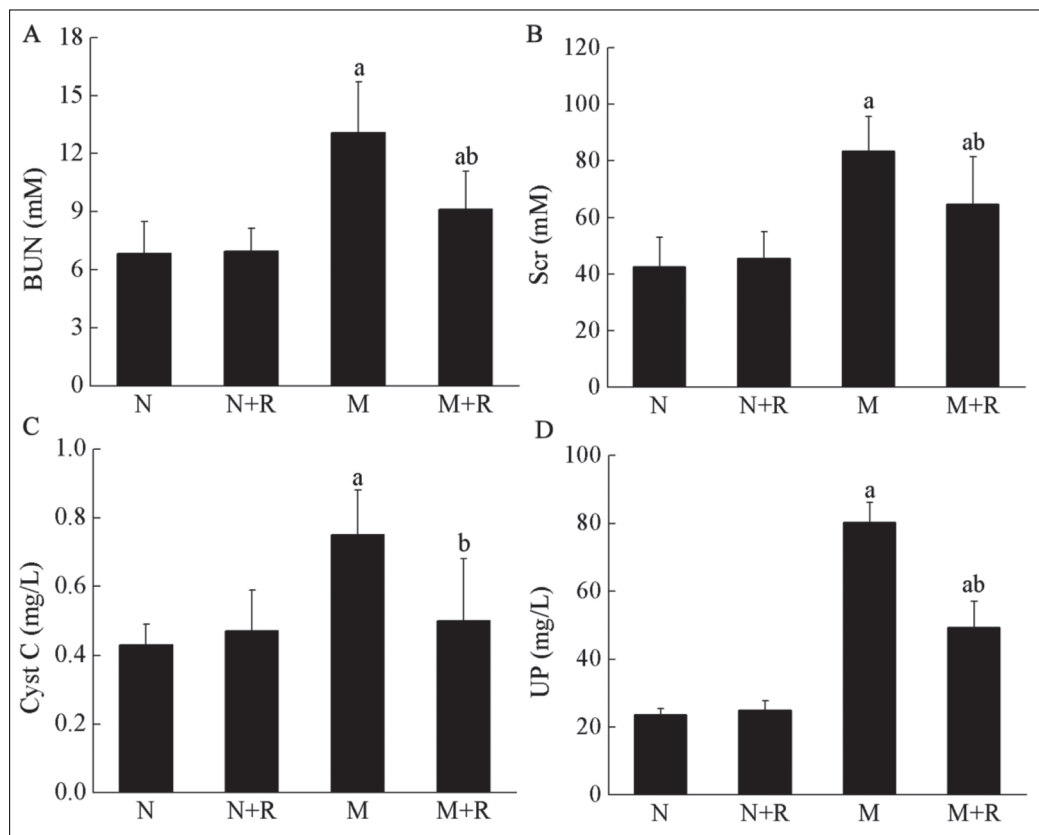
^a $p < 0.01$ vs. group N; ^b $p < 0.01$ vs. group M.

and luminal narrowing were found. Resveratrol reduced those pathological changes caused by DN. The data suggest that resveratrol can improve the renal tissue structure of rats with DN, but its effects on normal rats were not significant.

Resveratrol Treatment Reduced PERK Phosphorylation Levels

Endoplasmic reticulum stress can cause the increased level of p-PERK. As we can see from Figure 5A-B, resveratrol treatment on normal rats can slightly increase the ratio of p-PERK/PERK but showed no significant difference compared

with the p-PERK/PERK ratio in rats without resveratrol treatment ($p > 0.05$). The p-PERK/PERK ratio of the rats in M group was 2.4 times higher than that of the rats in N group. After resveratrol treatment for 6 weeks, the ratio of p-PERK/PERK in rats with DN was significantly lower than that of the rats in M group ($p < 0.05$) and showed no significant difference to that of the rats in N group ($p > 0.05$, only 1.1 times higher than that of the N group). Data suggest that resveratrol treatment can activate the PERK pathway of rats with DN, but it has no obvious effects on that of the normal rats.

**Figure 3.** The changes of the content of BUN, Scr, Cyst C and UP in rats of each group. ^a $p < 0.01$ vs. group N; ^b $p < 0.01$ vs. group M.

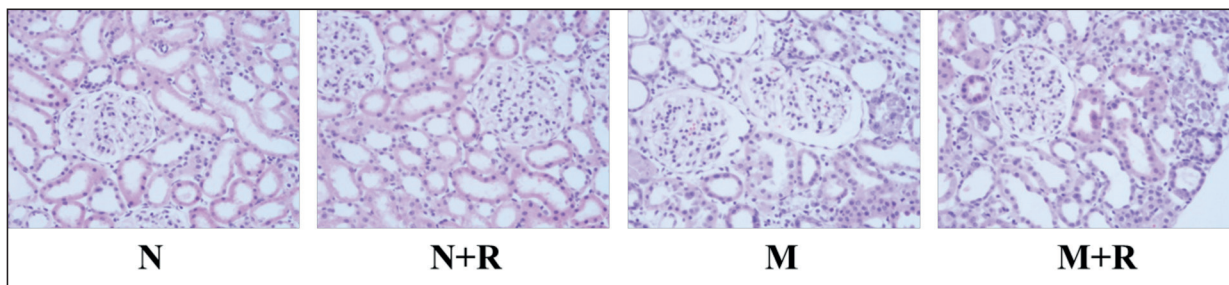


Figure 4. The pathological changes of renal tissue of rates in each group (HE $\times$ 400).

Resveratrol Treatment Reduced the Expression of GRP78, ATF4 and CHOP in the Renal Tissue of the Rats with DN

We examined the expression of GRP78, ATF4 and CHOP, which are in the downstream of endoplasmic reticulum stress signaling pathway, in renal tissue of the rats in each group. The expression levels of GRP78, ATF4 and CHOP were significantly up-regulated in the rats of the M group compared with those of the rats in N

group, while 6 weeks' resveratrol treatment significantly reduced the increased levels of GRP78, ATF4 and CHOP caused by DN ($p < 0.05$) (Figure 6). No significant difference was found in the expression levels of GRP78, ATF4, and CHOP between N group and N+R group ($p < 0.05$). Data suggest that resveratrol treatment can reduce the expression of GRP78, ATF4, and CHOP in rats with DN, but it has no effects on that of the normal rats.

Discussion

DN can significantly damage renal functions by causing various abnormal physiological and structural conditions, which in turn reduce the life quality of the patients^{19,20}. Various vulnerable groups suffered from the disease^{21,22}. No symptoms in the early stage of DN are shown and symptoms usually appear 5 to 10 years after the beginning of kidney damage²³. DN usually cannot be completely cured by any treatment methods except kidney transplantation. The prevention and the glycemic control are the better treatments for DN²⁴. In the ESRD, both dialytic treatment and the transplantation occur. However, the glycemic control always seems to be the better treatment²⁵. Moreover, it's important to consider the different stages of DN and microalbuminuria. Therefore, the aim of DN treatment is to control the complications and reduce the kidney damage progression rate. It has been reported that the progress of DN can be slowed down by improving the abnormal physiological conditions caused by diabetes, such as reduce the protein in urine and control the high and blood pressure levels²⁵. Agents that can target oxidative stress and inflammation to alleviate the damage caused by reactive oxygen species (ROS), were shown to be able to reduce renal damage in patients with DN. However, those

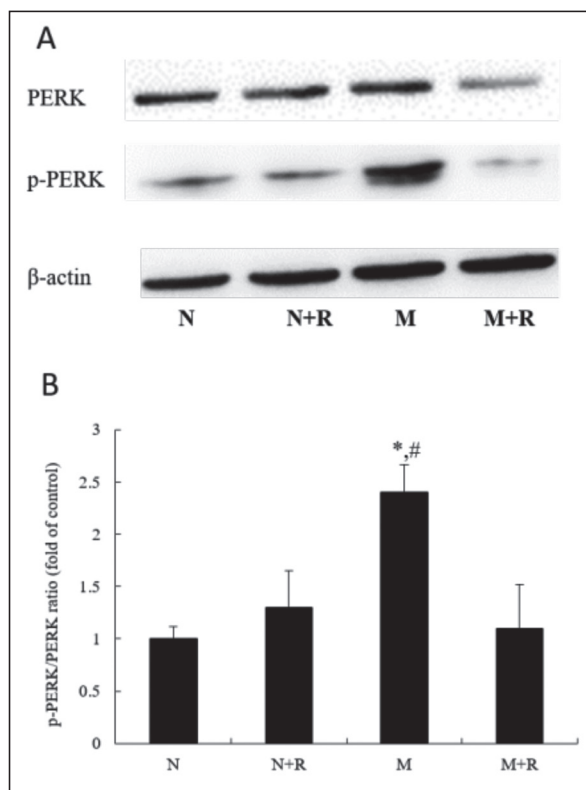


Figure 5. The detection of p-PERK and PERK in renal tissue of rats in each group. **A**, p-PERK and PERK protein in renal tissue detected by Western blot; **B**, The p-PERK/PERK ratio of the rats in each group. * $p < 0.01$ vs. group N; # $p < 0.05$ vs. group M+R.

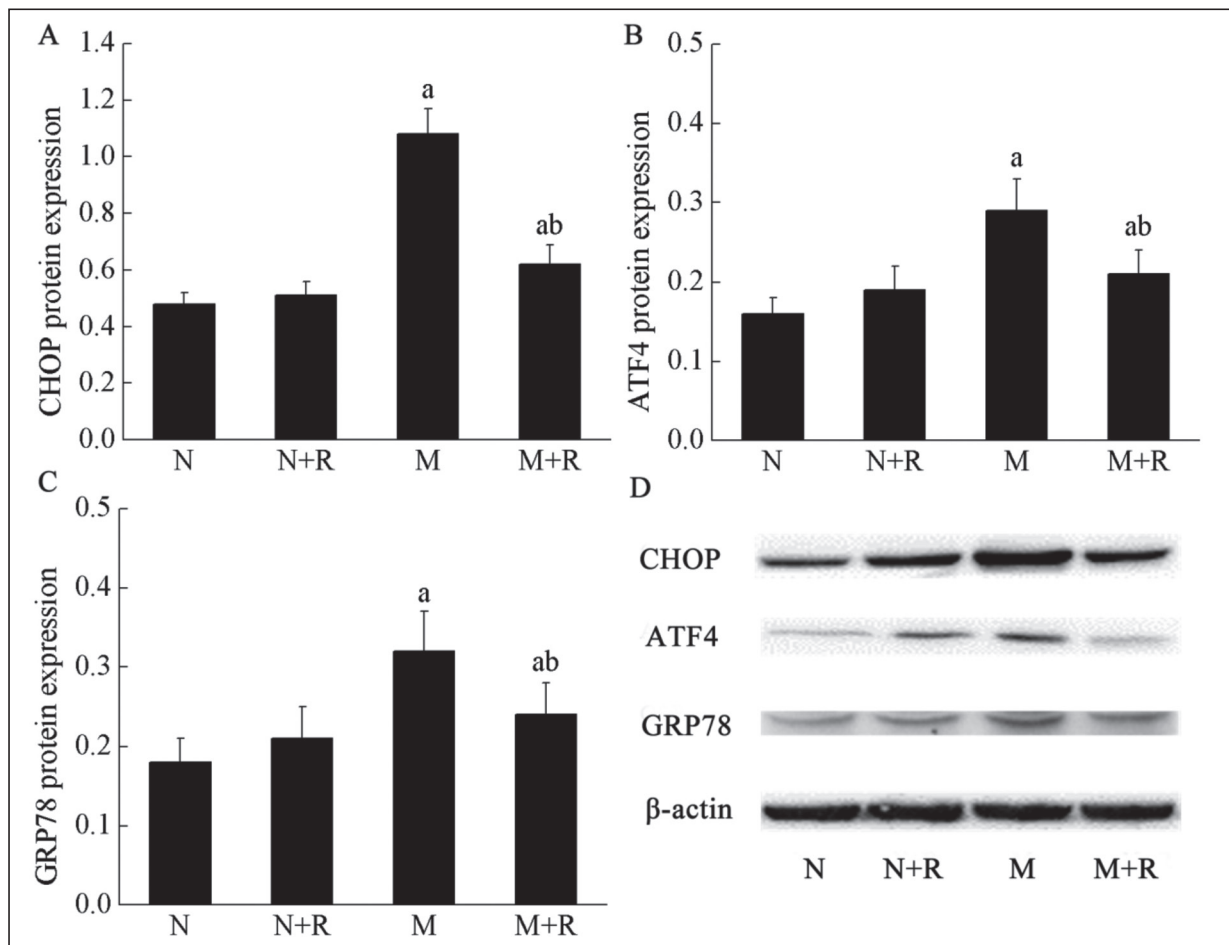


Figure 6. The expression of GRP78, ATF4 and CHOP in the renal tissues of rats in each group. **A-C**, Quantitative analysis of CHOP, ATF4 and GRP78 according to Western blot analysis, the bars represent mean $\pm$ SD; **D**, The expression of CHOP, ATF4, and GRP78 detected by Western Blot. ^a $p < 0.01$ vs. group N; ^b $p < 0.01$ vs. group M.

agents may cause increased albuminuria and cardiovascular events²⁶. As a natural phenolic compound, resveratrol was found to be able to function as antioxidants to protect the body from the damage caused by internal factors and external factors^{10,11}. Resveratrol treatment was also proved to be a safe and effective way in the treatment of various human diseases¹²⁻¹⁵. In our study, DN was found to be able to cause various abnormal physiological conditions, such as the increased body weight and urine protein (Tables I-II, Figures 1-3), and resveratrol treatment significantly improves those abnormal physiological conditions without significantly affects the normal conditions in rats without DN. Our data suggested that resveratrol treatment may be a safe and effective way in the treatment of DN. The normal tissue and cell structures are the basis for the various physiological and

biochemical processes, and the abnormality in tissue and cell structures may induce the changes in cell functions or even the development of disease. Previous studies have shown that renal tissue and cell morphology in patient with DN can significantly reduce the renal function²⁷. So the improvement of renal tissue and cell morphology will improve the renal function²⁷. Consistently with previous studies, the morphological changes renal tissue and cell, such as the enlarged glomerular, increased amount of glomerular mesangial matrix and tubule cell hypertrophy, swelling and luminal narrowing, were also observed in our work (Figure 4), indicating the successfully established DN rat model (Figure 4). After resveratrol treatment, the abnormal renal tissue and cell morphology in rats with DN were significant improved. Also, resveratrol treatment showed no significant

effects on the renal tissue and cell morphology in normal rats, indicating that resveratrol can improve the renal tissue and cell structure specifically in rats with DN. It has been well accepted that ER stress, which is related to the onset and development of various human disease, also plays a central role in the pathogenesis of DN¹⁷. Previous studies²⁸ have shown that the activated PERK pathway caused by the oxidative stress can induce ER stress. The increased ER stress is associated with the increased progression rate of DN and the decreased ER still usually can slow the progress²⁹. GRP78, ATF4, and CHOP are three ER stress-related factors that function in the downstream of PERK^{30,31}. The signaling pathway formed by those three ER stress-related factors and other related factors can be triggered by internal or external factors to regulate the expression of related genes, which in turn cause the increased ER stress and eventually lead the abnormal cell functions and even the development of some disease. In this research, increased protein levels of p-PERK (the activated form or PERK), GRP78, ATF4, and CHOP were found in rats with DN compared with those in rat with DN, indicating the increased ER stress induced by DN (Figures 5-6). After resveratrol treatment for 6 weeks, the protein level of p-PERK, GRP78, ATF4, and CHOP were also significantly reduced in rats with DN (Figures 5-6). However, no significant change in the expression of p-PERK, GRP78, ATF4 and CHOP was found in normal rat before and after resveratrol treatment (Figures 5-6). Our data suggest that resveratrol treatment can reduce the ER stress through PERK pathway specifically in rats with DN.

Conclusions

Resveratrol revealed a good therapeutic effect on DN in rats. Resveratrol treatment can reduce the increased level of PERK phosphorylation and endoplasmic reticulum stress-related factors (GRP78, ATF4, and CHOP) caused by DN. Resveratrol treatment can also improve the renal injury in rats with DN. Our data indicate that the protective effects of resveratrol on rats with DN are related to its function of endoplasmic reticulum stress regulation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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