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ORIGINAL ARTICLE

Resveratrol Protects Against Renal Damage *via* Attenuation of Inflammation and Oxidative Stress in High-Fat-Diet-Induced Obese Mice

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Abstract—Oxidative stress and inflammation play an important role in the chronic kidney disease associated with obesity. Resveratrol (RSV) has been reported to exhibit a wide range of biological activities including antioxidant and anti-inflammatory properties. The objective of the present study was to investigate the effects of RSV on renal inflammation and oxidative stress in obese mice induced by high-fat diet. Male C57BL/6 mice were induced to have nephropathy associated obesity by high-fat diet for 12 weeks. After 8 weeks of feeding, oral supplementation with 100 mg RSV/kg body weight/day was applied with the high-fat-diet feeding for another 4 weeks. The results showed that RSV treatment protected against renal damage induced by high-fat diet, as evidenced by the decreased serum creatinine and urea nitrogen levels, alleviation of glomerular damage, and tubular vacuolization. In addition, RSV enhanced the antioxidant enzyme activity; improved the expression of genes related to inflammation; and decreased the malondialdehyde, tumor necrosis factor-α, and interleukin-6 concentrations in the kidney of high-fat-diet mice. In conclusion, RSV could alleviate renal damage in obese mice induced by high-fat diet *via* suppressing inflammation and oxidative stress.

KEY WORDS: resveratrol; inflammation; oxidative stress; high-fat diet; obesity; mice.

INTRODUCTION

Obesity is the principal and escalating health problem in the industrialized world, and it is also becoming widespread in developing countries [1]. In recent years, numerous studies suggested a close relationship between obesity and renal dysfunction [2–4]. Obesity has been proven to increase a high risk for chronic kidney disease (CKD) [3]. Also, CKD is becoming as an important health threat because it is not only a predictor for the progression to end-stage renal failure but also an independent risk for cardiovascular mortality [5, 6]. Although the mechanism of CKD associated with obesity is multifactorial, an abnormal inflammatory response and oxidative stress in the kidney play a crucial role. Previous studies observed in obese patients and experimental animals that overexpression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, and high levels of reactive oxygen species (ROS) occurred in the kidney [1, 7, 8]. TNF-α deficiency attenuated renal fibrosis, glomerulosclerosis, inflammation, and oxidative stress in high-fat diet (HFD)-fed wild-type mice, as evidenced by the improved structure and function, decreased gene expression

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of pro-inflammatory cytokines, and redox status favoring antioxidants [8]. Therefore, the attenuation of inflammation and oxidative stress in the kidney may be a potential treatment for nephropathy associated with obesity.

Resveratrol (RSV, trans-3,5,4′-trihydroxystilbene) is a polyphenol found in many plants with anti-inflammatory, antioxidant, and anti-tumor properties. RSV has been shown to improve renal damage in various situations. Indeed, supplementation of RSV has been demonstrated to prevent diabetic nephropathy in mice and rats, probably at least in part through modulating inflammatory response and antioxidant defense systems [9–11]. In addition, RSV has been suggested to regulate the renal inflammatory response in mice induced by lipopolysaccharide, as indicated by the inhibition of cytokine release and TLR4 activation [12]. In a recent report, it showed that RSV treatment prevented HFD-induced renal damages in mice at least partially through the enhanced lipolytic genes [13]. However, limited information about the effects of RSV on the renal damage in obese mice induced by HFD is available.

Previous studies observed that C57BL/6 mice on a HFD developed renal functional and pathological abnormalities similar to those observed in patients with obesity [3, 14, 15]. The evidence suggests that this model is accepted as a suitable nephropathy-associated obesity model for human. Therefore, the objective of the present study was to determine the effects of RSV on the renal inflammatory response and oxidative stress in C57BL/6 mice induced by HFD.

MATERIAL AND METHODS

Animal Treatments

Male C57BL/6 mice with a body weight of 19–21 g were purchased from the Animal Multiplication Centre of Qinglong Mountain (Nanjing, China). All mice were housed in individual cages under standard condition with controlled humidity (40–60%) and temperature (20–24 °C), 12/12 light/dark cycles, and access to food and water *ad libitum*. After 1 week of adaption, mice were divided into three groups: (1) control (CON) group: mice were fed with normal diet (TP23302; fat 10%, carbohydrate 71.0%, protein 19.0%; Trophic Animal Feed High-Tech Co., Ltd., Nantong, China) for 12 weeks. From week 9 to week 12, mice were oral fed with 0.5% carboxymethylcellulose sodium (CMC-Na, diluted in 0.9% normal saline; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China); (2) HFD group: mice were fed with HFD (TP23300; fat 60%, carbohydrate 20.6%, protein 19.4%; Trophic Animal Feed High-Tech Co., Ltd., Nantong, China) for 12 weeks. From week 9 to week 12, mice were oral fed with 0.5% CMC-Na; (3) HFD-RSV group: mice were fed with HFD for 12 weeks. From week 9 to week 12, mice were oral fed with 100 mg RSV/kg body weight/day (99%; TCI Co., Ltd., Tokyo, Japan) diluted in 0.5% CMC-Na. The HFD-induced obese mice model was based on the model reported by Li *et al.* [16]. These doses of RSV were selected according to the previous studies [17, 18]. The food consumption and body weight were measured daily and weekly, respectively. The experimental duration was 12 weeks.

At the end of experiment, animals were anesthetized after overnight fasting. Blood samples were collected from eyeballs for biochemical analysis. Kidneys were rapidly harvested, weighed, and processed. The renal index was obtained by dividing the wet kidney weight by mice weight and multiplying by 100. One half of the unilateral kidney was fixed in 4% buffered paraformaldehyde (pH 7.4) at 4 °C overnight and embedded in paraffin for morphological staining. The remaining half and the contralateral kidney samples were frozen in liquid nitrogen immediately for subsequent evaluation.

Biochemical Analysis

Serum was isolated from blood by centrifugation (2000×*g* at 4 °C for 15 min). In order to assess renal function in mice, urea nitrogen (UN; catalog no. C103-2) and creatinine (Cr; catalog no. C011-1) were measured by commercial kits (Nanjing Jiangcheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Morphological Analysis

The kidney samples were embedded in paraffin. Cross sections of the segments were cut at a thickness of 5 μm and stained with hematoxylin and eosin. Stained areas were viewed using an optical microscope (Nikon Eclipse 80i; Nikon, NY, USA) with a magnifying power of × 200.

Measurements of TNF-α and IL-6

The kidney concentrations of TNF-α (catalog no. E-EL-M0049c) and IL-6 (catalog no. E-EL-M0044c) were determined by enzyme-linked immunosorbent assay (ELISA) using commercial assay kits (Elabscience

Biotechnology Co., Ltd., Wuhan, China) according to the protocols of the manufacturer.

The Analysis of Redox Status

The activities of total superoxide dismutase (T-SOD; catalog no. A001-1), glutathione peroxidase (GPX; catalog no. A005), and glutathione (GSH; catalog no. A006-1) and malondialdehyde (MDA; catalog no. A003-1) levels were determined using the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The protein concentrations in the kidney homogenate were quantified following the Bradford method [19]. All the results were normalized against protein content in each sample for inter-sample comparison.

Quantitative RT-PCR Analysis

Total RNA was isolated from a snap-frozen kidney using TRIzol reagent (TaKaRa Biotechnology Co. Ltd., Dalian, China) according to the manufacturer's instructions. The RNA integrity was checked on 1% agarose gel with ethidium bromide staining. The RNA concentration and purity were determined from OD260/280 readings (ratio > 1·8) using a spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA). After then, total RNA (1 μg) was reverse-transcribed into complementary DNA (cDNA) using the PrimeScript™ RT Reagent Kit (TaKaRa Biotechnology Co. Ltd., Dalian, China) according to the manufacturer's guidelines.

The primer of IL-10 [20], IL-6 [21], TNF-α [20], adhesion G protein-coupled receptor E1 (F4/80) [22], integrin alpha X (CD11c) [22], monocyte chemotactic protein 1 (MCP-1) [21], toll-like receptor 4 (TLR4) [23], tumor necrosis factor receptor-associated factor 6 (TRAF6) [23], beta-actin (β-actin) [23] genes were used according to previous studies. All primers are shown in Table 1. Expression of mRNA was quantified *via* real-time PCR using the SYBR® Premix Ex Taq™ Kit (Takara Biotechnology Co. Ltd., Dalian, China) and the QuantStudio®5real-time PCR Design & Analysis system (Applied Biosystems, USA). The reaction mixture was prepared using 2 μL of cDNA, 0.4 μL of forward primer, 0.4 μL of reverse primer, 10 μL of SYBR Premix Ex Taqe (TaKaRa Biotechnology Co. Ltd., Dalian, China), 0.4 μL of ROX Reference Dye (TaKaRa Biotechnology Co. Ltd., Dalian, China), and 6.8 μL of double-distilled water. The PCR consisted of a pre-run at 95 °C for 30 s and 40 cycles of denaturation at 95 °C for 5 s, followed by a 60 °C annealing step for 30 s. The conditions of the melting curve analysis were as follows: one cycle of denaturation at 95 °C for 10 s, followed by an increase in temperature from 65 to 95 °C at a rate of 0.5 °C/s. Each sample was run in duplicate and melt curve analysis was performed to validate the specificity of the PCR-amplified product. After normalization against the reference gene β-actin, the relative levels of mRNA expression were calculated using the $2^{-\Delta\Delta Ct}$ method [24]. The values of control group were used as a calibrator.

Statistical Analysis

The mouse was considered as the experimental unit for all analysis ($n = 10$). All data were verified to meet assumptions of normality and homogeneity of variance. All data were analyzed using the SPSS statistical software (version 16.0, SPSS Inc., Chicago, IL). Statistical significance was determined by ANOVA followed by Tukey's multiple means comparison test. The results are reported as mean ± standard error. Significance (p value) was evaluated at 0.05.

Table 1. Sequences for Real-time PCR Primers

Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)
TNF-α	GGCAGGTCTACTTTGGAGTCATTGC	ACATTCGAGGCTCCAGTGAATTCGG
CD11c	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTC
IL-6	AGTTGCCTTCTTGGGACTGA	CCACGATTTCCCAGAGAAC
TLR4	TCAGAGCCGTTGGTGTATCTT	CCTCAGCAGGGACTTCTCAA
MCP-1	TCAGCCAGATGCAGTTAACGC	TGATCCTCTTGTAGCTCTCCAGC
F4/80	GAGTGGAATGTCAAGATGTTA	CAGTGGAAGAAGAGAAGC
TRAF6	GCCGAAATGGAAGCACAG	GGGCTATGGATGACAACAGG
IL-10	TGCTGCCTGCTCTTACTGAC	AGAAAGTCTTCACCTGGCTGA
β-actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA

TNF-α tumor necrosis factor alpha, *CD11c* integrin alpha X, *IL-6* interleukin 6, *TLR4* toll-like receptor 4, *MCP-1* monocyte chemotactic protein 1, *F4/80* adhesion G protein-coupled receptor E1, *TRAF6* tumor necrosis factor receptor-associated factor 6, *IL-10* interleukin-10, *β-actin* beta-actin

Table 2. The Body Weight and Kidney Weight in Mice

Item	CON	HFD	HFD-RSV
Initial body weight (g)	20.24 ± 0.32	20.70 ± 0.29	19.92 ± 0.23
Final body weight (g)	25.12 ± 0.18	30.04 ± 0.90*	28.86 ± 0.68
Body weight gain (g)	4.89 ± 0.25	9.34 ± 0.80*	8.94 ± 0.63
Kidney weight (g)	0.36 ± 0.01	0.44 ± 0.02*	0.40 ± 0.01
Renal index (%)	1.45 ± 0.03	1.49 ± 0.04	1.39 ± 0.03

Data are presented as the mean ± standard error, $n = 10$. Significant difference is depicted as $^{*}p < 0.05$ when compared with the CON group. CON, mice fed a normal diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD, mice fed a high-fat diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD-RSV, mice fed a high-fat diet and were oral fed with 100 mg resveratrol /kg body weight/day

RESULTS

Growth Performance and Kidney Weight

As expected, at the end of week 12, the final body weight, body weight gain, and absolute kidney weight of mice were significantly increased by HFD feeding ($p < 0.05$, Table 2). However, 4 weeks of RSV treatment did not affect the above three parameters in the HFD-RSV group compared with the HFD group ($p > 0.05$). There was no significant difference in the relative weight of the kidney among different treatments ($p > 0.05$).

Serum Parameters

The concentrations of Cr and UN were significantly increased in the serum of HFD group compared with the CON group ($p < 0.05$, Fig. 1a, b). The Cr and UN levels in the serum of HFD-RSV group were decreased by RSV treatment compared with the HFD group ($p < 0.05$).

Histological Analysis

As shown in Fig. 2, the CON group had normal kidney morphology (Fig. 2a), while hypertrophy, enlarged mesangial area, and increased renal tubular vacuolar changes observed in the HFD group (Fig. 2b). RSV decreased the HFD-induced glomerular volume and tubular changes (Fig. 2c).

Cytokine Concentrations

As shown in Fig. 3, mice in the HFD group had an increased in IL-6 and TNF-α concentrations in the kidney compared with those in the CON group ($p < 0.05$, Fig. 3a, b). Oral supplementation with RSV decreased the renal

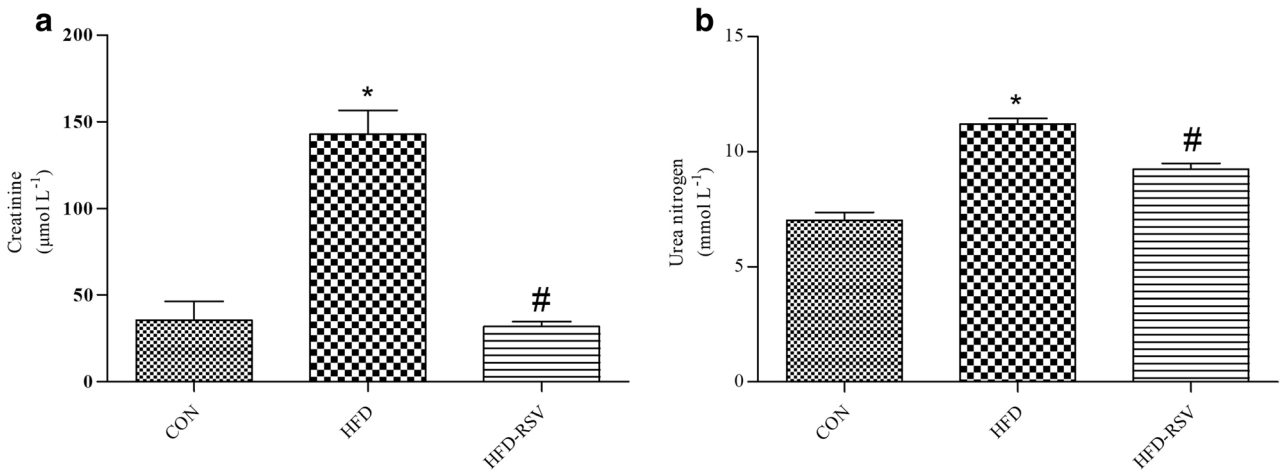

Fig. 1. The serum creatinine (**a**) and urea nitrogen (**b**) in mice. The column and its bar represented the means value and standard error, $n = 10$, respectively. Significant difference is depicted as $^{*}p < 0.05$ when compared with the CON group, $^{\#}p < 0.05$ when compared with the HFD group. CON, mice fed a normal diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD, mice fed a high-fat diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD-RSV, mice fed a high-fat diet and were oral fed with 100 mg resveratrol /kg body weight/day.

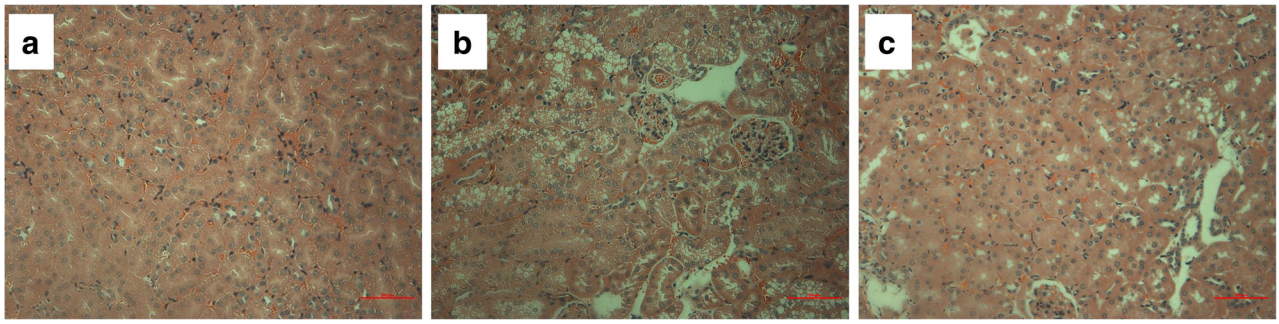

Fig. 2. The renal histology of mice (HE staining, original magnification: × 200). **a** CON. **b** HFD. **c** HFD-RSV. CON, mice fed a normal diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD, mice fed a high-fat diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD-RSV, mice fed a high-fat diet and were oral fed with 100 mg resveratrol/kg body weight/day.

TNF-α and IL-6 concentrations in the HFD-RSV group when compared with the HFD group ($p < 0.05$).

Redox Homeostasis

Compared with the CON group, HFD significantly increased the MDA concentration and decreased the T-SOD and GPX activities in the kidney ($p < 0.05$, Fig. 4a–c). The MDA concentration, T-SOD, and GPX activities in the kidney were significantly attenuated ($p < 0.05$) in HFD-RSV group compared with the HFD group. However, the GSH level in the kidney of mice was similar among all groups ($p > 0.05$, Fig. 4d).

Gene Expression Related to Inflammation

As shown in Fig. 5, HFD significantly increased ($p < 0.05$) the gene expression of TLR4, CD11c, MCP-1, and F4/80 in the kidney of the HFD group compared with the CON group. RSV treatment significantly decreased ($p < 0.05$) the mRNA expression of TLR4, CD11c, MCP-1, F4/80, TNF-α, and IL-6, whereas increased ($p < 0.05$) the mRNA expression of IL-10 in the HFD-RSV group compared with the HFD group.

DISCUSSION

Emerging evidence demonstrated that obesity is an independent risk for development and progression of CKD [3, 25]. Obesity itself could induce renal damage, which may culminate in end-stage renal disease. RSV has been reported to alleviate many diseases associated with obesity such as nonalcoholic fatty liver disease, type 2 diabetes, and cardiac hypertrophy [9, 26, 27]. However, whether

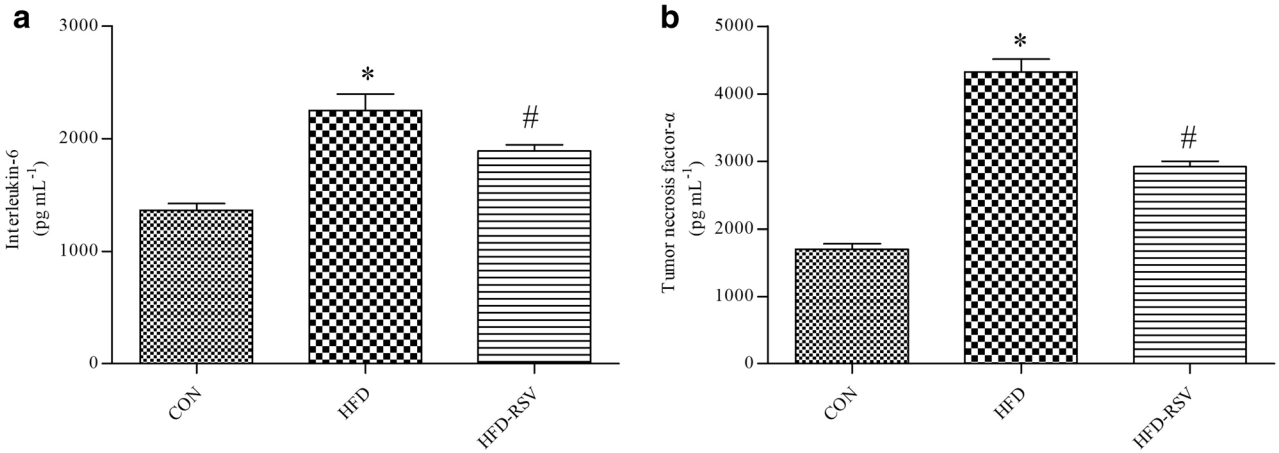

Fig. 3. The cytokine levels of kidney in mice. The column and its bar represented the means value and standard error, $n = 10$, respectively. Significant difference is depicted as $^{*}p < 0.05$ when compared with the CON group, $^{\#}p < 0.05$ when compared with the HFD group. CON, mice fed a normal diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD, mice fed a high-fat diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD-RSV, mice fed a high-fat diet and were oral fed with 100 mg resveratrol/kg body weight/day.

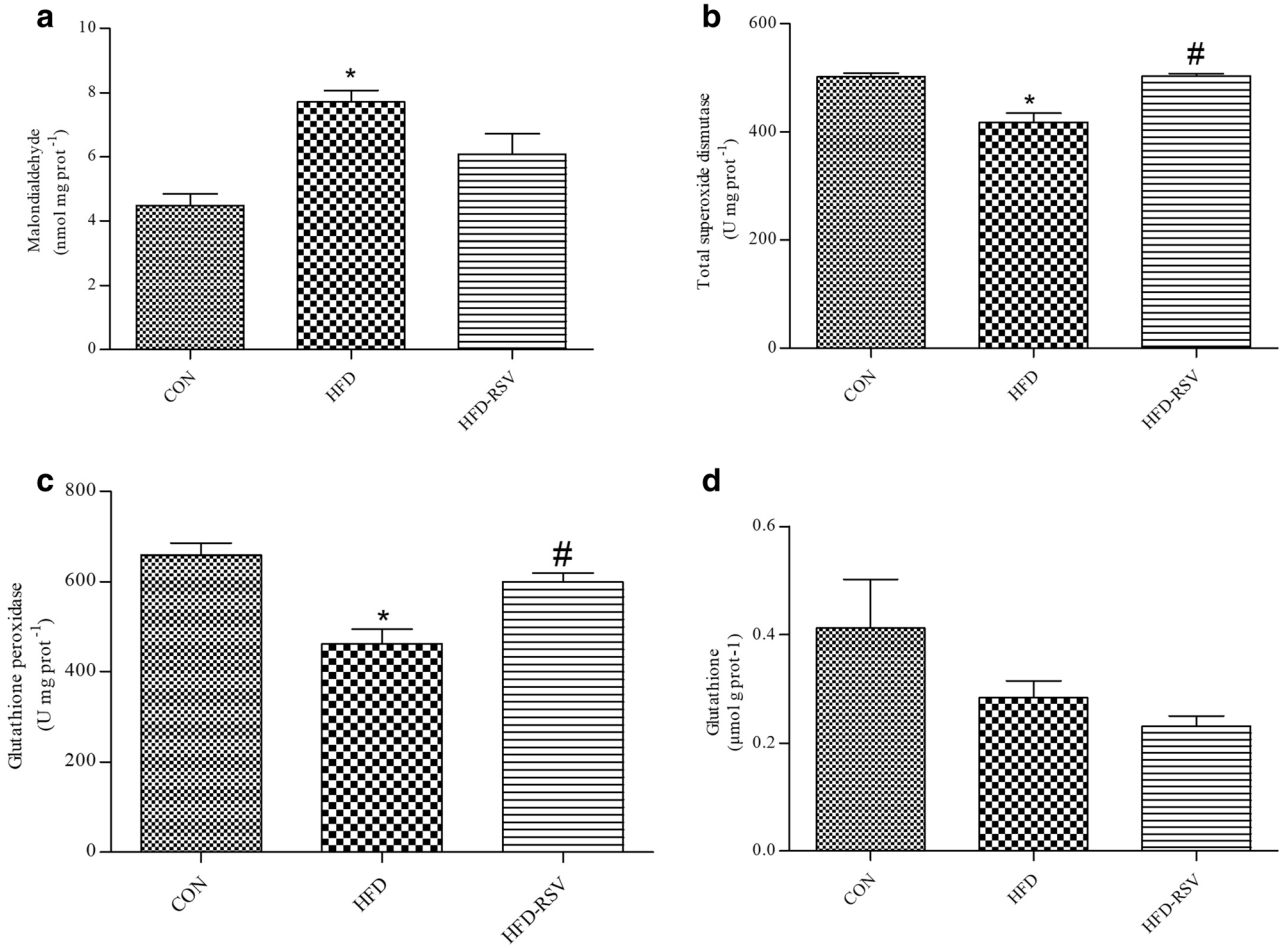

Fig. 4. The redox status of kidney in mice. The column and its bar represented the means value and standard error, $n = 10$, respectively. Significant difference is depicted as $^{*}p < 0.05$ when compared with the CON group, $^{\#}p < 0.05$ when compared with the HFD group. CON, mice fed a normal diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD, mice fed a high-fat diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD-RSV, mice fed a high-fat diet and were oral fed with 100 mg resveratrol/kg body weight/day.

RSV could exert the protective effects on nephropathy induced by obesity is still unclear. In the present study, we showed that HFD provoked an overt obesity characterized by the increased body weight gain. In addition, the kidney weight was increased by HFD feeding. We also found that HFD induced renal damage evidenced by the elevated serum Cr and UN levels, and impaired renal morphology structure. Additionally, oxidative stress, increased pro-inflammatory cytokine production, and infiltration of inflammatory cells in HFD mice kidney were also observed. These data indicated that the nephropathy-associated obesity model was successfully established. As expected, aforementioned results except the weight of body and kidney were reversed by RSV treatment in different levels, suggested that RSV ameliorated the renal damage in obese mice induced by high-fat diet partly due to the improvement of redox and immune status.

It is well known that inflammation and oxidative stress play vital roles in renal injury associated with obesity. The balance of pro- and anti-inflammatory cytokines plays an important role in human and animal health. If the balance was destroyed, it also would contribute to renal damage. We found that HFD increased the TNF-α and IL-6 protein levels, and numerically decreased the IL-10 gene expression in the kidney, similar to the studies by Pan et al. [28] and Zhou et al. [13], suggesting that the renal inflammation happened in the HFD mice. It is expected that oral RSV increased the mRNA expression of IL10, and decreased the TNF-α and IL6 gene expression and protein levels in the HFD-RSV group. Interestingly, we found that the increased TLR4 mRNA

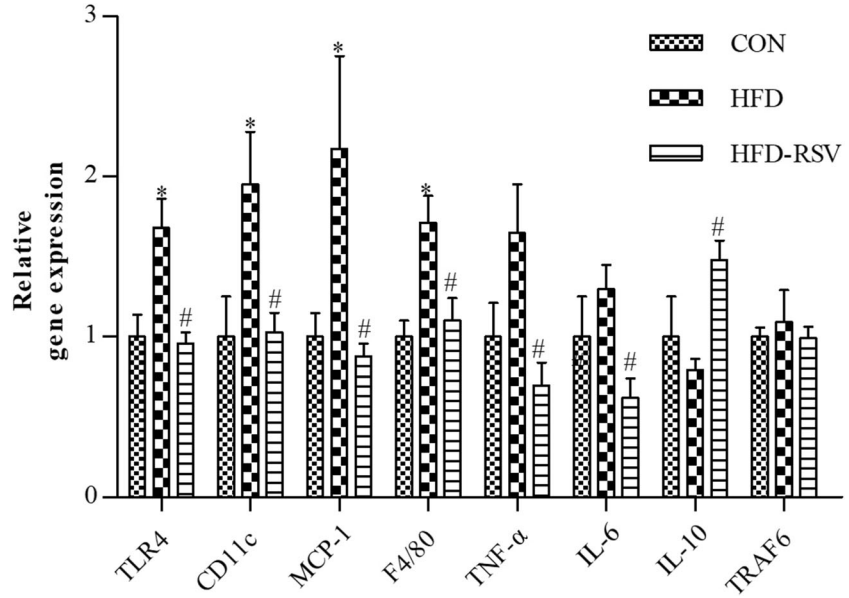

Fig. 5. The expression of genes related to inflammatory response in the kidney of mice. The column and its bar represented the means value and standard error, $n = 10$, respectively. Values are expressed in arbitrary units. The mRNA level of each target gene for the CON group is assigned a value of 1 and normalized against β-actin. Significant difference is depicted as $^{*}p < 0.05$ when compared with the CON group, $^{\#}p < 0.05$ when compared with the HFD group. CON, mice fed a normal diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD, mice fed a high-fat diet and were oral fed with 0.5% carboxymethylcellulose sodium; HFD-RSV, mice fed a high-fat diet and were oral fed with 100 mg resveratrol/kg body weight/day.

expression in the HFD mice was decreased by RSV. To the best of our knowledge, TLR4 plays a key role in renal inflammation. Once activated, the downstream pathways such as MyD88- and TRIF-signaling are initiated and culminate in pro-inflammatory cytokine production, such as TNF-α and IL6. It has been reported that the inhibition of TLR4 activity could suppress inflammation in kidney macrophages and *in vitro* [12]. Moreover, IL-10 is a potent inhibitor of pro-inflammatory cytokines and chemokines [29, 30]. Thus, in the present study, RSV suppressed the inflammation in the kidney in the HFD mice may be partly due to the inhibited TLR4 mRNA expression and increased IL-10 gene level. MDA is an end product of lipid peroxidation, and considered to be a reliable marker of oxidative stress [31]. The antioxidant defense systems in cells including enzymatic and non-enzymatic antioxidants can cooperatively convert ROS into non-toxic substrate. Consistent with the results by Pan et al. [28] and Zhang et al. [32], we also found that HFD mice had higher MDA concentration and lower levels of antioxidants in the kidney than those in the control group. These results suggested that oxidative stress had affected the kidney and damage had occurred. However, RSV attenuated the abnormal MDA and antioxidant levels induced by high-fat diet. In addition, the production of pro-inflammatory cytokines could lead to oxidative stress, which may further increase the inflammatory response, setting a vicious cycle. Similar observations were found in obese rats induced by high-fat diet in which RSV ameliorates renal damage partly by inhibiting the oxidative stress and inflammatory response [28]. In streptozotocin-induced diabetic rats, RSV also abrogated renal damage evidenced by the enhanced antioxidant defense (*e.g.*, SOD and catalase activities) [33], the reduced lipid peroxidation [33, 34], and the normal immune response [35]. In the present study, the increased antioxidant levels and inhibited inflammatory response may account for renoprotective impact of RSV in the obese mice induced by high-fat diet.

In addition, renal inflammation is associated with macrophage infiltration. Macrophage infiltrating into kidney tissue triggers inflammation by producing pro-inflammatory cytokines [12]. During renal inflammation, macrophages are divided into classically activated, pro-inflammatory M1 and alternatively activated, anti-inflammatory M2 type dependent on the phenotype and function [36, 37]. M1 macrophages produce pro-inflammatory mediators, such as TNF-α, IL-6, and MCP-1 [38, 39]. M2 macrophages are characterized by high expression of IL-10 [38, 39]. CD11c [40] and F4/80 [41] are well-known markers of M1 and macrophages, respectively. Previous studies have supported that number and phenotypes of macrophages are critical determinants of inflammation in obese mice [22, 41]. To the best of our

knowledge, in the present study, it is the first time to determine the effects of RSV on renal macrophages infiltration in HFD obese mice. We found that the altered macrophages markers including the CD11c, F4/80, IL-10, and MCP-1 in the kidney of HFD obese mice were reversed by RSV treatment. The results suggested that RSV may exert its anti-inflammatory effect by attenuating macrophage recruitment and facilitating macrophage phenotypic switch from pro-inflammatory M1 to anti-inflammatory M2 in the kidney of HFD obese mice. Moreover, the abovementioned results were supported by the downregulation of the protein expression of TNF-α and IL6. In addition to the recruited monocytes into mouse tissues, local proliferation of macrophages is the primary source of tissue macrophages. Thus, RSV may also play an important role in renal macrophage proliferation. Further work is needed to clarify this potential action of RSV.

In conclusion, RSV treatment alleviated renal damage in HFD obese mice by attenuating inflammation and oxidative stress. Therefore, RSV may serve as a potential therapeutic molecule for obesity-induced kidney injury. However, the precise molecular mechanisms behind the role of RSV in the regulation of renal damage induced by high-fat diet need to be further investigated.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that there is no conflict of interest.

Ethical Approval. All of the procedures involving mice were carried out in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, China. The experiment performed in the present study was approved by the Animal Care and Use Committee of Nanjing Agricultural University.

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