Resveratrol: A new approach to ameliorate hyperhomocysteinaemia-induced renal dysfunction

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Abstract. Hypertension is a common cause of kidney injury and renal damage occurs earlier and is more serious in patients with hypertension and hyperhomocysteinaemia (HHCY). Folic acid (FA) is widely used to ameliorate the organ damage caused by HHCY. However, the effective dose of FA remains controversial and certain studies have suggested that FA increases the risk of cancer. Therefore, it is necessary to identify a safe, effective drug. Resveratrol (RSV) is a natural polyphenol antioxidant. Therefore, the present study explored the effects of RSV on renal damage in spontaneously hypertensive rats (SHRs) with HHCY and its potential underlying mechanism. SHRs were divided randomly into control, HHCY, HHCY + FA and HHCY + RSV groups. Blood pressure, plasma homocysteine, indexes of oxidative stress [serum malondialdehyde (MDA) and superoxide dismutase (SOD) levels] and indexes of renal function [glomerular filtration rate (GFR) and urinary albumin creatinine ratio (UACR)] were assessed. The mRNA and protein expression levels of nephrin and NAPDH oxidase (NOX)2 and NOX4 were detected via reverse transcription-quantitative PCR and western blotting. The results demonstrated that there was no significant difference in BP (blood pressure) among the groups, while the levels of homocysteine (HCY) in the HHCY intervention groups were significantly increased compared with the control. Both FA and RSV decreased the level of HCY, but the decrease was more obvious in the HHCY + FA group. Compared with the control the serum SOD levels and GFR were significantly decreased in the HHCY group, whereas the serum MDA levels and UACR were significantly increased. Moreover, the NOX2 and NOX4 expression levels were significantly increased, whereas those of nephrin were significantly decreased in the HHCY group. The changes caused by HHCY were significantly counteracted in both the HHCY + FA and HHCY + RSV groups and the antioxidant effect was markedly stronger in the HHCY + RSV group. In conclusion, RSV, like FA, potentially improved the renal function damage aggravated by HHCY in SHRs. Furthermore, RSV improved renal function mainly via the inhibition of oxidative stress. RSV may be a potential safe and effective treatment for HHCY-induced hypertensive renal damage.

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Introduction

Homocysteine (HCY) is an important intermediate product of methionine and cysteine metabolism. HCY is normally metabolized *in vivo* and the HCY concentration is usually low (1). However, MTHFR gene mutation or decreased enzyme activity, as well as lack of folic acid, vitamin B6, vitamin B12 and other factors may affect HCY metabolism, which can lead to hyperhomocysteinaemia (HHCY) (2). Hypertension can damage renal function and when combined with HHCY renal damage is worse and occurs earlier (3). Zhao *et al* (4) reported that the synergistic effect of hypertension and HHCY increases the risk of all-cause mortality in middle-aged and older adults in the United States. In China, ~75% of patients with hypertension have HHCY (5) and the renal damage in these patients is worse compared with hypertensive patients without HHCY (6). Therefore, it is necessary to study the mechanism by which HHCY aggravates hypertensive renal damage to identify effective treatments.

The pathological underlying mechanism of HHCY-induced renal disease is complex and numerous factors have been implicated, such as oxidative stress, endoplasmic reticulum stress, inflammation and hypomethylation (7). A previous study demonstrated that oxidative stress is more severe in patients with hypertension with HHCY than in patients without HHCY (8). Moreover, our previous study demonstrated that HHCY aggravates hypertensive renal damage due

to the abnormal expression of NADPH oxidase (NOX)2/NOX4 caused by oxidative stress (9). Furthermore, folic acid (FA) was reported to improve renal damage in spontaneously hypertensive rats (SHRs), which was caused by HHCY (10). However, the effective dose of FA remains controversial and one study has suggested that FA increases the risk of cancer (11). Therefore, it is important to find a safe, effective drug to improve renal function damage worsened by HHCY.

A diet rich in fruits and vegetables is beneficial to health, and an epidemiological study has reported that it has a positive role in preventing kidney disease and renal cancer (12). Resveratrol (RSV) is a polyphenolic antioxidant found in the extracts of peanuts, grapes and plant roots. RSV has various biological activities, including antioxidant, anti-inflammatory, anticancer, antiproliferative and angio-regulatory effects (13). Numerous studies have examined the effects of RSV on antioxidant stress, which demonstrated that it has vascular protective and anti-aging effects (14,15). RSV also significantly decreases the serum HCY levels in rats (16) and reverses HCY-induced oxidative stress (17). Using this information, it was hypothesized that RSV, as a natural antioxidant, may improve hypertensive renal damage aggravated by HHCY.

Therefore, the aim of the present study was to use a HHCY-associated hypertension animal model to investigate whether RSV could reduce renal injury via the inhibition of oxidative stress, and to compare the efficacy of RSV and FA.

Materials and methods

Animal experiments. The animal care and experimental procedures in the present study complied with the regulations of and was approved by the Medical Ethics Committee of Shandong Provincial Qianfoshan Hospital (Jinan, China). Male SHRs (age, 8-10 weeks; weight, 180-200 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and were housed in the animal room of Shandong Provincial Qianfoshan Hospital Medical Research Centre. The SHRs were kept in eight cages under a 12-h light/dark cycle at 23-24˚C with a humidity of 45-55%. All animals were allowed free access to standard rodent food and water. The SHRs were acclimated to their environment for 1 week. Subsequently, the animals were randomly divided into four groups of eight SHRs as follows: i) Control; ii) HHCY; iii) HHCY + FA; and iv) HHCY + RSV. HHCY was induced by administering 2% DL-HCY (5 ml/kg; cat. no. H4628; Sigma-Aldrich; Merck KGaA) twice a day intraperitoneally for 12 weeks. From the fifth week, the SHRs in the HHCY group were treated with 0.9% saline (0.5 ml/day) by gavage, those in the HHCY + FA group were treated with FA (0.4 mg/kg/day; cat. no. F7876; Sigma-Aldrich; Merck KGaA) and the HHCY + RSV group was treated with RSV (30 mg/kg/day; cat. no. SRT501; MedChemExpress) for 8 weeks. Throughout the experiment, the control SHRs were given only an equal volume of 0.9% saline (0.5 ml/day). After 12 weeks of treatment, the SHRs were anaesthetised with pentobarbital sodium (40 mg/kg; Sigma-Aldrich; Merck KGaA) and 1 ml blood was then collected via cardiac puncture and the kidney tissues were dissected. Finally, all SHRs were sacrificed using pentobarbital sodium (150 mg/kg). Death was verified by the cessation of breathing and heartbeat.

Blood pressure (BP) measurement. The BP of conscious SHRs was assessed using the tail cuff method and a non-invasive BP system (cat. no. BP-2010A; Softron Beijing Biotechnology Co., Ltd.) as previously described (18). The BP of the SHRs in each group was assessed before the start of the experiment and after 4, 8 and 12 weeks of the experiment. Each time, the BP was determined three times and then averaged.

Plasma HCY and serum oxidative stress biomarker analysis. At week 4, ~1 ml of rat tail blood was collected and used to detect HCY levels and assess if the SHR HHCY model had worked. The plasma HCY and creatinine levels were analysed using a Cobas8000 automatic biochemistry analyser (Roche Diagnostics) with Cobas 8000 data manager (Version 1.06.05.0516; Roche Diagnostics). Superoxide dismutase (SOD) is an important antioxidant enzyme (19). Malondialdehyde (MDA) is the end-product of lipid oxidation and reflects the degree of oxidative stress (19). The serum MDA and SOD levels were quantified using commercial kits (MDA Assay Kit; cat. no. A003-1; SOD Assay Kit; cat. no. A001-3; Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions.

Detection of renal function indexes. After 12 weeks of treatment, SHRs were placed individually in metabolic cages for urine collection. Serum and urine biochemistry were determined using an automatic biochemical analyser (Roche Diagnostics) with Cobas 8000 data manager (Version 1.06.05.0516, Roche Diagnostics). The urinary albumin creatinine ratio (UACR) and glomerular filtration rate (GFR) were used as the two main indicators of renal function. UACR and GFR were determined as follows: UACR=urinary microalbumin/urinary creatinine; and GFR=(urine creatinine/plasma creatinine) x urine volume/body weight.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the kidney tissue samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Subsequently, complementary (c)DNA was synthesised using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) according to the manufacturer's protocol. The mRNA expression levels were analysed using an ViiA 7 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the SYBR Green real-time PCR reagent (Roche Diagnostics). The thermocycling conditions for qPCR were as follows: Initial denaturation at 95˚C for 10 min; 40 cycles of denaturation at 95˚C for 15 sec, and annealing and elongation at 60˚C for 60 sec; and a final extension at 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec. The relative change in mRNA expression levels was determined using the $2^{-\Delta\Delta Cq}$ method (20) with GAPDH as the internal reference gene. The qPCR primer sequences (BioSune Biotechnology) of the target genes were as follows: NOX2 forward (F), 5'-CTGCCAGTG TGTCGGAATCT-3' and reverse (R) 5'-TGTGAATGGCCG TGTGAAGT-3'; NOX4 F, 5'-ATGTTGGGCCTAGGATTG TGT-3' and R, 5'-TCCTGCTAGGGACCTTCTGT-3'; nephrin

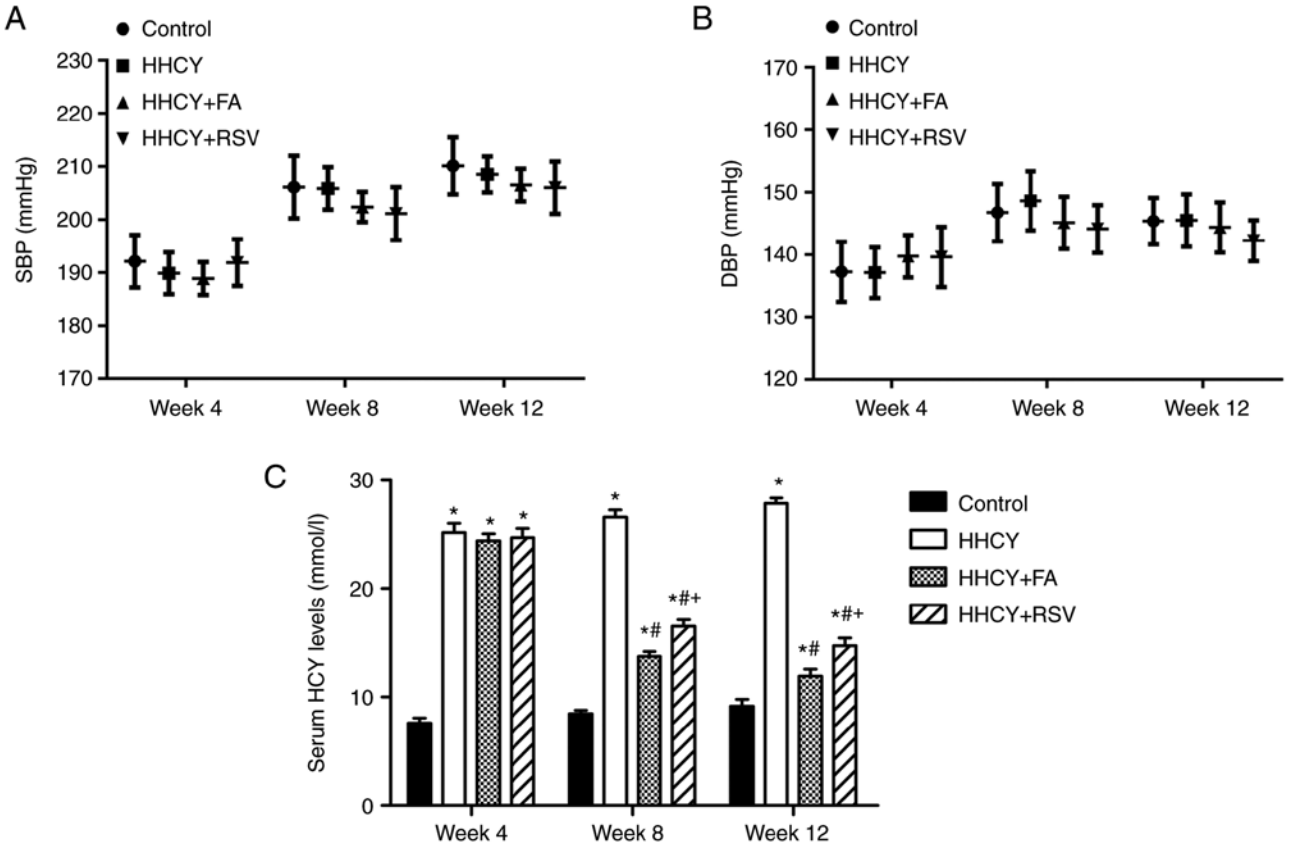

Figure 1. Levels of plasma HCY, SBP and DBP. (A and B) BP levels and (C) serum HCY levels in the control, HHCY, HHCY + FA and HHCY + RSV groups at weeks 4, 8 and 12. Data are presented as the mean ± SE (n=8). *P<0.05 vs. control; #P<0.05 vs. HHCY; and +P<0.05 vs. HHCY + FA. BP, blood pressure; HCY, homocysteine; HHCY, hyperhomocysteine; FA, folic acid; RSV, resveratrol; SBP, systolic BP; DBP, diastolic BP.

F, 5'-CCTGACCATCCTGGCCAACTC-3' and R, 5'-ATCTTC CAGCCTCTCTCCTTCCT-3'; and GAPDH F, 5'-CCCCCA ATGTATCCGTTGTG-3' and R, 5'-TAGCCCAGGATGCCC TTTAGT-3'.

Western blotting. Total protein was extracted from renal tissue using the RIPA protein extraction solution (Beyotime Institute of Biotechnology; cat. no. P0013B). Protein concentrations were determined by BCA Protein Assay Kit (Beyotime Institute of Biotechnology; cat. no. P0012). The loading amount was calculated according to the standard of 50 μg protein in each lane. Total protein was separated using SDS-PAGE on a 10% gel and separated proteins were transferred onto PVDF membranes. Subsequently, the membranes were blocked with 5% skimmed milk at room temperature for 2 h. The membranes were then incubated with the primary antibodies at 4°C overnight. After three washes using TBS with 0.05% Tween-20 (TBST), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (cat. no. SA00001-2; 1:9,000; Proteintech Group, Inc.) at room temperature for 1 h. After three more washes with TBST, the protein bands were visualised using the Ultra High Sensitivity ECL Kit (cat. no. E412-01; Vazyme Biotech Co., Ltd.) and the ChemiDoc™ Touch Gel Imaging System (Bio-Rad Laboratories, Inc.). Finally, the protein bands were semi-quantified using ImageJ software (V1.4.3.67; National Institutes of Health) and were normalised to GAPDH. The primary antibodies used were as follows: anti-GAPDH (cat. no. ab181602, 1:10,000), anti-NOX2 (cat. no. ab31092, 1:1,000), anti-NOX4 (cat. no. ab133303, 1:4,000) and anti-nephrin (cat. no. ab216341,1:200), which were all purchased from Abcam.

Statistical analysis. All data are presented as the mean ± SE and were analysed using SPSS version 24.0 (IBM Corp.). Groups were statistical compared using one-way ANOVA followed by the Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference. All experiments were performed at least three times.

Results

RSV reduces HCY levels in the SHR HHCY model, but to a lesser extent compared with FA. Fig. 1A and B present the systolic and diastolic BP data for the control, HHCY, HHCY + FA and HHCY + RSV groups at weeks 4, 8 and 12. The results demonstrated that there was no significant difference in BP among the groups.

After 4 weeks of intraperitoneal injection of DL-HCY in the HHCY, HHCY + FA and HHCY + RSV groups, the HCY levels (25.14±0.85, 24.35±0.66 and 24.66±0.84 μmol /l, respectively) were significantly higher compared with the control (7.58±0.47 μmol/l). There was no significant difference between the HHCY, HHCY + FA and HHCY + RSV groups. Subsequently the HHCY, HHCY + FA and HHCY + RSV groups were treated with DL-HCY intraperitoneally for a

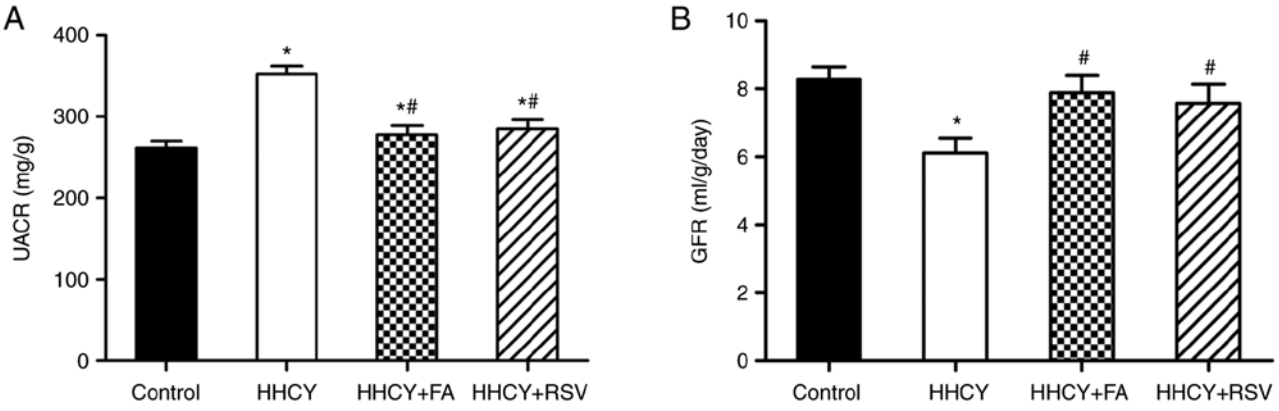

Figure 2. FA and RSV improve the HHCY-induced UACR increase and GFR decrease. (A) UACR and (B) GFR in the control, HHCY, HHCY + FA and HHCY + RSV groups. Data are presented as the mean ± SE (n=8). *P<0.05 vs. control; #P<0.05 vs. HHCY. UACR, urinary albumin creatinine ratio; GFR, glomerular filtration rate; HHCY, hyperhomocysteine; FA, folic acid; RSV, resveratrol.

further 8 weeks. The serum HCY levels in the HHCY group markedly increased in a time-dependent manner (week 8, 26.54±0.66 μmol/l; week 12, 27.81±0.5 μmol/l). Meanwhile, from week 5, FA was administered to the HHCY + FA group and RSV was administered to the HHCY + RSV group. The serum HCY levels were significantly lower in the HHCY + FA and HHCY + RSV groups compared with the HHCY group at both week 8 and week 12 (week 8, 13.73±0.47 and 16.52±0.58 μmol/l; and week 12, 11.92±0.63 and 14.72±0.72 μmol/l in the HHCY + FA and HHCY + RSV groups, respectively). Furthermore, compared with that in the HHCY + RSV group, the serum HCY levels in the HHCY + FA group was significant decreased at weeks 8 and 12 (Fig. 1C).

FA and RSV improve the HHCY-induced UACR increase and GFR decrease. The results demonstrated the the UACR was significantly higher in the HHCY group (351.97±9.69 mg/g) compared with the control (261.35±8.65 mg/g) (Fig. 2A). FA and RSV treatment both significantly reduced the UACR compared with the HHCY group. However, there was no significant difference between the HHCY + FA (277.34±11.52 mg/g) and HHCY + RSV (284.99±10.75 mg/g) groups. Furthermore, compared with the control (8.27±0.37 ml/g/day), the GFR of the HHCY group decreased significantly (6.11±0.44 ml/g/day) (Fig. 2B). However, even though the GFR significantly increased in the HHCY + FA (7.89±0.51 ml/g/day) and HHCY + RSV (7.57±0.57 ml/g/day) groups, there was no significant difference between the two groups. Therefore, these results demonstrated that both FA and RSV treatment may have significantly decreased the HHCY-induced UACR increase and improved the HHCY-induced GFR decrease.

FA and RSV inhibit HHCY-induced oxidative stress. The serum SOD concentration in the HHCY group (353.52±13.17 U/ml) was significantly decreased compared with the control (436.33±11.23 U/ml). However, the SOD concentration in the HHCY + FA (428.67±12.14 U/ml) and HHCY + RSV (446.63±10.69 U/ml) groups demonstrated a significant reversal of the HHCY-induced reduction, compared with the HHCY group (Fig. 3A). Moreover, the MDA concentration was significantly higher in the HHCY group (9.41±0.59 nmol/ml) compared with the control (7.72±0.83 nmol/ml). However, compared with the HHCY group, FA and RSV treatment significantly reduced the increase in MDA induced by HHCY (HHCY + FA, 7.65±0.81 nmol/ml; HHCY + RSV, 6.56±0.73 nmol/ml) (Fig. 3B). Furthermore, compared with the HHCY + FA group, the HHCY + RSV group exhibited significantly different results for both SOD and MDA.

FA and RSV inhibit HHCY-induced NOX2 and NOX4 mRNA and protein expression. Compared with the control, the NOX2 and NOX4 mRNA and protein expression levels in the HHCY group were significantly increased (Fig. 3C-E). Compared with the HHCY group both FA and RSV treatment significantly inhibited the HHCY-induced NOX2 and NOX4 mRNA and protein expression. Furthermore, the NOX2 and NOX4 mRNA expression levels in the HHCY + RSV group were significantly weaker compared with the HHCY + FA group. However, there was no significant difference in the protein expression levels of NOX2 and NOX4 between these two groups.

FA and RSV treatment reverse the HHCY-induced decrease in nephrin expression levels. RT-qPCR demonstrated that nephrin mRNA expression levels were significantly decreased in the HHCY group compared with the control. Compared with the HHCY group both FA and RSV treatment significantly reversed this change in nephrin mRNA expression levels (Fig. 4A). Furthermore, compared with the control, nephrin protein expression levels were significantly reduced in the HHCY group, which was significantly reversed via FA or RSV treatment (Fig. 4B and C). However, there was no significant difference in nephrin mRNA and protein expression levels between the HHCY + FA and HHCY + RSV groups.

Discussion

Hypertension is a common cause of renal damage and HHCY aggravates renal function impairment in patients with hypertension (21). In China, a large proportion of patients with hypertension also have HHCY and most of these individuals have renal damage, which may be related to insufficient dietary FA (3). An intervention study demonstrated that FA reduces

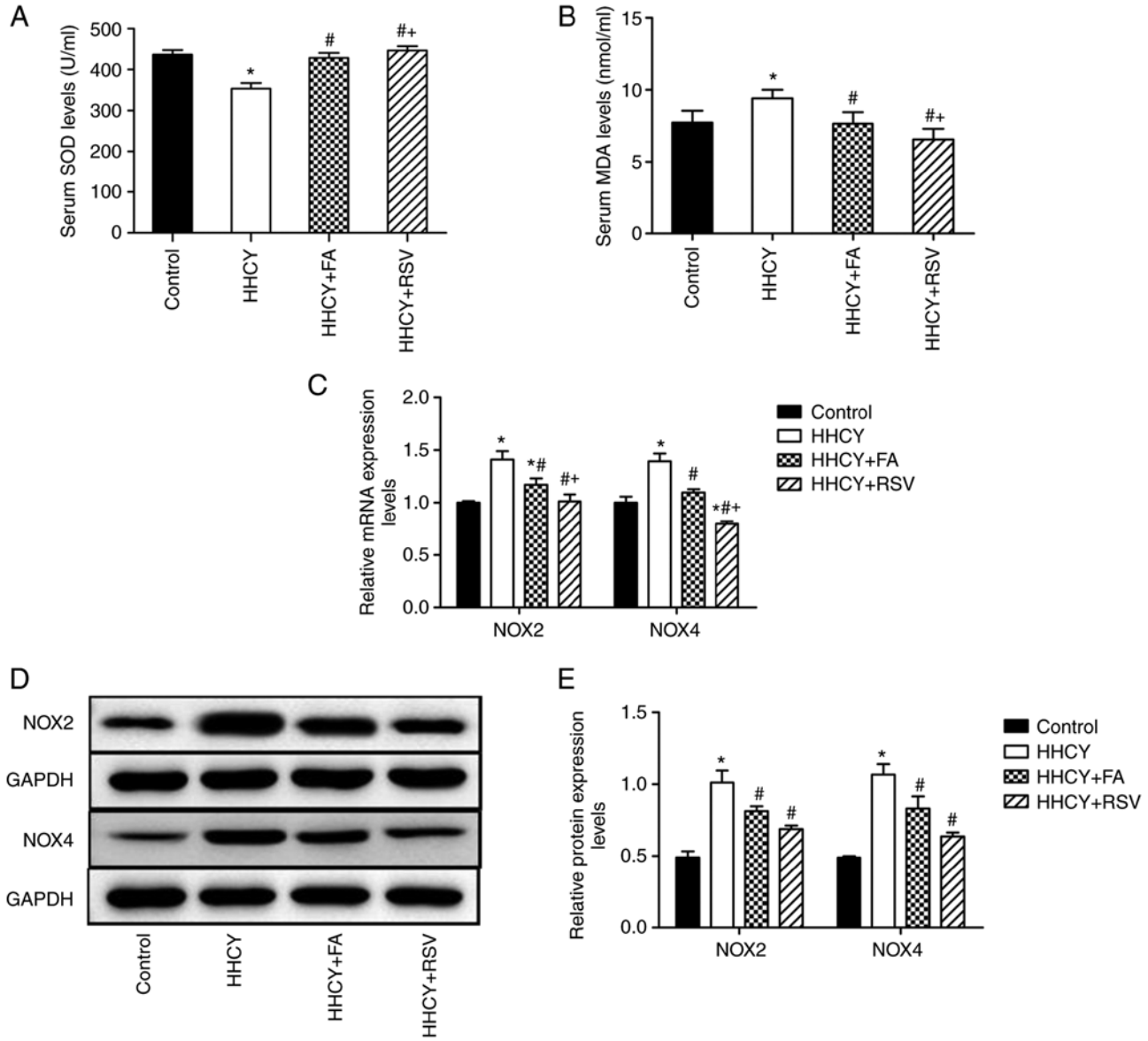

Figure 3. FA and RSV inhibit HHCY-induced oxidative stress. (A) Serum SOD and (B) MDA levels in the control, HHCY, HHCY + FA and HHCY + RSV groups. (C) Relative NOX2 and NOX4 mRNA expression levels. (D) Representative western blots of NOX2 and NOX4 protein expression levels and the (E) densitometric data following normalisation with the endogenous control, GAPDH. Data are presented as the mean ± SE (n=8). *P<0.05 vs. control; $^{\#}$P<0.05 vs. HHCY; $^{+}$P<0.05 vs. HHCY + FA. SOD, superoxide dismutase; MDA, malondialdehyde; HHCY, hyperhomocysteine; FA, folic acid; RSV, resveratrol; NOX, NADPH oxidase.

HCY and improves renal function (7). However, the effective dose of FA has not been determined and certain studies have suggested that FA increases the risk of cancer (11,22). Therefore, it is necessary to identify an effective drug with minimal side-effects that can improve the hypertensive renal damage that is aggravated by HHCY.

In our previous study it was demonstrated that HHCY aggravates hypertensive renal damage via the activation of oxidative stress (9). The natural antioxidant RSV improves diabetic kidney damage and delays kidney aging (23,24). Moreover, it has previously been confirmed that RSV can reduce HCY levels (16). Therefore, RSV may be a potential treatment for HHCY-induced hypertensive renal damage. In the present study, SHRs were treated with intraperitoneal DL-HCY for 12 weeks to establish a hypertensive rat model with HHCY. The serum HCY levels in the HHCY group markedly increased in a time-dependent manner, which indicated that the model had been established.

In the present study, RSV and FA were used as treatments in the HHCY group and changes were observed in BP, serum HCY levels, oxidative stress and renal function. The results demonstrated that there was no significant change in BP in any of the experimental groups throughout the experiment, which conflicts with previous reports on the anti-hypertensive effects of RSV (25,26). A study showed that feeding SHRs with food containing RSV (4 g/kg) meant that 146 mg/kg/day RSV was available, which decreased the blood pressure of the SHRs significantly (27). The results in the present study may be different because of the dosage, the mode of administration and the effective concentration of RSV.

The results of the present study demonstrated that compared with the control the UACR, an important index of early renal

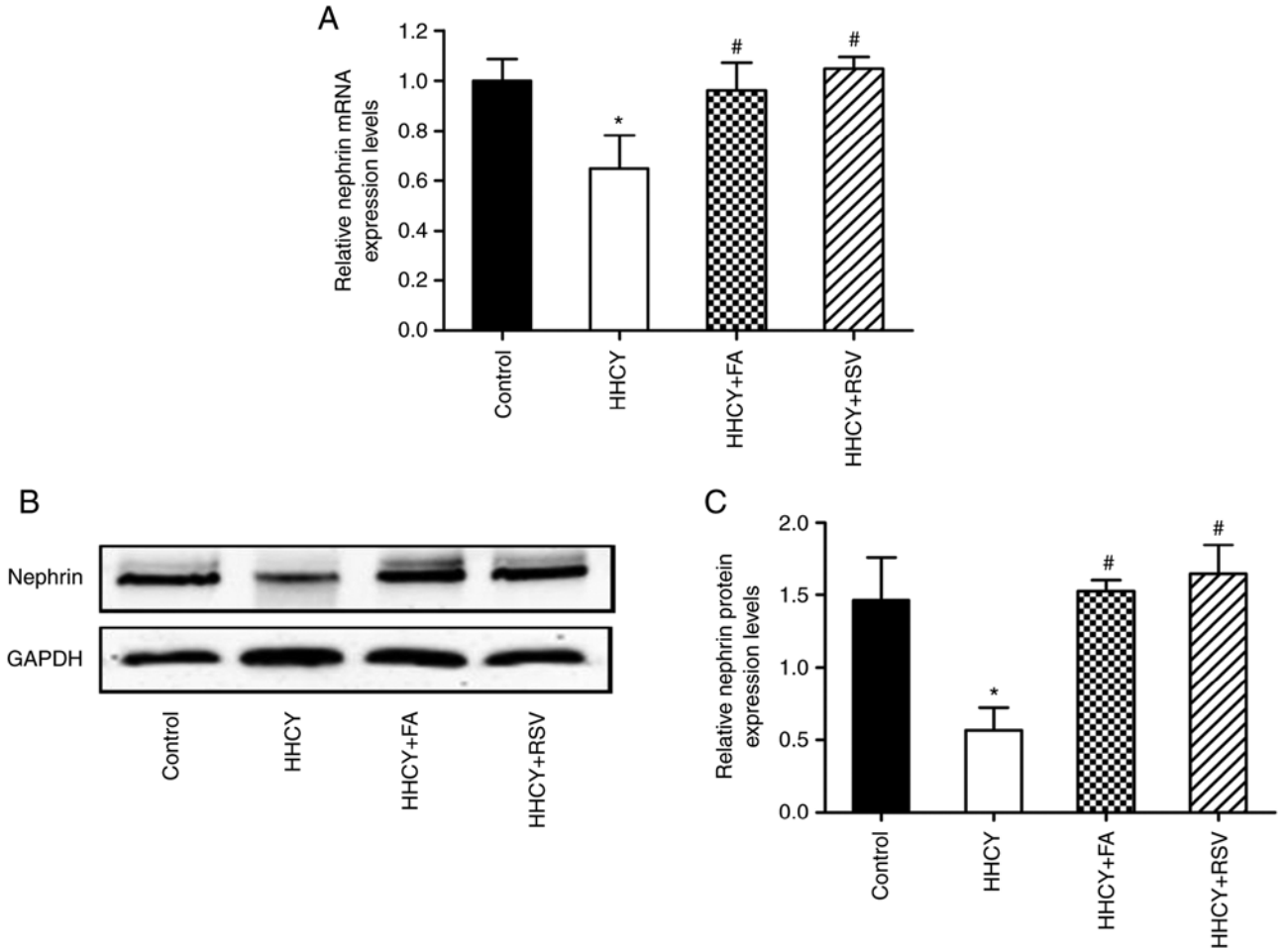

Figure 4. FA and RSV reverse the HHCY-induced decrease in nephrin mRNA and protein expression levels. (A) Relative nephrin mRNA expression levels. (B) Representative western blots of nephrin and GAPDH protein expression levels, GAPDH served as markers and the (C) densitometric data following normalisation with the endogenous control, GAPDH. Data are presented as the mean ± SE (n=8). *P<0.05 vs. control; #P<0.05 vs. HHCY. HHCY, hyperhomocysteine; FA, folic acid; RSV, resveratrol.

damage, was significantly higher in the HHCY group, whereas the GFR was significantly lower. Consistent with a Mendelian randomisation study (28), a high HCY level potentially lead to a decreased GFR in the present study. The present study also demonstrated that RSV significantly ameliorated the increase in UACR and decrease in GFR caused by HHCY; however, there was no significant difference compared with the FA treatment group. Furthermore, the significant change in GFR in the HHCY group was potentially related to the decreased mRNA and protein expression levels of nephrin, a podocyte gap molecule that serves an important role in maintaining the glomerular filtration barrier (29,30). HHCY reduces nephrin expression in kidney tissue (31), which was also confirmed in our previous study (9). In the present study, RSV supplementation increased nephrin expression levels and significantly improved the GFR in SHRs with HHCY; however there was no significant difference compared with the FA treatment group.

It was also previously observed that HHCY aggravated hypertensive renal damage and its underlying mechanism are related to oxidative stress (9). RSV reduces the expression and activity of NOXs in endothelial cells (32). In the present study, HHCY resulted in decreased serum SOD and increased MDA levels; however, treatment with FA or RSV significantly counteracted these effects. Compared with the control, NOX2 and NOX4 mRNA and protein expression levels in the HHCY group were significantly increased, which was offset by the FA and RSV treatments. In the present study, the rats in the control group were SHRs, and a previous study confirmed that SHR themselves are in a state of oxidative stress (33). RSV, as an antioxidant, can improve the oxidative stress of SHRs, which explains the phenomenon of the expression of NOX4 mRNA being lower in HHCY + RSV group than that of the control group. Furthermore, it was demonstrated that compared with the HHCY + FA group, the indexes related to oxidative stress (SOD, MDA and the mRNA expression levels of NOX2 and NOX4) improved more significantly in the HHCY + RSV group. However, there was no significant difference for the protein expression levels of NOX2 and NOX4 between the HHCY + FA and HHCY + RSV groups. As previous stated, mRNA is the template for protein synthesis, but due to differential translation, protein degradation, contextual confounders among other reasons, protein may not be present in proportional quantities (34). This may explain why there was no difference in the expression of NOX2 and NOX4 proteins between the HHCY + RSV and HHCY + FA groups.

Furthermore, the results of the present study demonstrated that RSV reduced the serum HCY levels, which is consistent with previous research (16). However, this change was not as significant as for FA treatment, as there was no significant difference between FA and RSV in terms of improving renal function. These results suggested that RSV potentially improved the renal damage induced by HHCY via a mechanism other than that of reducing serum HCY levels.

In summary, RSV, like FA, improved the renal damage aggravated by HHCY in SHRs. Furthermore, RSV potentially reduced the renal damage, not only by decreasing HCY levels but also, and mainly, by reducing oxidative stress. We consider that RSV alone or in combination with FA may become a safe and effective new choice to improve hypertensive renal damage aggravated by HHCY in the clinic. However, certain limitations need to be explored in future studies. For example, the effect of different doses of RSV is unclear and future studies should examine the effect of combined treatment with FA and RSV on improving hypertensive renal damage aggravated by HHCY.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RX, NG and QCH conceived and designed the experiments. QCH and XZ performed the experiments. QCH, XZ and PC analysed the data. RX, NG and PC provided reagents and advice. XZ was a major contributor in writing the manuscript. XZ and QCH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocol was established according to the ethical guidelines and was approved by the Medical Ethics Committee of Shandong Provincial Qianfoshan Hospital (approval no. 2020-S292).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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