



SIRT1/NOX1 pathway mediated ameliorative effects of rosmarinic acid in folic acid-induced renal injury

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Abstract

Background and purpose: Renal injury is a serious disorder that can be caused by some diseases or agents. Rosmarinic acid (RA) is a natural and safe compound with powerful antioxidant and anti-inflammatory properties. In this study, the ameliorative effects of RA were assayed in folic acid (FA)-induced renal injury by involving the SIRT1/NOX1 pathway.

Experimental approach: Thirty-six male C57/BL6 mice were divided into 6 groups (n = 6) including control, vehicle, FA, RA, FA + RA 50, and FA + RA 100. After 10 days, blood urea nitrogen (BUN), creatinine, and oxidative stress were measured. The expression of SIRT1 and NOX1 proteins was evaluated by western blot. Also, histopathological alterations were assayed by H&E and PAS staining methods.

Findings/Results: BUN and creatinine were significantly higher in the FA group compared to the control group; however, their levels decreased after RA treatment in both doses. A significant decrease was observed in swelling, necrosis, and desquamation of tubular epithelial cells in the FA + RA 50 and FA + RA 100 groups compared to the FA group. RA in the animals receiving FA increased SIRT1 expression and the levels of GSH and SOD compared to the FA group. RA in the animals receiving FA showed a significant decrease in NOX1 expression and MDA level compared to the FA group.

Conclusion and implications: The findings declared that the administration of RA has positive effects against renal damage induced by FA. The effect might result from involvement in the SIRT1/NOX1 pathway and thereby attenuation of oxidative stress.

Keywords: Folic acid; NOX1; Renal failure; Rosmarinic acid; SIRT1.

INTRODUCTION

Kidneys are vital organs for balancing ions and eliminating waste products, drugs, and toxins. (1). Etiologically, hypertension, chronic inflammation, diabetes, environmental toxins, and prolonged metabolic diseases can lead to renal injury (2). Several medications including antibiotics, antivirals, antifungals, analgesics (such as non-steroidal anti-inflammatory drugs), chemotherapeutic agents, and toxic doses of drugs also damage renal epithelial cells *via* the activation of the inflammatory cascade and induction of reactive oxygen species (ROS) (3,4).

Folic acid (FA) is known as vitamin B9 (5) and is commonly used as a complementary drug. However, low doses of FA (usually less than 10 mg/day) are beneficial, some documents have shown that high doses of FA are toxic (6,7). The literature reported kidney injury induced by FA and described the concepts of “renal folate toxicity” and “folate nephropathy” (8). FA-induced kidney disease can lead to acute kidney disease (AKI) or chronic kidney disease (chronic FA-nephropathy).

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Indeed, FA is stored in the endosomal vesicles of epithelial cells in proximal tubules. When the vesicles are fused with mitochondria can lead to mitochondrial oxidative stress, inflammation, and apoptosis (9). Therefore, FA-induced kidney disease can mimic the symptoms of human kidney disease and be highly reproducible (10). Today, FA at the dose of 250 mg/kg is used to induce kidney injury as an animal model (11). Silent information regulator-two 1 (SIRT1), as a critical regulator of mitochondrial biogenesis, (12) is a member of class III nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase which plays an essential role in the attenuation of apoptosis, aging, and oxidative stress (13,14). Reduction in ROS-induced NAD⁺ content can diminish SIRT1 activity (15). NADPH oxidase (NOX) can lead to tissue damage with contribution to ROS production after catalyzing substrate molecules (16). Multiple studies have shown the role of SIRT1 in the anti-inflammatory and anti-stress pathways (13,14,17). In this context, SIRT3 was significantly blocked after FA-induced AKI (18). SIRT1 decreases NOX production, and SIRT1 inhibition leads to the upregulation of NOX subunits (19). Recent research has demonstrated that the antioxidant activity of SIRT1 is achieved *via* the inhibition of ROS production and the stimulation of antioxidant enzymes such as superoxide dismutase (SOD) and catalase. Moreover, its ability to mitigate stress is mediated by inhibiting the activity of NOX (20). SIRT1 increases the activity of important antioxidant enzymes like SOD and glutathione peroxidase (GSH), which are vital elements of the cellular antioxidant defense system. Consequently, SIRT1's ability to boost the performance of the enzymes leads to a decrease in ROS levels and an improvement in the antioxidant capacity of cells (21).

Rosmarinic acid (RA) is a polyphenolic compound, and like other natural compounds, is found in a variety of plants and their derived bioactive combinations and has antioxidant properties (22,23). Several biological functions, including antioxidant, antimicrobial, anti-inflammatory, and antifungal (22,24) properties make the RA a suitable candidate for the treatment of several diseases such as gastritis

(24), pulmonary ischemia/reperfusion injury (25), kidney injuries (26), and nonalcoholic steatohepatitis (22). Another study confirmed that RA can protect kidneys from diabetic glomerular deterioration and nephropathy (27). Also, it is suggested that RA is well-tolerated and safe for use in healthy individuals (28).

Limited information is currently available on the correlation between RA, SIRT1, and NOX1 in tissues. In a rat model of diabetic nephropathy, treatment with RA increased the expression of SIRT1 and decreased the level of NOX1 (29). RA also inhibits NOX1 expression and activity in human retinal pigment epithelial cells, thereby reducing inflammation and oxidative stress (30). In addition, the anti-inflammatory and anti-apoptotic role of RA has been reported on nonalcoholic steatohepatitis by SIRT1/NF κ B pathway (22). Accordingly, this study was designed to assess the effects of RA on FA-induced renal injury, focusing on the SIRT1/NOX1 pathway.

MATERIALS AND METHODS

Chemicals and reagents

RA (536954-5G), Bradford assay kit, and the stains of hematoxylin and eosin were purchased from Sigma-Aldrich Company (USA). FA and sodium bicarbonate (NaHCO₃) were obtained from Cayman Chemical Company (Ann Arbor, MI, USA) and Sigma-Aldrich Company (USA), respectively. Blood urea nitrogen (BUN) and creatinine kits were provided from Man Company (Iran). Polyvinylidene fluoride (PVDF) membranes were purchased from Roche Diagnostics GmbH Company (Mannheim, Germany). The periodic acid Schiff (PAS) stain (ab150680), primary antibodies monoclonal anti-SIRT1 (ab189494), polyclonal anti-NOX1 (ab131088), and polyclonal anti- β -actin (ab8227) were provided from Abcam Company (USA) SOD (ZB-SOD-96A), MDA (ZB-MDA-96A), and GSH (ZB-GSH-96A) kits were purchased from Zellbio Company (Germany).

Animals

This study was conducted using 36 male C57/BL6 mice aged 5-7 weeks, which were purchased from the Pasteur Institute (Tehran,

Iran). The animals were kept in an animal room (Animal Center of Qom University of Medical Sciences) with controlled conditions including a temperature of 23 ± 1 °C, humidity of $55 \pm 10\%$, and a 12-h light/12-h dark cycle (31). The mice had access to fresh drinking water ad libitum. They also had free access to standard laboratory chow (Pars Animal Feed Company, Tehran, Iran). Before the experiment, the mice were acclimatized to the laboratory conditions for a week. All the animal experiments were approved and performed according to the guidelines of the Animal Ethics Committee of Qom University of Medical Sciences (Ethics No. IR.IAU.SRB.REC.1402.018).

Experimental design

The animals were divided into 6 groups, with 6 animals in each group, including 1-control group receiving normal saline by gavage; 2- vehicle group receiving NaHCO₃ (0.2 mL of 0.3 M solution) intraperitoneally as a vehicle (placebo) for FA; 3- FA group receiving FA intraperitoneally at the single dose of 250 mg/Kg to induce renal injury (10); 4- RA group receiving RA at the dose of 100 mg/kg/day by gavage for 10 days (32); 5- FA + RA 50 group receiving RA at the dose of 50 mg/kg/day by gavage for 10 days and FA at the single dose of 250 mg/Kg 60 min after the first dose administration of RA (32); 6- FA + RA 100 group receiving RA dose of 100 mg/kg/day by gavage for 10 days and FA at the single dose of 250 mg/Kg 60 min after the first dose administration of RA.

At the end of experimental period, the animals were anesthetized using sodium pentobarbital (35 mg/kg, intraperitoneally), and blood samples were taken from the heart for biochemical assays. After euthanasia and make an abdomen midline incision, both right and left kidneys were removed. Right kidney was fixed in 10% formalin solution for histopathology assessment, while left kidney was stored at the temperature of -80 °C for molecular evaluations.

Assessment of renal function

For evaluating renal function, the levels of BUN and creatinine were measured according

to the manufacturer's instructions for the respective kits.

Histopathology examinations

Hematoxylin and eosin staining

To evaluate histological factors, the kidneys were maintained with a 10% formalin solution and embedded in paraffin after routine processing. The sections were then stained with hematoxylin and eosin (H&E) staining. The digital images of slices were captured through an Olympus DP70 digital camera (Olympus Optical Co, Ltd., Tokyo, Japan). The tubular diameter was evaluated and analyzed using the Image J software (Bethesda, Maryland, United States). The histological findings were interpreted by an expert pathologist, blinded to the group definitions. Moreover, the slices were meticulously scrutinized for abnormalities such as pyknotic nuclei, tubular cell swelling, the presence of red blood cells within tubules, cellular detachment, and tubular necrosis.

Periodic acid-Schiff staining

Periodic acid-Schiff (PAS) staining was performed on the sections with 4- μ m thick (34). Briefly, the PAS reaction included the removal of paraffin, followed by periodic acid immersion for 8-10 min and then thorough rinsing with distilled water. Next, the slices were placed in Schiff's reagent for 15-20 min. After rinsing with tap water for 5 min, the slices were stained with Harris's hematoxylin solution for 1 min, rewashed, and embedded in entellan (107961, Merck KGaA). Finally, the qualitative alterations in the structure of kidney tissue were evaluated.

Western blot analysis

To determine the expression levels of SIRT1 and NOX1 proteins, western blot analysis was conducted using the method previously described (23). In brief, the concentration of proteins was measured using the Bradford assay kit. The proteins were separated and transferred to PVDF membranes. Then, the membranes were blocked with 5% skim milk for 2 h and incubated overnight at 4 °C with primary antibodies against SIRT1, NOX1, and β -actin. After washing, the horseradish peroxidase-conjugated secondary antibody (1:7000, Cell

Signaling) was added to the membranes. The membranes were then washed and incubated with Amersham-enhanced chemiluminescence (ECL) reagents in a darkroom. Afterward, the membranes were exposed to an X-ray film and visualized using the ECL reagent and detected with the ECL detection system (Image Lab™ Touch Software, BIO-RAD, USA). Image J software (version IJ 1.46r, NIH, USA) was used to determine the intensity of the bands, and the relative expression of proteins was normalized to β -actin (23).

Measurement of SOD activity, MDA, and GSH levels in the renal tissue

Tissue samples, weighing approximately 100 mg, were gently homogenized in a buffer containing 1 mM EDTA and 10 mM Tris-HCl (pH 7.4), followed by centrifugation at 5000 rpm for 15 min at 4 °C. Finally, the supernatants were used to assess SOD activity, MDA, and GSH levels according to the supplier's instructions for the respective kits.

Statistical analysis

All data were presented as mean \pm SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's post-test using SPSS (version 25, IBM,

USA). P -values < 0.05 were considered statistically significant.

RESULTS

Effect of RA on renal dysfunction in FA-induced renal injury

Findings showed that BUN and creatinine serum levels were significantly higher in the FA group compared to the control, vehicle, and RA groups; however, RA treatment in both doses decreased the levels of BUN and creatinine compared to the FA group, significantly (Fig. 1).

Effect of RA on renal tissue damage in FA-induced renal injury

As shown in Fig. 2A, the H&E staining of renal tissue showed normal kidney structure with Malpighian corpuscles and glomerulus in the control, vehicle, and RA groups. The proximal convoluted tubules (PCT) were lined with pyramidal epithelial cells with central rounded nuclei and eosinophilic cytoplasm. Also, the distal convoluted tubules (DCT) were lined with a relatively large number of cuboidal epithelial cells. The lumens of the DCT were wider than those of the PCT; their nuclei were round and the cytoplasm was less acidophilic.

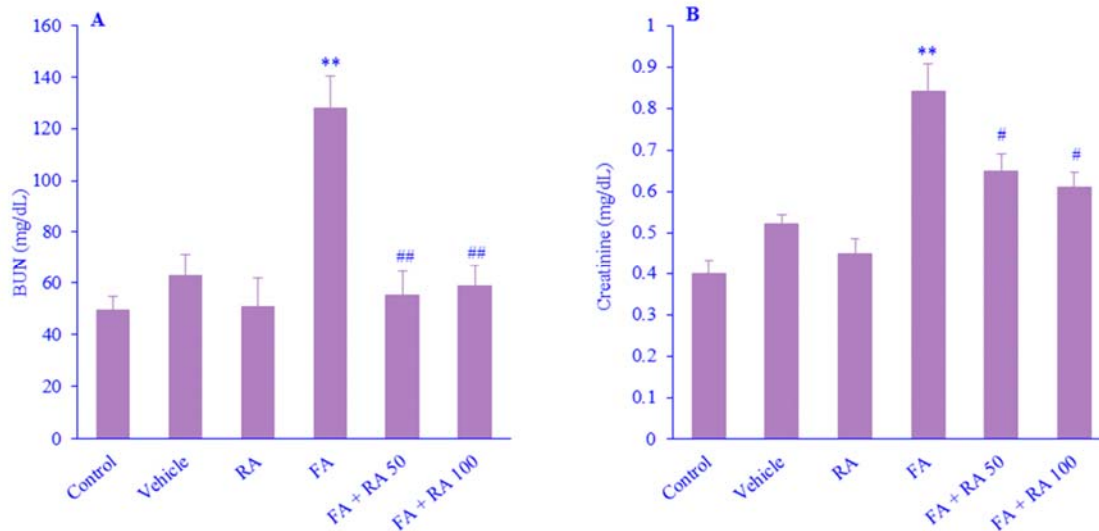


Fig. 1. The effect of RA (50 and 100 mg/kg) on the serum levels of (A) BUN and (B) creatinine in FA-induced renal injury. The vehicle group received NaHCO₃ as FA solvent. FA group received a single dose of FA (250 mg/Kg). Data were expressed as mean \pm SEM, n = 6. ** $P \leq 0.01$ indicates a significant difference as compared to the control, vehicle, and RA groups; # $P \leq 0.05$ and ## $P \leq 0.01$ versus the FA group. FA, Folic acid; RA, rosmarinic acid; NaHCO₃, sodium bicarbonate; BUN, blood urea nitrogen.

The FA group exhibited degenerative changes such as dilated tubular lumina and vacuolation with the loss of lining epithelium. Shrunken glomeruli and the dilatation of capsular space were also observed.

The infiltration of mononuclear cells and interstitial hemorrhage were significantly present, along with desquamated epithelial cell

debris disseminated into the tubular lumen (Fig. 2A).

Distal tubule diameter significantly increased in the FA group compared to the control, vehicle, and RA groups, whereas, distal tubule diameter significantly decreased in the FA + RA 50 and FA + RA 100 groups compared to the FA group (Fig. 2B).

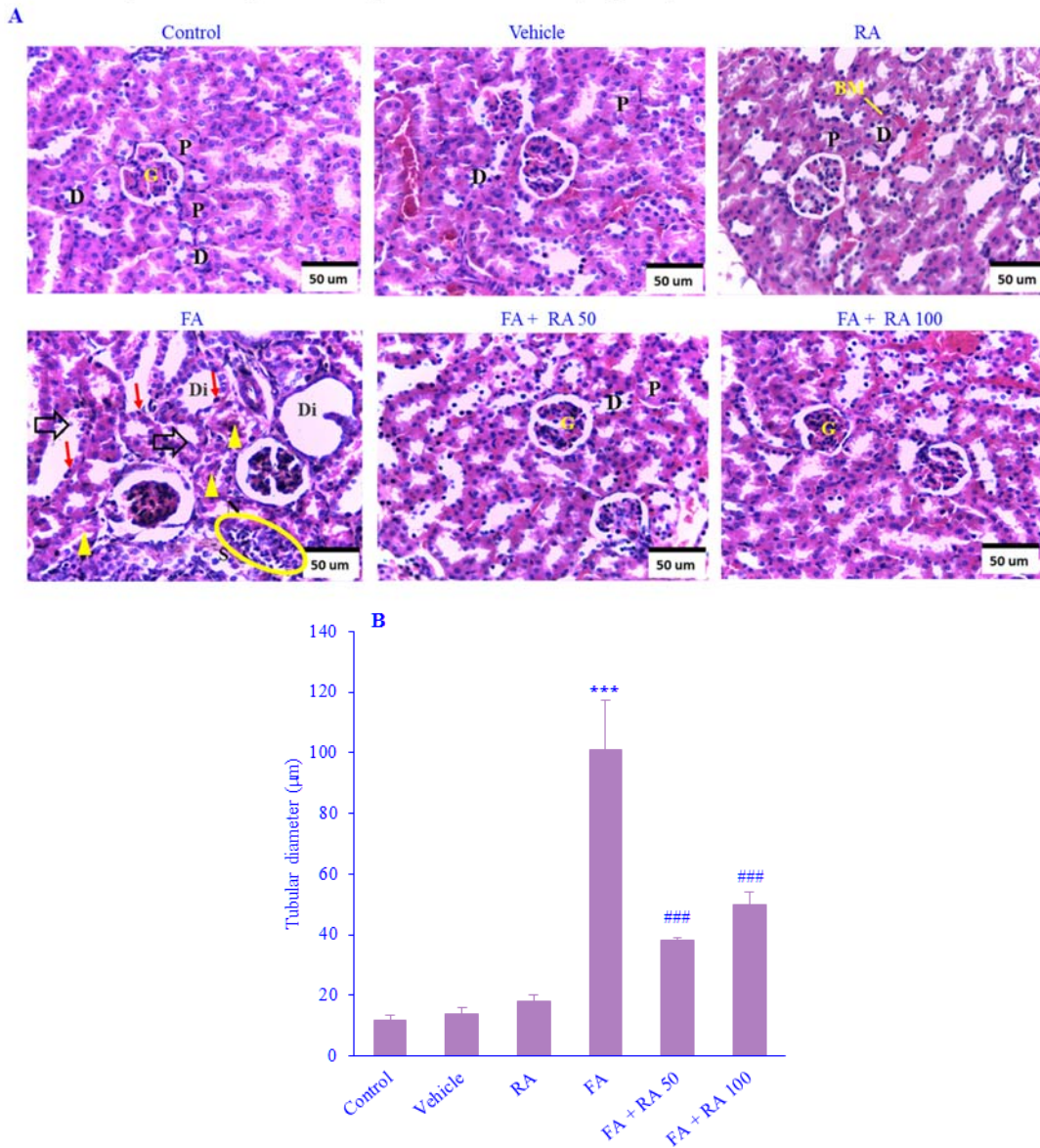


Fig 2. The RA effect (50 and 100 mg/kg) on renal tissue (hematoxylin and eosin) in FA-induced renal injury. (A) The images of renal tissue with magnification 400× and (B) the tubular diameter in all experimental groups. The vehicle group received NaHCO₃ as FA solvent. FA group received a single dose of FA (250 mg/Kg). Data were expressed as mean ± SEM, n = 6. ****P* ≤ 0.001 indicates a significant difference compared to the control, vehicle, and RA groups; ###*P* ≤ 0.001 versus FA group. G, glomerulus; D, distal tubules; P, proximal tubules; Di, dilated renal tubules; BM, tubular basement membranes; yellow arrowheads, disseminated epithelial cells; black arrows, detached tubular cells; N, tubular necrosis; S, swollen tubular cells; red arrows, pyknotic nuclei; oval circle, infiltrations of mononuclear cells; FA, folic acid; RA, rosmarinic acid; NaHCO₃, sodium bicarbonate.

The FA + RA 50 and FA + RA 100 groups also showed decreased areas of necrosis and desquamation compared to the FA group. In the FA + RA 50 group, there were some areas of necrosis and detached renal tubular epithelium, but most tubules were normal, and regenerating epithelial cells could be seen intermittently. The FA + RA 100 group had normal tubular structure with the majority of tubules appearing normal. The FA + RA 100 group had less tissue damage compared to the FA + RA 50 group, with smaller areas of tubular epithelial necrosis and desquamation (Fig. 2A).

PAS staining revealed the thickening of the glomerular basement membrane, dilated tubular lumina, vacuolation with the loss of lining epithelium, tubular epithelial cell degeneration, and necrosis in the FA group. Other histological changes such as shrunken glomeruli and the dilatation of capsular space were seen in Malpighian corpuscles (Fig. 3). Nevertheless, mice receiving RA treatment exhibited a significant improvement in the renal histological damage induced by FA compared to the FA group (Fig. 3).

Effect of RA on the expression of renal SIRT1 and NOX1 in the FA-induced renal injury

Western blotting findings detected a noteworthy reduction in the protein expression of SIRT1 in the FA group compared to the control, vehicle, and RA groups. Nevertheless, there was a considerable increase in the SIRT1 expression in the FA + RA 50 and FA + RA 100 groups compared to the FA group (Fig. 4A and 4B). On the contrary, NOX1 protein expression significantly increased in the animals with FA alone in comparison to the control, vehicle, and RA groups. However, the FA + RA 50 and FA + RA 100 groups exhibited a decrement in NOX1 expression distinctly compared to the FA group (Fig. 4A and 4C).

Effect of RA on renal antioxidant defense in FA-induced renal injury

The FA group showed a significantly higher level of MDA, an indicator of lipid oxidation, compared to the control, vehicle, and RA groups. While, the FA + RA 50 and FA + RA 100 groups had decreased levels of MDA compared to the FA group, as illustrated in Fig. 5A.

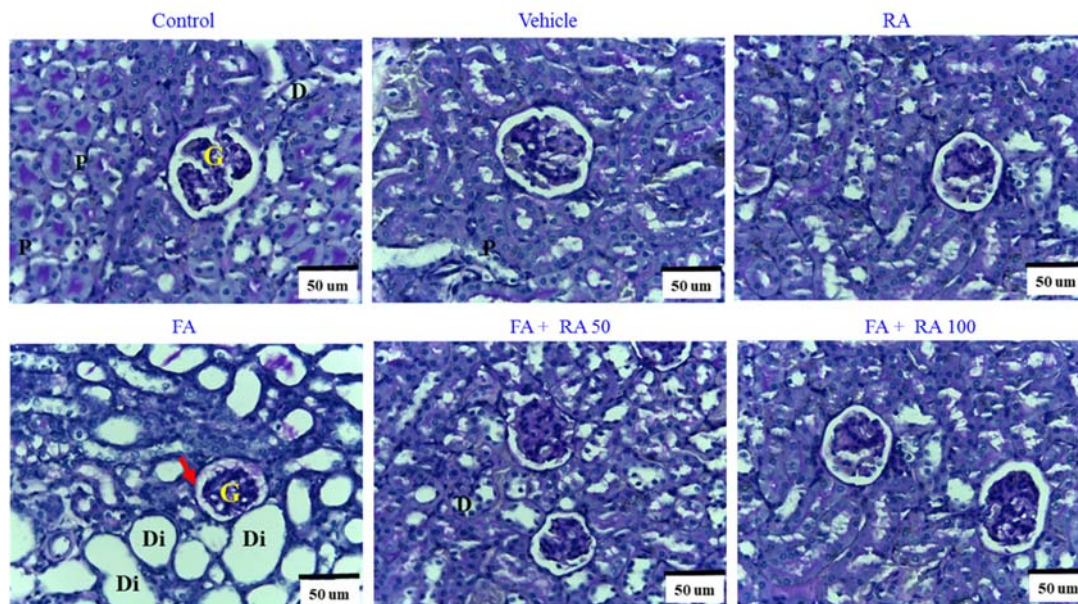


Fig 3. The effect of RA (50 and 100 mg/kg) on renal tissue stained by PAS staining (magnification 400×) in FA-induced renal injury. The vehicle group received NaHCO₃ as FA solvent. FA group received the single dose of FA (250 mg/Kg). G, Glomerulus; D, distal tubules; P, proximal tubules; Di, dilated renal tubules; red arrow, tubular basement membranes; FA, folic acid; RA, rosmarinic acid; NaHCO₃, sodium bicarbonate; PAS, periodic acid-Schiff.

As presented in Fig. 5B, the activity level of SOD enzyme in the renal tissue decreased in the FA group compared to the control group, although it was not significant. While, after treatment with RA, a remarkable increase was seen in the FA + RA 50 and FA + RA 100 groups as compared with the FA group (Fig. 5B).

The tissue levels of GSH had a significant decrease in the FA group in comparison to the control, vehicle, and RA groups; however, it was seen a significant increase in GSH tissue levels in the FA + RA 100 group compared with the FA group, as indicated in Fig. 5C.

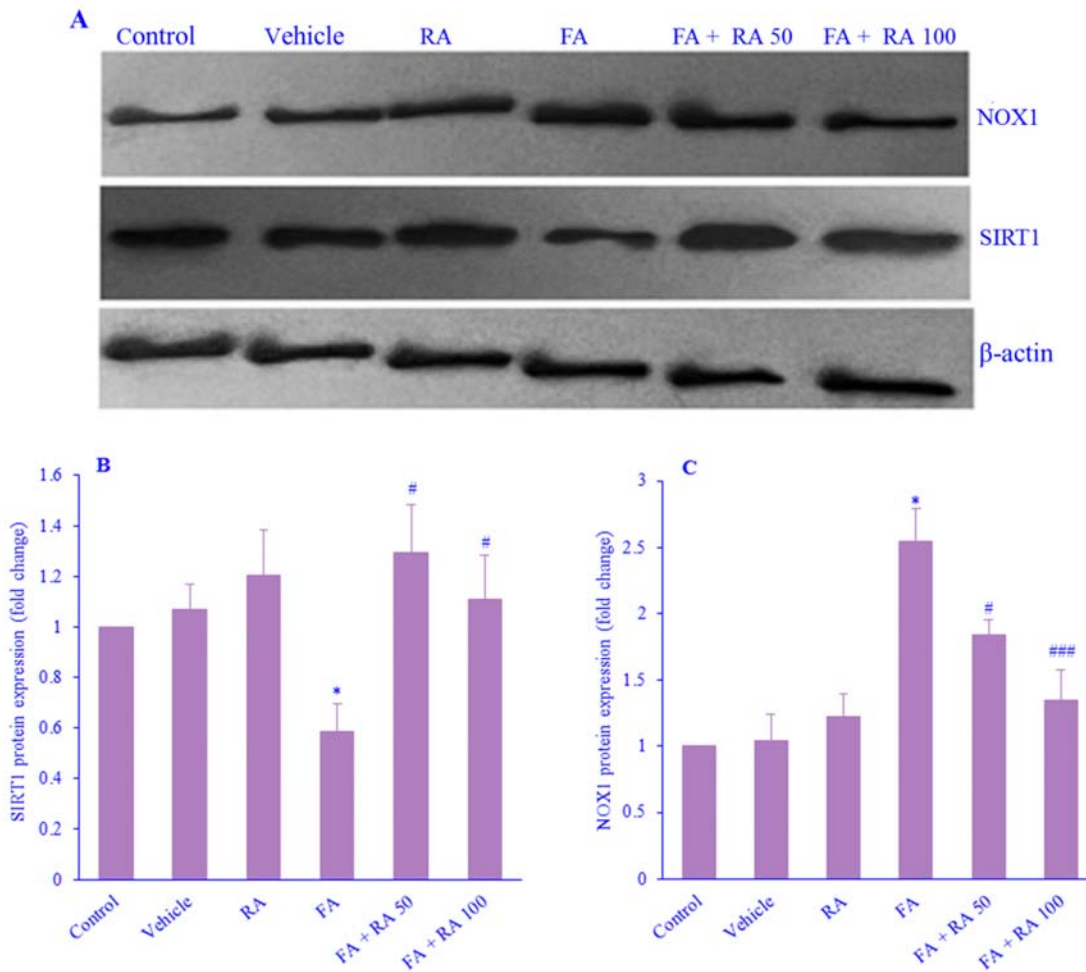


Fig 4. The RA effect (50 and 100 mg/kg) on the expression of renal SIRT1 and NOX1 in FA-induced renal injury. (A) A, the bands of western blotting; (B) and (C) the expression of SIRT1 and NOX1 proteins, respectively. The vehicle group received NaHCO₃ as FA solvent. FA group received a single dose of FA (250 mg/Kg). The data were presented in mean ± SEM, n = 6. **P* ≤ 0.05 indicates a significant difference compared to the control, vehicle, and RA groups; #*P* ≤ 0.05 and ###*P* ≤ 0.001 versus FA group. FA, Folic acid; RA, rosmarinic acid; NaHCO₃, sodium bicarbonate.

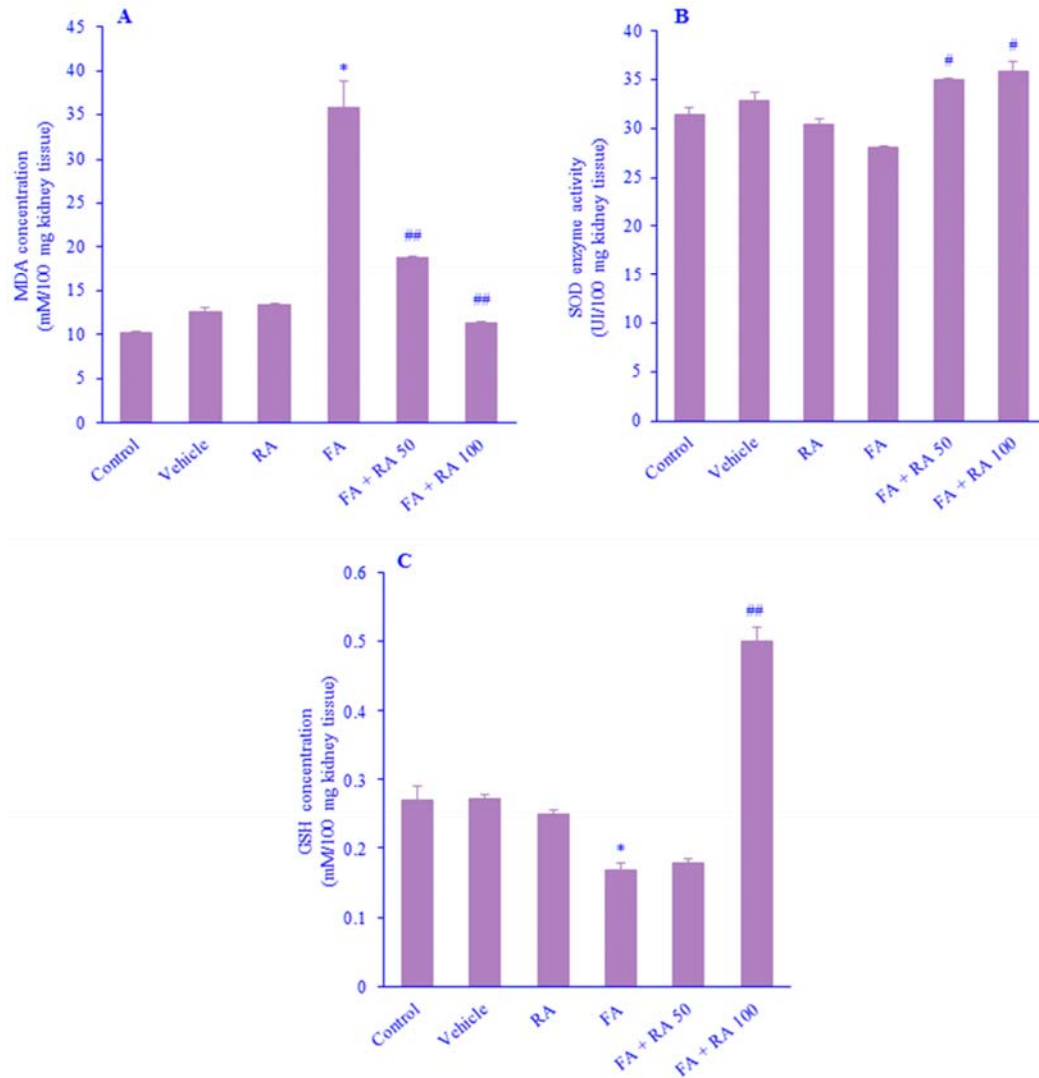


Fig. 5. The RA effect (50 and 100 mg/kg) on (A) MDA concentration; (B) SOD enzyme activity; (C) GSH concentration in the kidney tissue in FA-induced renal injury. The vehicle group received NaHCO₃ as FA solvent. FA group received a single dose of FA (250 mg/Kg). The data were presented in mean \pm SEM, n = 6. * $P \leq 0.05$ indicates significant difference compared to the control, vehicle, and RA groups; # $P \leq 0.05$ and ## $P \leq 0.01$ versus FA group. FA, Folic acid; RA, rosmarinic acid, NaHCO₃; sodium bicarbonate; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione.

DISCUSSION

In this study, the results showed that RA lowered BUN and creatinine as well as attenuated swelling, necrosis, and desquamation of tubular epithelial cells in the FA-induced kidney injury. RA treatment also increased SIRT1 protein expression concomitant with the significant depletion of NOX1 protein expression and enhanced renal antioxidant defense by increasing GSH level and SOD activity and decreasing MDA level.

To the best of our knowledge, this was the first study that evaluated the ameliorative effects of RA on FA-induced mice renal injury through activating the SIRT1/NOX1 pathway and decrementing oxidative stress.

According to previous documents, kidney injury induced by FA is considered a standard model for studying kidney diseases (11,35). In the model, FA leads to high concentrations of BUN and creatinine as considerable indicators for renal function (10). Numerous studies, on the other hand, have suggested that natural

products containing phenolic compounds can be used to mitigate the negative impacts of medications (31,36). This study determined that the BUN and creatinine levels were significantly increased in the FA group. However, RA suppressed the increased levels of BUN and creatinine. These results suggested that RA administration improves kidney function loss in FA-induced kidney injury. These results are consistent with other studies and confirm that RA could alleviate the increased levels of creatinine and BUN caused by FA (37,38).

In this study, extensive degenerative changes such as dilated tubular lumina, vacuolation with loss of the lining epithelium, and necrosis were seen in the FA group; nevertheless, mice that received RA treatment exhibited a significant improvement in the renal histological changes compared to those in the FA group. These findings are consistent with earlier works about RA effects in the amelioration of renal injuries (26,27,39). The adverse impact of FA on the structure of the kidneys may result from the abundance of folate receptors present in the proximal tubules (9). The proximal tubules are the primary sites for reabsorption and active transport due to their significant apical endocytic apparatus. The high doses and longtime administration of FA can cause the excessive accumulation of FA and store it for a long time in the kidney (40,41). FA can be conserved as cell membrane impermeable folate derivatives (42). Notably, when folate is distributed in all cellular compartments, it specifically affects the function of critical organelles and processes necessary for cell survival. Mitochondrial involvement in the processes is particularly significant and can result in mitochondrial abnormalities and oxidative stress (43-46). In addition, dihydrofolate reductase mediates the conversion of folate to tetrahydrofolate, which requires NADPH for it (47). Thus, the accumulation of folate in the kidneys can strictly compromise the cellular antioxidative systems and lead to significant redox imbalance and oxidative stress within the kidney (35,48). Several studies have linked FA-induced renal damage or nephrotoxicity to oxidative stress (49,50). In this study, the expression of SIRT1

and NOX1 indicated that RA supplementation activated the SIRT1/NOX1 pathway in RA-treated animals. Prior research demonstrated that RA can increase SIRT1 expression and regulate the inflammatory-oxidative stress cycle through the SIRT1/NF κ B signaling pathway (22). It is known that SIRT1 reduces NOX production (15). Studies have also confirmed that inhibiting SIRT1 leads to an increase in NOX subunits. SIRT1 is important in several cellular processes and relies on NAD⁺ for its activity (15). In the same way, increasing NAD⁺ levels induced by ROS can diminish SIRT1 activity (15). Abduh's study has well demonstrated the antioxidative effects of SIRT1 and reported that modulating SIRT1 and Nrf2 by RA decreases oxidative stress and attenuates chlorpyrifos-induced oxidative stress in rat kidneys (38). Consequently, the remarkable impact of RA on mice with kidney injury induced by FA (35) could be attributed to the activation of the SIRT1/NOX1 pathway and thereby the attenuation of renal oxidative stress. In this study, RA at the dose of 50 mg/kg led to more increase in SIRT1 expression in comparison to the dose of 100 mg/kg, non-significantly resulted from the different doses of RA. It seems that the different doses of the drug can show antithetical effects.

The accumulation of free oxygen radicals in renal cells triggers a protective mechanism involving various antioxidant enzymes, including SOD and GSH (51). The direct toxic effect of FA can also reduce the activity of one or more antioxidant systems, leading to an increase in lipid peroxidation and oxidative injury, which ultimately worsens renal damage (52). The present findings showed a significant increase in the MDA level and a decrease in the levels of GSH and SOD in the FA group compared to the control group, consistent with the histological findings indicating widespread and massive tubular necrosis manifested through cortical tissue. However, the administration of RA interrupted the vicious cycle by increasing the levels of SOD and GSH while decreasing the level of MDA. This action may be due to the high protective capability of RA against ROS (26,53) along with its ability to stimulate the endogenous antioxidant defense system in renal cells (52).

Similar results have been obtained in rats pre-administered with RA and rosemary extract against diethylnitrosamine-initiated nephrotoxicity (39). Among polyphenols, RA is particularly effective in protecting cortical tubular cells from oxygen radical-induced injury by preventing the generation of oxidized products (54). Cells typically produce small amounts of pro-oxidative agents, which are counteracted by enzyme and antioxidant systems such as glutathione and SOD. In the kidneys, the mitochondrial respiratory system and enzymes like NOX primarily generate ROS. There are different NOX isoforms including NOX1, NOX2, and NOX4, that contribute to oxidative stress production, impair vascular function, and prompt renal fibrosis (55). According to another research, there is an increase in the levels of NOXs in both acute and chronic kidney disease, leading to the overproduction of ROS (56). Therefore, the FA-induced renal injury model in the present study exhibited that the overproduction of ROS in kidney tubules may link with NOXs, resulting in the enhancement of mitochondrial ROS. Consequently, it seems that SIRT1 and NOX1 can initiate a cycle of ROS production. Overall, RA could diminish MDA levels and rise SOD and GSH levels, probably through the increase of SIRT1 and then the decrease of NOX1.

CONCLUSION

The results obtained from this study revealed that the administration of RA through the activation of SIRT1/NOX1 pathway could reduce oxidative stress and enhance antioxidant power in damaged kidneys. Therefore, RA has an improved effect against FA-induced renal damage.

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Conflict of interest statement

The authors declared no conflicts of interest in this study.

Authors' contributions

M. Mottaghi performed the literature review and experiment; A. Eidi assisted in data collecting and analysis; F. Heidari performed the experiment and wrote the manuscript; T. Komeili-Movahhed performed molecular evaluations; A. Moslehi designed the study, analyzed data, and revised manuscript. All authors approved the final version of the manuscript.

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