IJKD KIDNEY DISEASES

Grape Seed Extract for Reduction of Renal Disturbances Following Reperfusion in Rats

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Introduction. Proanthocyanidines in grape seed extract (GSE) possess a wide array of pharmacological and biological actions, including anti-inflammatory, antioxidant, free radical scavenging, and vasodilatory properties as well as inhibition of phospholipase A2, cyclooxygenase, and lipooxygenase enzymes. The aim of this study was to examine the effects of the oral administration of GSE on renal disturbances due to reperfusion injury in rats.

Materials and Methods. Thirty-two male Sprague-Dawley rats were divided into 4 groups. They received a standard diet for two weeks. During this period, one group also received normal saline and GSE (50 mg/kg) daily. At the beginning of day 14, the rats in 2 groups underwent surgery and bilateral renal ischemia, and one group had sham operation. Urine and blood samples were taken and the kidneys were removed for histologic and enzyme studies. The control group did not receive any solutions and did not have surgery.

Results. The increased amount of plasma creatinine concentration induced by reperfusion injury was improved by GSE administration. In addition, urine osmolality increased in the GSE group in comparison with the reperfusion injury only group. The degrees of histological damages and oxidative stress that had increased following reperfusion injury were also significantly lower with GSE administration.

Conclusions. Oral supplementation of GSE for 2 weeks may decrease histologic damages and oxidative stress, and as a result, may reduce kidney function disturbances following reperfusion injury.

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INTRODUCTION

Reperfusion injury is one of the most common causes of acute kidney injury. Disturbances induced by reperfusion injury include arterial endothelium damages, tubular epithelium damages, and inflammation.[1-3] Endothelial damages, lead to the constriction of the renal arteries by increasing the production of vasoconstrictor agents such as adenosine and endothelin and decreasing vasodilator agents, such as nitric oxide and prostaglandins. On the other hand, with the increase of the bonding molecules and linking the leucocytes, platelets and erythrocytes lead to vascular congestion that collectively result in the sustained reduction of the renal blood flow during reperfusion period.[1,2,4] Tubular damages can be seen as necrosis, apoptosis, or intracellular vacuolization. Inflammation of the intracellular

space is induced by the activation of the complement system, production of cytokines and chemokines, and infiltration of the leucocytes into the tissue of the kidneys. With increased production of such substances as reactive oxygen species (ROS) and proxy nitrite, these can exaggerate arterial and tubular damages.[1,3]

Black grape seed extract (GSE) contains flavonoids such as proanthocyanidin oligomers, which include catechin and epicatechin. It has been demonstrated that the antioxidant property of proanthocyanidin oligomers is nearly 50 times greater than that of vitamins C and E.[6,5] In addition to the antioxidant and free radical scavenging properties, proanthocyanidin possess several other effects such as vasodilatory, anti-inflammatory, endothelial nitric oxide synthase (NOS) activating and inducible NOS inhibiting, inhibition of cyclooxygenase, lipoxygenase, proliferation of T lymphocytes, infiltration of neutrophils, apoptosis and necrosis, platelet aggregation, and increased capillary permeability.[6-13] Furthermore, it has been demonstrated that using proanthocyanidin decreases renal damages following gentamicin, cisplatin, and methotrexate administration.[14-16]

This study was designed to examine the effect of the oral administration of GSE on kidney function parameters, histological damages, and oxidative stress in kidney tissues following 30 minutes of ischemia and reperfusion for 24 hours.

MATERIALS AND METHODS

Experimental Animals and Induction of Renal Ischemia

This experimental study was conducted on 32 male Sprague Dawley rats (weight range, 250 g to 320 g) which were housed in a room at suitable temperature with a 12-hour light-dark cycle and free access to water and standard pellet chow. Throughout the study, all protocols and codes issued by the Ethics Committee of the Ministry of Health of Iran for using labortory animals were observed.

The rats were randomly divided into 4 groups (n = 8 in each group). In the reperfusion and GSE groups, the rats were kept for 13 days in ordinary cage, during which they received water or GSE (50 mg/kg/d) through gavage, respectively. At the end of this period, the rats were anesthetized with pentobarbital sodium (50 mg/kg to 60 mg/kg, intraperitoneally) and the arteries and veins of both kidneys were simultaneously clamped for 30 minutes. Then, incision area was sutured and the 24-hour reperfusion period started. At the beginning of the last 6 hours of this period, the rats were transferred to a metabolic cage and their urine samples were collected, and at the end of the reperfusion period, plasma samples were obtained from the descending aorta. Next, the right kidney was first removed and after snap freeze in liquid nitrogen, it was moved to the -70°C freezer in order to measure its oxidative stress parameters (malondialdehyde and ferric reducing ability of plasma [FRAP]). Then, the left kidney was fixed in 10% formaldehyde to be studied histologically. The same protocol was followed for a sham group that did not receive GSE, except for the fact that during the operation, the arteries and veins of both kidneys remained intact. The control group did not receive any substances or operation.

Preparation of Black Grape Seed Extract

After extracting the black grape (*Vitis vinifera*) juice, the obtained seed and peel were dried in shade, milled, and turned into powder. Then, 500 mL of 70% ethanol was added for every 500 g of the powder and mixed in the rotary at room temperature for one day. The obtained mixture was again rotated in a 40°C incubator. With gradual reduction of temperature, the mixture turned into a thick black solution which was easily soluble in water.[17]

Measurement of Plasma and Urine Parameters

Plasma creatinine and plasma and urine osmolality were measured by auto-analyzer devices (Technicon, RA-1000, USA) and osmometer (Osmomat 010, Gonotec, Germany), respectively. For evaluating the status of oxidative stress, malondialdehyde and FRAP values in kidney tissue samples were measured. These parameters were measured, as explained in our previous study,[18] through the Ohakawa[19] and Benzie[20] methods, respectively, as summarized below.

Measurement of malondialdehyde. After adding cold phosphates buffered saline to samples under a layer of ice, they were homogenized through a homogenizer (Fisher Scientific, Loughborough, UK). Next, a suspension containing acid acetic,

thiobarbituric acid, and sodium dodecyl sulfate was prepared in each tube. Then, 200 µL of the homogenized sample was added to the tubes intended for testing while 200 µL of different concentrations of the standard sample was added to the standard tubes. The tubes were heated in a water bath at 95°C for 60 minutes. After that, 4 mL of n-Butanol was added to each tube and after centrifuging at 4000 rpm for 10 minutes, the light absorption of upper layer was measured at 532 nm. Tetraethoxypropane was used as the external standard.

Measurement of FRAP. Initially, by mixing acetate buffer, chloride ferric, and 2,4,6-tris(2-pyridyl)-1,3,5-triazine solution, the FRAP detector was provided. Then, standard solution of $FeSO_4$ $7H_2O$ in serial dilutions were provided. After adding FRAP detector and tissue extract to each of the tubes intended for testing and standard to the standard tubes, the intensity of the obtained stain in 593-nm wavelength was read against the blank.

Histopathological Examination

At the end of each experiment, the left kidneys of the rats were fixed in 10% formaldehyde and after processing and immersion in paraffin; the slices (5 µm) were stained in hematoxylin-eosin. The degree of renal histopathological damage in terms of increase in Bowman space, increase in the number of erythrocytes in glomerular capillaries, tubular cell necrosis and their exfoliation into tubular lumens, vascular congestion, and intratubular proteinaceous casts was measured. Scoring the level of histological damages was done as zero for no damage, 1 for 1% to 20% damage, 2 for 21% to 40%, 3 for 41% to 60%, 4 for 61% to 80%, and 5 for 81% to 100%. The total histopathological score was calculated which was equal to all scores of different damages in each group.[18,21,22]

Statistical Analyses

All data were presented as mean ± standard error, and statistical analyses were done by using the SPSS software (Statistical Package for the Social Sciences, version 16.5, SPSS Inc, Chicago, Ill, USA). Between-group comparisons of kidney function parameters and oxidative stress values were done using the 1-way analysis of variance and Duncan post hoc test. The LSD test was also used for determining the exact *P* value. The comparison of total histopathological score between the groups was made by nonparametric Kruskal-Wallis and Mann-Whitney tests. A *P* value less than .05 was considered significant.

RESULTS

Kidney Function

Figure 1 shows that there were no significant differences between the sham and control groups in plasma creatinine concentration. However, ischemia for 30 minutes and reperfusion for 24 hours resulted in significant increases in creatinine concentrations in the reperfusion group ($P < .001$). Administration of GSE for 2 weeks decreased plasma creatinine concentration in the GSE group in comparison with the reperfusion group ($P < .001$), so that its value reached the same level as the sham group and there were no significant differences between them.

Urine osmolality in the control and sham groups were similar (Figure 2); however, its value in the reperfusion group significantly decreased in comparison with the sham group ($P < .001$). Administration of GSE could increase urine osmolality in the GSE group in comparison with the reperfusion group ($P < .001$), and reached to the same level in the sham group so that there were no significant differences between them.

Renal Oxidative Stress Indexes

Malondialdehyde values of kidney tissues in the control and sham groups were not significantly different (Figure 3); however, bilateral occlusion

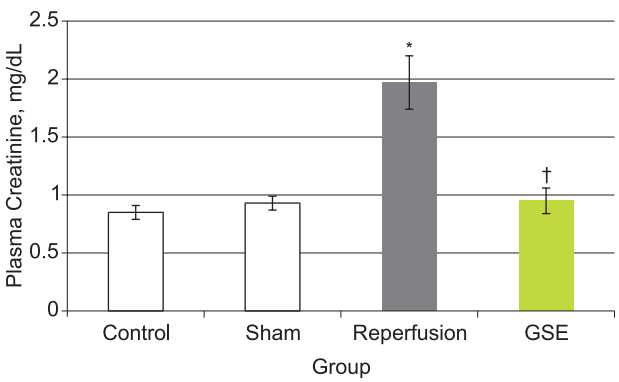

Figure 1. Plasma creatinine concentration following the reperfusion period in rats pretreated with saline (reperfusion group) or grape seed extract (GSE group), as compared to the rats with sham surgery (sham group) or without any operation (control group).
*$P < .001$ as compared with the sham group.
†$P < .001$ as compared with the reperfusion group.

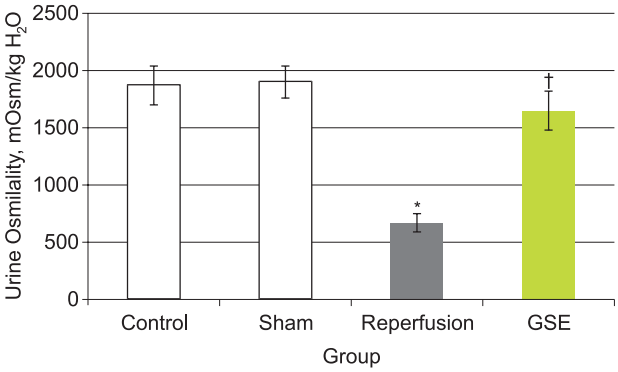

Figure 2. Urine osmolality following the reperfusion period in rats pretreated with saline (reperfusion group) or GSE (GSE group), as compared to the rats with sham surgery (sham group) or without any operation (control group).
*P < .001 as compared with the sham group.
†P < .001 as compared with the reperfusion group.

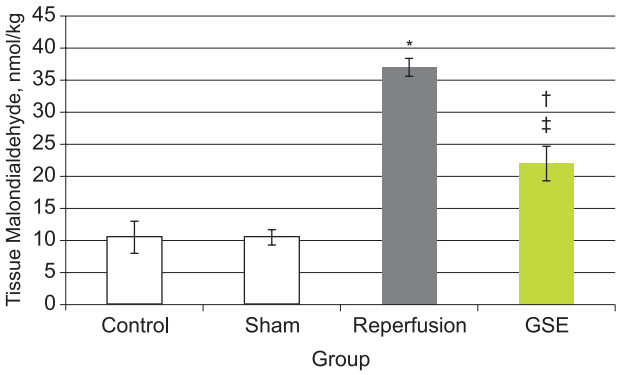

Figure 3. Tissue malondialdehyde following the reperfusion period in rats pretreated with saline (reperfusion group) or GSE (GSE group), as compared to the rats with sham surgery (sham group) or without any operation (control group).
*P < .001 as compared with the sham group.
†P < .01 as compared with the sham group.
‡P < .001 as compared with the reperfusion group.

of the renal arteries and veins for 30 minutes and reperfusion for 24 hours increased the level of malondialdehyde in the reperfusion group in comparison with the sham group (P < .001). Administration of GSE could significantly decreased malondialdehyde values in the GSE group as compared with its corresponding values in the reperfusion group (P < .001), yet it was still significantly higher than the sham group (P < .01). The FRAP value of the kidney tissues in the control group was not different from the sham group (Figure 4), but its value in the reperfusion group was significantly lower than the sham group (P < .05). Administration of GSE could increase the level of FRAP in the GSE group in comparison with the reperfusion group (P < .01). The FRAP in

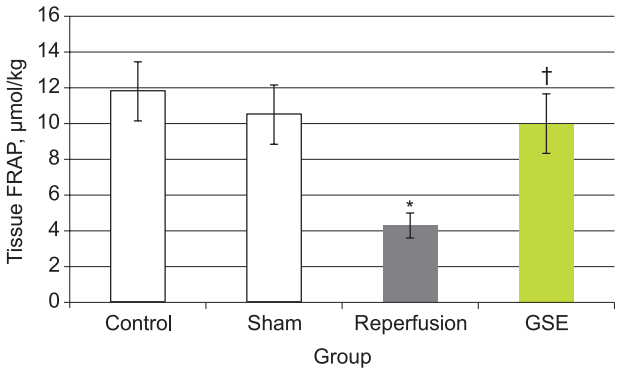

Figure 4. Tissue ferric reducing ability of plasma (FRAP) following the reperfusion period in rats pretreated with saline (reperfusion group) or GSE (GSE group), as compared to the rats with sham surgery (sham group) or without any operation (control group).
*P < .05 as compared with sham group.
†P < .01 as compared with the reperfusion group.

this group reached the same level as that in the sham group.

Renal Histological Damages

The histological status in the control group was similar to that in the sham group. No histological damages were observed in the kidneys procured from the sham group rats (Figure 5). In the reperfusion group, the Bowman space was enlarged and the number of erythrocytes decreased in the glomerular capillaries (Figure 5B). In addition, cells in the proximal tubules walls showed severe damage, vacuolization, and exfoliation into the lumens. All these damages had a lower score in the GSE group in comparison with the reperfusion group (Table).

In the external medulla of the reperfusion group, cellular damages in the tubular segments of pars recta (S3) and the thick ascending limb of loop of Henle were severe. The severity of these damages decreased to some extent in the GSE group (Figures 5E and 5F). Also, GSE administration somehow improved vascular congestion and intratubular proteinaceous casts in the GSE group in comparison with reperfusion group (Figure 6). Also, in the internal medulla, reperfusion injury led to moderate vascular congestion and intratubular proteinaceous casts in the reperfusion group in comparison with the sham group, but they were less obvious in the GSE group (Table).

The total histopathological score in the reperfusion group was 43 (Table), which was

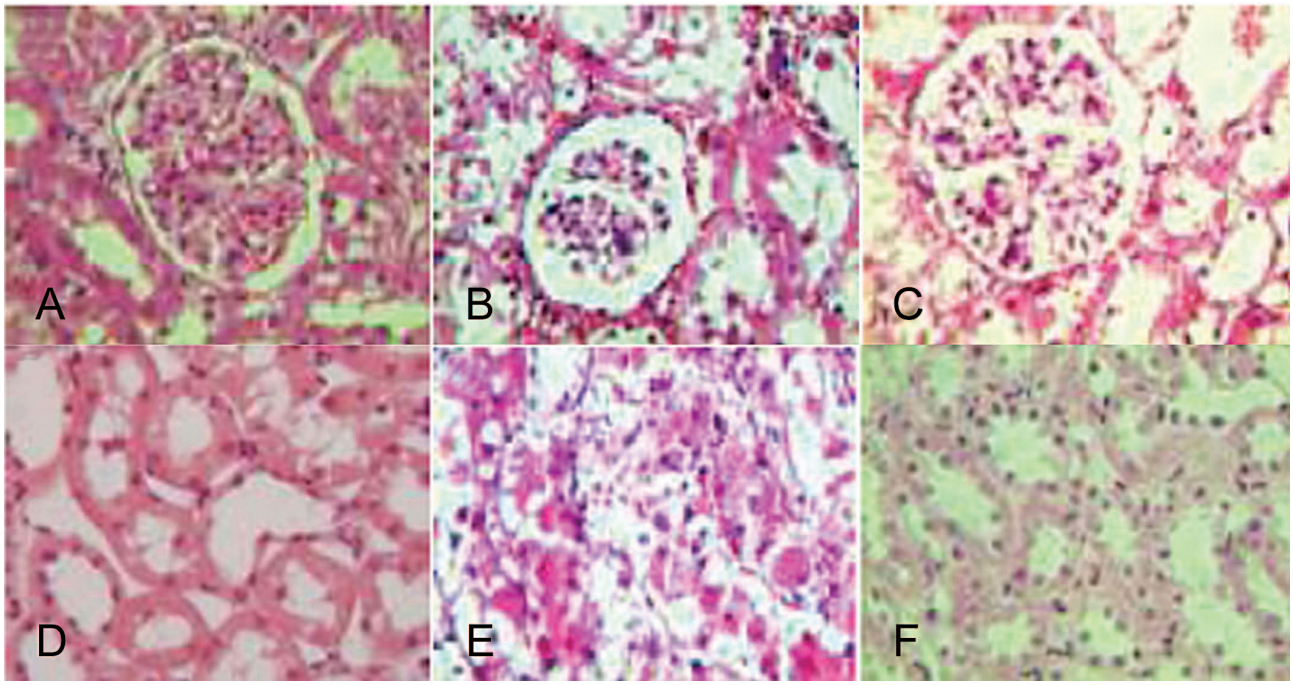

Figure 5. Renal cortex for Bowman space widening; **A**, sham group; **B**, reperfusion group; and **C**, GSE group. Outer medulla for tubular cells necrosis; **D**, sham group, **E**, reperfusion group; and **F**, GSE group (hematoxylin-eosin, × 400).

significantly higher in comparison with the scores in the sham group ($P < .001$). Although this score was lower (18) in the GSE group ($P < .01$), yet it was still different from that in the sham group ($P < .001$).

Renal Histopathological Scores Following Bilateral Reperfusion Injury and Impact of Oral Grape Seed Extract (GSE) Administration

Histopathology Groups	Rat Groups		
	Sham	Reperfusion	GSE
Cortex			
Bowman space enlargement	0	5	2
Proximal tubule injury	0	3	1
Thick ascending limb injury	0	2	1
Reduced number of erythrocytes in glomerular capillaries	0	5	1
Intracellular vacuolization	0	4	2
Outer medulla			
Pars recta (S3) injury	0	4	1
Thick ascending limb injury	0	5	2
Vascular congestion	0	5	2
Intratubular proteinaceous casts	0	4	2
Inner medulla			
Vascular congestion	0	3	2
Intratubular proteinaceous casts	0	3	2
Total histopathological score*	0	43	18

*The total score of the reperfusion and GSE groups were significantly higher than that of the sham group (P < .001 for each). The total score of the reperfusion group was also significantly higher than that of the GSE group (P < .001).

DISCUSSION

This study was conducted to investigate the effect of GSE on the changes of plasma creatinine concentration, renal concentrating ability, histological damages, and oxidative stress in the kidneys following bilateral renal ischemia for 30 minutes and reperfusion for 24 hours. The examination of the cortical and medullary regions showed that renal reperfusion injury resulted in evident necrosis in renal tubular cells, increased Bowman space and cell vacuolization, decreased number of erythrocytes in glomerular capillaries, and led to formation of intratubular casts and medullar congestion in the reperfusion group (Table). The main factor in establishing of different tubular damages due to reperfusion injury is the intensity of intracellular adenosine triphosphate depletion. In cell necrosis, severe cellular adenosine triphosphate depletion following ischemia leads to complete disruption in metabolic activity of cells, activation of inflammatory processes, and decreased activity of calcium pumps. This leads to mitochondrial damages, increased production

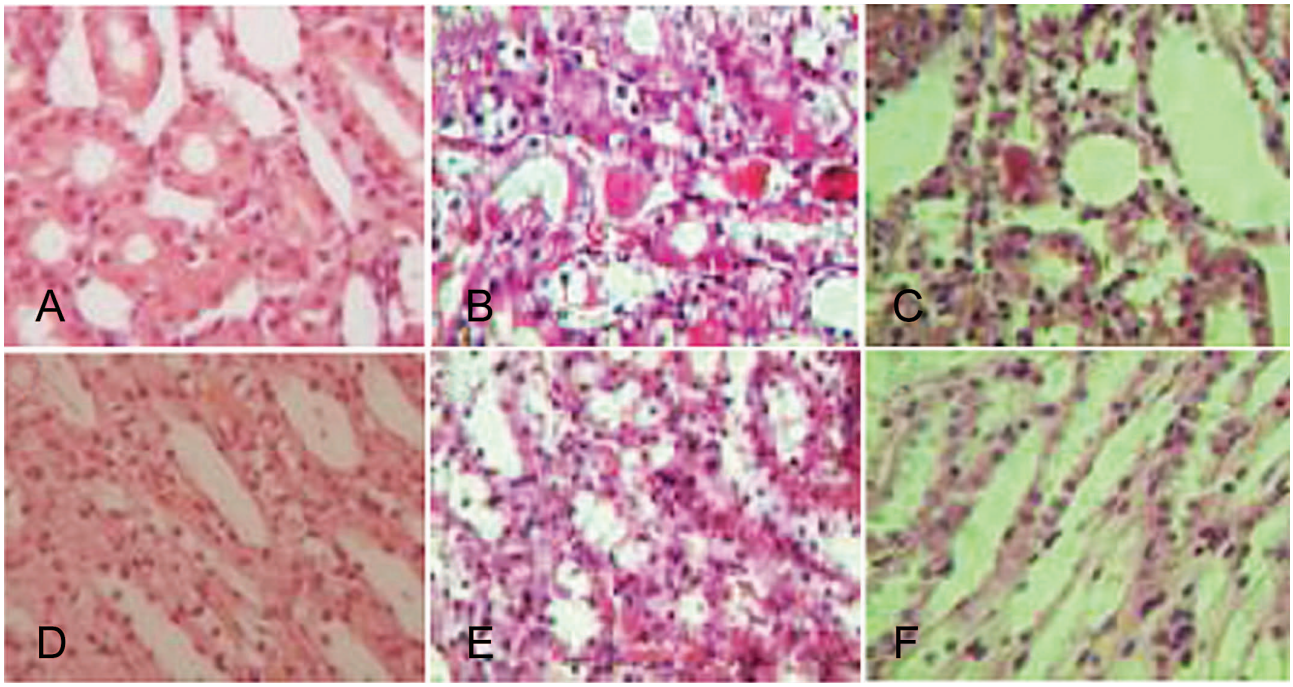

Figure 6. Renal outer medulla showing intratubular casts and vascular congestion; **A** and **D**, sham group; **B** and **E**, reperfusion group; and **C** and **F**, GSE group (hematoxylin-eosin, × 400).

of ROS, and increased intracellular calcium, which result in the destruction of proteins, DNA, and cell membranes together by activating phospholipases, proteases, endonucleases, and peroxidation of lipids and proteins. Resulting in inflation and explosion of cells, this causes the spread of damage to the adjacent cells.[1,23,24]

The increase in cytosolic calcium, in addition to having a constrictive effect on arteries, results in tubular epithelium cells necrosis by activating phospholipases, endonucleases, and proteases, breaking the cellular skeleton, and intervening in mitochondrial energy metabolism.[24] The three main targets in cells which are attacked by the ROS are lipids, proteins, and DNA; ROS react with unsaturated fatty acids and cause lipid peroxidation and inactivate them by attacking the -SH and the $-NH_2$ groups of proteins. In addition, ROS cause DNA fragmentation by changing the structure and chemical properties of DNA.[25] It is noteworthy that renal reperfusion injury enhances the expression of inducible NOS enzyme in tubules and the production of NO. Increased production of NO by inducible NOS, on the one hand, results in its rapid reaction with $°O_2^-$ and leads to production of highly reactive $CNOO^-$ that with a greater power than ROS induce direct oxidant damages and nitrosylation of proteins in cells,[26] and, on the other hand, inhibiting endothelial NOS and decreasing the production of NO in the endothelial layer of arteries, ROS remove its moderating effects on vasoconstrictor substances, such as angiotensin II, catecholamines, and adenosine, and plays a role in increasing renal arteries constrictions and further decreases in renal blood flow.[27,28]

In this study, histopathological damages and oxidative stress in kidney tissues were shown to decrease following GSE consumption. Ray and colleagues showed that GSE could reduce necrosis and apoptosis due to the consumption of high doses of acetaminophen in rat liver cells.[8] They concluded that GSE could induce such effects via blockade of DNA fragmentation by calcium and magnesium-dependent endonuclease and increasing the expression of antiapoptotic proteins $Bcl\text{-}X_L$. It seems that GSE also decreases the level of necrosis and apoptosis through its antioxidant and free radical scavenging effects. Jia and colleagues demonstrated that free radicals scavenging and antioxidant effects of GSE acted by decreasing the expression of MAPK and NF-κB.[29] Oxidative agents produced in the cells by activating of this two factors result in the activation of the signaling pathways of such processes as apoptosis, bonding

the cells to each other, proliferation, inflammation, and cells response to stress.[30,31] Other researchers have shown that GSE restores level and activity of antioxidant enzymes such as glutathione peroxidase, glutathione transferase, superoxide dismutase, and catalase in kidneys, liver, and heart of rats that had received cisplatin.[15] Additionally, it has been shown that GSE has endothelial NOS activating and indicible NOS inhibiting effects.[12,13] As it was mentioned earlier, one of the problems following renal reperfusion injury is the activation of inducible NOS and inhibition of endothelial NOS, which increases oxidative stress and damages renal blood flow.[26] Therefore, GSE might be involved in decreasing oxidative stress in kidney tissues and improving renal blood flow in this way, as well.

In the present study, it was also shown that renal reperfusion injury results in increased plasma creatinine concentration and decreased urine osmolality, which indicates the decreased cleaning and concentrating activity of kidneys. Through administration of GSE, the levels of these two parameters in the GSE group became closer to their corresponding values in the sham group. This was due to the decreased amount of cellular damages and oxidative stress as well as improved renal blood flow in this group.

CONCLUSIONS

Oral administration of GSE reduces the amount of cellular damages and oxidative stress, and as a result, it improves the function of kidneys in acute kidney injury induced by reperfusion injury in rats. Although the exact mechanism of the renal effects of GSE is not currently known and can be a topic for future studies, one or several of the processes explained above might be responsible in this regard.

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CONFLICT OF INTEREST

None declared.

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