

## Research Article

Velid Unsal\*, Engin Kolukcu, Fatih Firat and Fikret Gevrek

# The protective effects of sinapic acid on acute renal ischemia/reperfusion injury

## Sinapik asidin akut renal iskemi/reperfüzyon hasarı üzerine koruyucu etkileri



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### Abstract

**Objectives:** The aim of this research was to investigate whether sinapic acid (SA) can alleviate oxidative damage, apoptosis, and inflammation in I/R induced renal injury.

**Methods:** A total of 24 male rats were randomly separated into four groups as six rats in each group. Group 1 (Sham), Group 2 (I/R), Group 3 (I/R + SA, 10 mg/kg), Group 4 (I/R + SA, 20 mg/kg). In order to evaluate kidney function serum BUN, Cr, and AST were measured in an autoanalyzer. SOD, GSH-Px, MDA, PC and NO oxidative stress parameters were measured with spectrophotometric methods and TNF- $\alpha$ , IL-1 $\beta$ , IL-6, KIM-1 and NGAL parameters were measured with the ELISA method. In addition, H&E method and immunohistochemical examinations were performed for histological evaluations of kidney tissue.

**Results:** SA significantly decreases the increase in kidney damage, inflammation, oxidative stress, cell death and restore the decrease in antioxidant enzyme activities ( $p < 0.05$ ). Pre-treatment of the rats with SA reduces kidney dysfunction and morphological changes.

**Conclusions:** The development of oxidative stress and lipid peroxidation seems to be the leading factors that accelerate inflammation and cell death during renal IRI. The antioxidant, anti-inflammatory, and anti-apoptotic features of SA displayed a renoprotective effect.

**Keywords:** apoptosis; kidney; KIM-1; NGAL; oxidative stress; sinapic acid.

### Öz

**Giriş:** Bu araştırmanın amacı, I/R kaynaklı böbrek hasarında sinapik asidin (SA) oksidatif hasarı, apoptozu ve inflamasyonu hafifletip hafifletemeyeceğini araştırmaktır.

**Yöntemler:** Toplam 24 erkek sıçan her grupta 6 adet sıçan olacak şekilde rastgele 4 gruba ayrıldı. Grup 1 (Sham), Grup 2 (I/R), Grup 3 (I/R + SA, 10 mg/kg), Grup 4 (I/R + SA, 20 mg/kg). Böbrek fonksiyonunu değerlendirmek için serum BUN, Cr ve AST otoanalizörde ölçüldü. SOD, GSH-Px, MDA, PC ve NO oksidatif stres parametreleri spektrofotometrik yöntemlerle, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, KIM-1 ve NGAL parametreleri ELISA yöntemiyle ölçüldü. Ayrıca böbrek dokusunun histolojik değerlendirmeleri için H&E yöntemi ve immünohistokimyasal incelemeler yapıldı.

**Bulgular:** SA, böbrek hasarı, inflamasyon, oksidatif stres, hücre ölümündeki artışı önemli ölçüde azaltır ve antioksidan enzim aktivitelerindeki azalmayı geri kazandırır ( $p < 0.05$ ). Sıçanların SA ile ön tedavisi böbrek fonksiyon bozukluğunu ve morfolojik değişiklikleri azaltır.

**Sonuç:** Oksidatif stres ve lipid peroksidasyonunun gelişimi, renal IRI sırasında inflamasyonu ve hücre ölümünü hızlandıran önde gelen faktörler gibi görünmektedir. SA'nın antioksidan, anti-inflamatuar ve anti-apoptotik özellikleri, renoprotektif bir etki göstermiştir.

**Anahtar Kelimeler:** Apoptozis; Böbrek; KIM-1; NGAL; Oksidatif stres; Sinapik Asit.

\*Corresponding author: Velid Unsal, Faculty of Health Sciences and Central Research Laboratory, Mardin Artuklu University, Mardin, Turkey, E-mail: velidunsal@gmail.com. <https://orcid.org/0000-0003-1415-0563>

Engin Kolukcu, Department of Urology, Faculty of Medicine, Gaziosmanpasa University, Tokat, Turkey

Fatih Firat, Department of Urology, Tokat State Hospital, Tokat, Turkey

Fikret Gevrek, Department of Histology and Embryology, Faculty of Medicine, Gaziosmanpasa University, Tokat, Turkey

## Introduction

It is emphasized with increasing evidence that inflammation, oxidative stress, necrosis and apoptosis have adverse effects in the pathophysiological onset and progress of renal ischemia-reperfusion (I/R) injury [1–3]. Oxidative stress is a result of the increased free radical formation and/or decreased physiological activity of the antioxidant defense against free radicals [4]. The downregulation of antioxidant enzymes such as Superoxide dismutase (SOD), Glutathione peroxidase (GSH-px) is believed to be responsible for the pathophysiology of IR injury. Malondialdehyde (MDA) is a lipid peroxidation marker whose concentration directly reflects the degree of lipid peroxidation. In addition, Protein Carbonyl (PC) is an indicator of protein oxidative injury [5, 6]. Inflammation is a prevalent anomaly in renal I/R and plays a significant role in its pathophysiology [7, 8]. Kidney Injury Molecule-1 (KIM-1), Neutrophil Gelatinase-Associated Lipocalin (NGAL), are suggested to be the early markers of acute renal failure [9, 10]. The anti-inflammatory, antioxidant and anti-apoptotic properties of sinapic acid are well-documented [11]. Despite the progress made in the treatment of renal I/R injury, alternative therapies have some uncertainties. Although there are significant data showing the protective antioxidant, anti-inflammatory and anti-apoptotic properties of SA, the protective effect of SA against kidney injury caused by I/R has not been researched adequately. The aim of this research was to investigate whether SA can alleviate oxidative damage, apoptosis, and inflammation in I/R-induced kidney injury.

## Materials and methods

### Animals and renal I/R model protocol

Male Wistar albino rats (290–360 g, 12–14 weeks old) were obtained from Gaziosmanpaşa University Experimental Medicine Research Unit. They were accommodated in standard environmental conditions with temperature control and free access to food and water. The rats were not fed a day before the surgery. Animals were provided care in line with the National Institutions. All animals procedures were followed in accordance with ethical rules. The study was approved by Gaziosmanpaşa University Animal Experiments Local Ethics Committee (2019-HADYEK-43). Prior to the surgery, the rats were administered a combination of 10 mg/kg 2% xylazine hydrochloride and 50 mg/kg ketamine hydrochloride intramuscularly (i.m.) for anesthesia. After their abdominal regions were shaved, they were laid down on the operation table in the supine position. After the operation area was wiped with 10% povidone-iodine, with a 3 cm median incision on the upper abdomens, skin, subcutaneous tissue, fascia and periton were opened. The left kidney was found, the ureteropelvic junction was dissected, and the renal pedicle was accessed. Then, steel-alloy microvascular vein

clamps, which do not squeeze the tissue, were suitable for working on arteries and veins, used in veins with 0.4–1.0 mm length and whose pressure does not exceed 5 mm/Hg, were used. The rats were randomly divided into four groups, each comprised of six rats: The only laparotomy was applied to Group 1 (Sham group, n=6) for control purposes. Group 2 (I/R group, n=6) was not administered anything before the experiment. Upon laparotomy, the left kidney artery was clamped for 30 min. After opening the clamp, reperfusion was allowed for 60 min, and left nephrectomy was applied and the skin was closed. Group 3 (I/R + SA, 10 mg/kg, n=6) was given 10 mg/kg sinapic acid through gavage 1 h before the operation. Upon laparotomy, the left kidney artery was clamped for 30 min. After opening the clamp, reperfusion was allowed for 60 min, left nephrectomy was applied, and the skin was closed. Group 4 (IR + SA, 20 mg/kg, n=6) was given 20 mg/kg sinapic acid through gavage 1 h before the operation. Upon laparotomy, the left kidney artery was clamped for 30 min. After opening the clamp, reperfusion was allowed for 60 min, and left nephrectomy was applied, and the skin was closed [12–14]. During reperfusion, sterile saline (0.9%, wt/vol and 2 mL/kg/h) was given to the abdominal cavity as in the previous surgery in order to prevent the hypovolemic effects of the fluid lost after the surgery. This process was applied to each animal. Regarding renal tissue samples taken for histological studies, in all groups right after the dissection, the left kidney was removed, and the middle part was put in 10% neutral formaldehyde fixation liquid for histological analyses, and other parts were placed in a deep freezer at –80 °C in polyethylene tubes for biochemical analyses. At the finalizing phase of the experiment, blood was taken from the anesthesia intracardiac. In order to obtain serum, the blood samples taken were centrifuged at 3,280×g for 5 min. The blood samples were placed in a deep freezer at –80 °C.

### Preparation of renal tissues for biochemical analyses

The tissues were washed in a frosty, normal saline solution (0.9%). After the renal tissue was weighed, homogenate and supernatant samples were prepared. Some of the renal tissues were homogenized with a homogenizer with pH 7.4 Tris-HCl phosphate buffer. 10% homogenates were centrifuged at 4 °C at 3,208×g for 15 min. SOD, GSH-Px activities and PC levels in the supernatant were determined, Nitric oxide (NO) and MDA levels in the homogenate were studied.

### Chemicals

All materials used were in the analytical category. These materials were purchased from the chemical companies Merck, Sigma and Isolab.

### Oxidative stress markers and the biochemical test of the anti-oxidative status

**Measurement of SOD and GSH-Px activity of the tissue:** SOD enzyme activity was studied with the method modified by Sun et al. [15]. The principle of this method is based on the reduction of nitrobluete-trazolium (NBT) by the xanthine-xanthineoxidase system, which is a superoxide producer. One unit of SOD was defined as the amount causing 50.0% inhibition in the NBT reduction rate. Specific activity (U/g protein) = [U/mL/g/mL protein]. SOD activity was expressed as U/g protein.

GSH-Px activity was studied according to the method of Paglia et al. [16]. GSH-Px activity was calculated by reading the absorbance decrease at 340 nm during the oxidation of NADPH to NADP<sup>+</sup>. The GSH-Px activity was calculated using the molar absorption coefficient of NADPH ( $\epsilon_{\text{NADPH}}: 6,220 \text{ L mol}^{-1} \text{ cm}^{-1}$ ). GSH-Px activity (U/g protein) =  $(\Delta\text{OD}/\text{min}) \times 4,800/\text{protein concentration (mg/mL)}$  GSH-Px activity was expressed as U/g protein.

**Measurement of the MDA level of the tissue:** The study was performed according to Esterbauer method [17] which is a measurement method of lipid peroxidation. MDA, which reacts with thiobarbutyric acid at 90–95 °C, creates a pink chromogen. Fifteen minutes later, the absorbance of the rapidly cooled samples were spectrophotometrically read at 532 nm. Graph was drawn using stock standard solution (1,1,3,3 tetramethoxypropane). MDA (nmol/g wet tissue) =  $(\text{Sample OD}/\text{Standart OD}) \times (\text{Standard Concentration})$ . MDA level was expressed as mmol/g wet tissue.

**Measurement of protein carbonyl content and NO level in tissue:** The tissue PC was determined spectrophotometrically through the method based on the reaction of the carbonyl group to form 2,4-dinitrophenylhydrazine 2,4-(DNPH). 2,4-DNPH was originally a reactive used for proteins exposed to metal-catalyzed oxidation. During the calculations, the molar absorption coefficient  $s=22,000 \text{ M}^{-1} \text{ cm}^{-1}$  at 360 nm was taken for 2,4-DNPH. PC (nmol/mg protein) = Carbonyl (nmol/mL)/protein (mg/mL). The results were expressed as nanomole carbonyl per gram protein (nmol/mg protein) [18].

The method for measuring nitrite and nitrate levels in tissue was based on the Griess reaction. Total nitrite [nitrite + nitrate] was measured at 545 nm by the spectrophotometric method as a result of converting nitrate to nitrite with copper cadmium granules. A standard curve was created with sodium nitrite by making a series of serial dilutions ( $10^{-8}$ – $10^{-3} \text{ mol/L}$ ), linear regression was performed using the peak area from the nitrite standard. Unknown sample concentrations were calculated from the resulting equation. Results were expressed in micromoles per g wet tissue (mmol/g wet tissue) [19].

**Measurement of the protein amount of the tissue:** Initially, the protein contents of the homogenates and supernatants s were measured according to the Folin-Lowry method et al. [20]. Bovine serum albumin was used as a standard. The standard graph was obtained by using solutions prepared from bovine serum albumin, the concentration of which we know. Protein (mg/mL) = value read from graph  $\times$  Factor ( $\times 20$ ) [20].

### Acute kidney injury indicators and inflammation markers

After the renal tissue reserved for ELISA was weighed, supernatant samples were prepared. The renal tissue was homogenized with a homogenizer with 0.01 N pH=7.2–7.4 phosphate buffer. 10% homogenates were centrifuged at 4 °C at 3,208×g for 15 min. At the end of the procedure, the surface part was taken and the supernatant was obtained. KIM-1, NGAL, Tumor Necrosis Factor alpha (TNF- $\alpha$ ), Interleukin-1 $\beta$  (IL-1 $\beta$ ) and Interleukin-1 $\beta$  (IL6) levels in tissue supernatants were measured by using ELISA kits according to the instructions of the producer. All ELISA kits (Bioassay Technology laboratory brand) were procured by Atlas Biotechnology company (Ankara, Turkey). The protein amount in supernatants was measured, as mentioned before. The results were expressed as ng/protein. Besides, Blood Urea

Nitrogen (BUN), Creatinine (Cr) and Aspartate Aminotransferase (AST) parameters in the serums were measured in an autoanalyzer (Cobas e 601, Roche Diagnostics, USA).

### Histological procedures

At the end of the experiment, the kidney of the rats under anesthesia were removed and immediately placed in neutral (pH: 7.2) formalin solution with 4% buffer and kept there for 48 h for fixation. Five micrometer wide consecutive fine series of sections were taken from the blocked kidneys through rotary microtome (Leica RM2135, Germany). The kidney tissue sections taken were placed on ground frozen laminae for hematoxylin eosin staining and on Poly-L-Lizin laminae for immunohistochemical staining. They became preparats to be used for histopathological and immunohistochemical analyses.

### Hematoxylin-eosin staining and immunohistochemical analyses

Hematoxylin eosin stained kidney tissue preparats were analyzed with light microscope (Nikon Eclipse, Japan) histologically. The analyses were performed on an average of consecutive 5–6 sections belonging to each individual and in five different medullar and cortical regions in each section. Microscopic analyses were run as evaluations in terms of inflammation and general histological structure. All these procedures were carried out by a histologist with a coding system as a blind study. In order to determine apoptotic active caspase-3 (Acas-3) and anti-apoptotic (Bcl-2) protein expressions in the 5  $\mu\text{m}$  wide kidney tissue sections immunohistochemically, they were immunohistochemically stained with Acas-3 and Bcl-2 primary antibodies. Immune staining degrees were determined on the basis of the immune staining degree scoring criteria in Table 1 by NIS-Element program (Hasp ID: 6648AA61; Nikon) with 40x zooming in research light microscope (Nikon Eclipse 200; Nikon). For this purpose, immune-stained and unstained cells in the kidney in the five immunohistochemically stained five sections for each protein molecule were categorically counted according to staining reaction degrees. The weighted group average results obtained were converted to H-score values with the  $[\sum Pi(i + 1)]$  formula. In this formula,  $i$  represents the staining intensity score, while  $Pi$  represents the percentage of cells stained.

### Statistical analyses

In order to decide whether to use parametric or non-parametric analysis methods in this study, the data were first tested for normality. Therefore, Kolmogorov-Smirnov and Shapiro-Wilk tests were applied. As a result of the evaluation, it was seen that the data did not show a

**Table 1:** Rating criteria for immunohistochemical reactivity (staining degrees).

Score	Immune reactivity
0+	Negative staining
1+	Weak staining
2+	Medium staining
3+	Strong staining

normal distribution because the p values were less than 0.05. In line with this result, non-parametric tests were preferred. Therefore, Mann-Whitney U test was used for biochemical parameters. Following the homogeneity test of the variances, a comparison of immune staining H-scores values was made with Tukey HSD, which is one of the One-Way ANOVA Post Hoc multiple tests. The values of all variables measured were presented as mean  $\pm$  standard deviation (mean  $\pm$  SD).  $p < 0.05$  was considered statistically significant. GraphPad prism 9 program was used for the drawings of the graphs.

## Results

### Renal I/R-induced oxidative stress and inflammation reduced with SA

SOD, GSH-Px enzyme activities, MDA, NO and PC levels were determined in all groups. In comparison with the G1 group, a significant increase was observed in the MDA and PC levels of the G2 group rats ( $p < 0.05$ ), while a significant decrease was observed in the SOD and GSH-Px activities ( $p < 0.05$ ). While SA increased SOD and GSH-Px activity in G3 and G4 rats, it significantly decreased MDA and PC levels. The results indicate that SA reduced the oxidative stress (MDA and PC) induced by renal I/R. In addition, the results also show that SA has the potential to increase the activity of SOD and GSH-Px enzymes. The level of NO in renal tissue was significantly increased in rats exposed to renal I/R ( $p < 0.05$ , G2 vs. G1). The

increased tissue NOx levels significantly decreased in groups G3 and G4 (Table 2). Following renal I/R injury, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 levels of group G2 significantly increased compared to those of group G1 ( $p < 0.05$ ). SA treatment suppressed these inflammatory changes (G3 and G4,  $p < 0.05$ ). TNF- $\alpha$ , IL-1 $\beta$  and IL-6 levels slightly decreased after treatment with a low dosage of SA (10 mg/kg,  $p > 0.05$ ), but no significant difference was observed. However, with 20 mg/kg dosage of SA treatment, significant decrease was observed ( $p < 0.05$ ). The results indicate that cytokines induced by renal I/R are critical. Besides, they also show that cytokines have the capacity to reduce inflammation resulting from renal I/R injury (Table 3).

### SA modulates serum BUN, Cr and AST values

Thirty-minute renal ischemia and the following 1-h reperfusion caused injury in the kidneys. Kidney function tests were evaluated by measuring serum BUN, Cr and AST levels. BUN, Cr and AST levels of group G2 were significantly higher compared to group G1 ( $p < 0.05$ ), which suggested that there was considerable glomerular dysfunction. SA treatment led to a considerable decrease in these parameters ( $p < 0.05$ , G3 and G4) (Table 4). The data obtained from serum biochemical examination suggested that SA had a renoprotective effect in healing glomerular function.

**Table 2:** The effect of renal ischemia reperfusion (I/R) and SA on tissue SOD and GSH-Px activity, MDA, PC and NO levels.

Experimental groups	SOD, U/g protein, n=6	GSH-Px, U/g protein, n=6	MDA, nmol/g wet tissue, n=6	PC, nmol/mg protein, n=6	NO, mmol/g wet tissue, n=6
G1	45.3 $\pm$ 4.8 <sup>b,c,d</sup>	4 $\pm$ 0.43 <sup>b,c</sup>	5.1 $\pm$ 0.3 <sup>b,c,d</sup>	1.25 $\pm$ 0.20 <sup>b</sup>	0.42 $\pm$ 0.09 <sup>b,c</sup>
G2	28.1 $\pm$ 3.3 <sup>a,d</sup>	2.3 $\pm$ 0.39 <sup>a,c,d</sup>	8.6 $\pm$ 0.6 <sup>a,c,d</sup>	1.99 $\pm$ 0.21 <sup>a,c,d</sup>	0.59 $\pm$ 0.08 <sup>a,c,d</sup>
G3	35.8 $\pm$ 2.9 <sup>a,b</sup>	3 $\pm$ 0.18 <sup>a,c,d</sup>	7.1 $\pm$ 0.7 <sup>a,b,d</sup>	1.50 $\pm$ 0.12 <sup>b</sup>	0.47 $\pm$ 0.06 <sup>a,b,d</sup>
G4	40.6 $\pm$ 6.9 <sup>b</sup>	3.8 $\pm$ 0.2 <sup>b,c</sup>	6.28 $\pm$ 0.5 <sup>b,c</sup>	1.28 $\pm$ 0.17 <sup>b</sup>	0.44 $\pm$ 0.09 <sup>b</sup>

<sup>a</sup>Different from G1, <sup>b</sup>Different from G2, <sup>c</sup>Different from G3, <sup>d</sup>Different from G4. Data are expressed as mean  $\pm$  standard deviation (n=6). Values in the same rows with different letters (a–d) are statistically different ( $p < 0.05$ ). SOD, Superoxide dismutase; GSH-Px, Glutathione peroxidase; MDA, Malondialdehyde; PC, Protein carbonyl; NO, Nitric oxide.

**Table 3:** Concentrations of proinflammatory cytokines in the experimental groups.

Experimental groups	TNF- $\alpha$ , n=6, pg/protein	IL-1 $\beta$ , n=6, pg/protein	IL-6, n=6, pg/protein
G1	473.4 $\pm$ 61.8 <sup>b,c</sup>	151.8 $\pm$ 22.4 <sup>b,c</sup>	533.7 $\pm$ 65.3 <sup>b,c,d</sup>
G2	678.9 $\pm$ 112.8 <sup>a,d</sup>	252.5 $\pm$ 30.9 <sup>a,c,d</sup>	815.3 $\pm$ 42.1 <sup>a,d</sup>
G3	600.6 $\pm$ 29.3 <sup>a</sup>	208.3 $\pm$ 29.9 <sup>a,b</sup>	756.3 $\pm$ 26.6 <sup>a,d</sup>
G4	560.3 $\pm$ 41.4 <sup>b</sup>	176.4 $\pm$ 13.9 <sup>b</sup>	635.2 $\pm$ 57.4 <sup>a,b,c</sup>

<sup>a</sup>Different from G1, <sup>b</sup>Different from G2, <sup>c</sup>Different from G3, <sup>d</sup>Different from G4. Data are expressed as mean  $\pm$  standard deviation (n=6). Values in the same rows with different letters (a–d) are statistically different ( $p < 0.05$ ). TNF- $\alpha$ , Tumor necrosis factor alpha; IL-1 $\beta$ , Interleukin 1 beta; IL-6, Interleukin 6.

**Table 4:** Creatinine, BUN, AST concentrations in the experimental groups.

Experimental groups	Cr, mg/dL	BUN, mg/dL	AST, U/L
G1	0.91 ± 0.33 <sup>b,c,d</sup>	9.1 ± 1.1 <sup>b,c,d</sup>	92 ± 4.6 <sup>b,c</sup>
G2	1.76 ± 0.51 <sup>a,c,d</sup>	36.0 ± 4.1 <sup>a,c,d</sup>	263.5 ± 22.8 <sup>a,d</sup>
G3	1.22 ± 0.42 <sup>a,b,d</sup>	26.8 ± 3.6 <sup>a,b,d</sup>	204.3 ± 74.6 <sup>b</sup>
G4	1.13 ± 0.36 <sup>a,b,c</sup>	15.1 ± 1.3 <sup>a,b,c</sup>	153.5 ± 12.7 <sup>b</sup>

<sup>a</sup>Different from G1, <sup>b</sup>Different from G2, <sup>c</sup>Different from G3, <sup>d</sup>Different from G4. Data are expressed as mean ± standard deviation (n=6). Values in the same rows with different letters (a–d) are statistically different (p<0.05). Cr, Creatinine; BUN, Blood Urea Nitrogen; AST, Aspartate Aminotransferase.

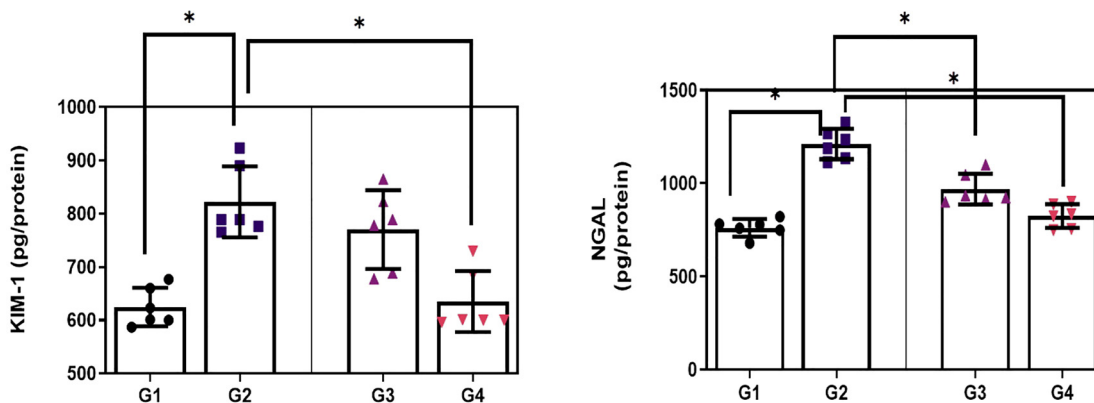
## I/R-induced acute renal injury reduced with SA

The early markers of acute renal injury were evaluated by measuring KIM-1 and NGAL levels. KIM-1 and NGAL kidney tissue levels of group G2 were significantly higher compared to group G1. SA treatment led to a considerable decrease in KIM-1 and NGAL parameters. The fact that SA treatment caused a decrease in KIM-1 and NGAL parameters suggested that it had a healing effect on acute renal injury (Table 5 and Figure 1).

**Table 5:** Renal ischemia reperfusion (I/R) and SA serum KIM-1 and NGAL levels.

Experimental groups	KIM-1, n=6, pg/protein	NGAL, n=6, pg/protein
G1	624.6 ± 36.2 <sup>b,c</sup>	760.3 ± 47.3 <sup>b,c</sup>
G2	822 ± 66.8 <sup>a,d</sup>	1,210.1 ± 81.2 <sup>a,c,d</sup>
G3	770.3 ± 73.8 <sup>a</sup>	969.5 ± 82.4 <sup>a,b,d</sup>
G4	690.5 ± 47.9 <sup>b</sup>	824.3 ± 164.0 <sup>b,c</sup>

<sup>a</sup>Different from G1, <sup>b</sup>Different from G2, <sup>c</sup>Different from G3, <sup>d</sup>Different from G4. The data are expressed as mean ± standard deviation (n=6). Values in the same rows with different letters (a–d) are statistically different (p<0.05). KIM-1, Kidney Injury Molecule-1; NGAL, Neutrophil Gelatinase-associated Lipocalin.

**Figure 1:** Comparative graphic representation of KIM-1 and NGAL levels. \*p<0.05.

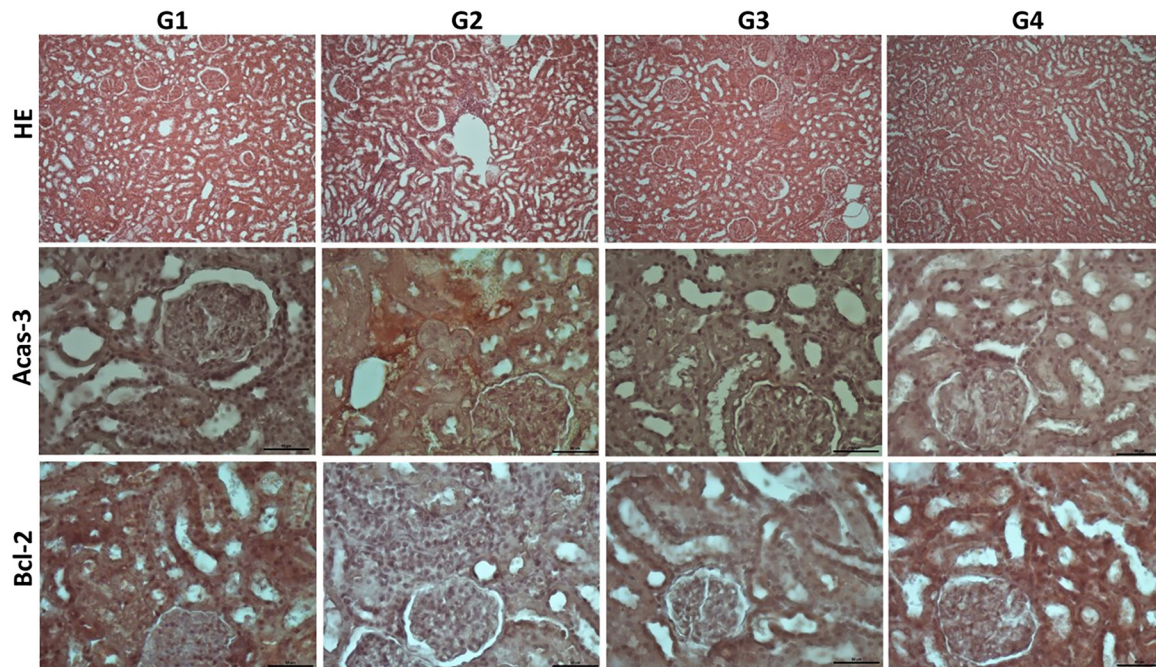
## Histopathological findings

In the general histopathological analyses, kidney parenchymal and stromal regions in group G1 had a normal histological appearance (Figure 2). A few tissue injuries were detected in renal tissue in group G2. These histological injuries were especially in some degenerative regions such as vascular dilatation, congestion, hemorrhage, congestion on the floor, edema and in the form of tubular dilatation and glomerular deformities (Figure 2). These histological injuries were observed to have decreased to a certain extent in both G3 and G4 groups.

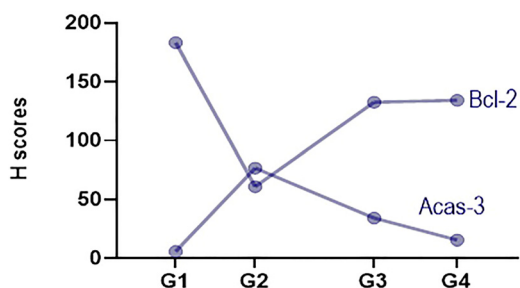
## Immunohistochemical results

H-score values of groups regarding analysis results of immunohistochemical staining of each apoptosis protein and microscopic representations of immunohistochemical stainings are presented in Figure 2. In the microscopic analyses of immunohistochemical expression of apoptotic protein Acas-3, it was observed that immune staining intensity was the highest in G2, and the lowest in G1 and G4, respectively, and that G3 had a moderate value compared to G2. In the statistical comparison of immune staining degree H-score values obtained, it was determined that G2 had the highest value (p<0.05) different from other groups, G1 and G4 had the lowest values similar to each other (p>0.05) and different from other groups (p<0.05), and G3 had a lower value than G2 different from other groups (p<0.05) (Figure 3).

In the analyses of immunohistochemical expression of anti-apoptotic protein Bcl-2, it was observed that immune staining intensity was the lowest in G2, and the highest in G1, G3 and G4, respectively. In the statistical comparison of the immune staining degrees H-Score values obtained, it was determined that G1 and G2 were different from all groups (p<0.05), and that G3 and G4 were similar to each



**Figure 2:** Microscopic representations of hematoxylin eosin and immunohistochemically stained renal tissue from each group. Top row hematoxylin eosin stained representations. It is seen that there is normal renal tissue in G1, renal tissue with evident histological injury and inflammation in G2, renal tissue with reduced histological injury and inflammation in G3 and G4, respectively (Hematoxylin eosin, 10XObj). Middle row shows Acas-3, and bottom row shows Bcl-2 immunohistochemical expressions. In the middle row, it is seen that while immune staining degree is the lowest in G1, the highest in G2, and reduced in G3 and G4, in the bottom row, there is exactly an opposite situation (IHC-AEC, Scale bar: 50  $\mu$ m).



**Figure 3:** Comparative graphic representation of immune staining H-score values of Acas-3 and Bcl-2 molecules. The letters given alongside the values indicate statistical (Tukey HSD) significance.

other ( $p > 0.05$ ) but different from the other two groups ( $p < 0.05$ ) (Figure 3).

## Discussion

In the study, the protective effects of SA treatment in the ischemic acute kidney injury rat model were examined. Our study is the first study to demonstrate that SA modulates kidney function and it plays a protective role in ischemic renal injury by healing kidney structural injury. In the

study, some factors such as kidney injury and kidney functions indicators, apoptotic markers/oxidative stress, and proinflammatory cytokines were determined. The increase in oxidative stress and the initiation of acute inflammatory responses triggered a pathological cascade, including large tubular injury, apoptotic cell death, and consequent kidney failure. The results of the study showed that SA treatment restored kidney function significantly and supported cell survival by targeting oxidative stress, inflammatory cytokines and apoptosis. Inflammation, oxidative stress or ROS production in the pathophysiology of renal I/R injury includes a complicated combination of autophagia, necrosis, apoptosis and immunological pathways. Therefore, the inhibition of oxidative stress, apoptosis, autophagia, necrosis and inflammation have been an important focus in IRI treatment [21–24]. In the study, MDA level, which is a product of lipid peroxidation, PC level, which is a product of protein oxidation, and NO level, which plays a role in physiological and pathological interactions, increased in IR group. The activity of the antioxidant enzymes SOD and GSH-Px were decreased. On the contrary, SA decreased MDA and PC levels by increasing SOD and GSH-Px activity (Table 2). This shows that SA restored the antioxidant system balance in the ischemic

kidney. In a research, both *in vivo* and *in vitro* studies were carried out in order to evaluate the antioxidant and cardio-protective potential of SA against I/R injury. In this study, it was verified by combined results obtained from both *in vivo* and *in vitro* experiments that I/R-induced cardiac dysfunction, injury, and oxidative stress were suppressed with SA treatment. In a rat model with bleomycin-induced lung fibrosis, SA significantly decreased the increased MDA levels (10 and 20 mg/kg) in lung tissues in comparison to the control group. In addition, it was also found that SA treatment (10 and 20 mg/kg) increased antioxidant enzymes such as CAT and GSH-Px, which have free radical eliminating properties [13]. It was once again confirmed that oxidative stress has a key role in renal I/R with the increase in ROS after I/R and decrease in antioxidant enzyme activities. These results are consistent with this study. Renal I/R injury is related to intrarenal inflammation [25]. In our study, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 significantly increased in I/R group (Table 3). Our results suggest the role of the overproduction ROS and proinflammatory cytokines in intense cellular and tissue injury by inducing oxidative damage of inflammatory cell infiltration and biological macromolecules [24]. Besides, our pathological findings also support these biochemical findings (Figure 2). Bin Jordan et al. [26] examined the activity of SA against Doxorubicin-induced cardiotoxicity. They demonstrated that in rats administered Doxorubicin, SA significantly regulated SOD activity and MDA level and that SA treatment decreased NF- $\kappa$ B expression and inhibited down-flow inflammatory cascade. They, therefore, reported that SA was an inhibitor of oxidative stress and inflammatory cytokines. In this study, SA treatment in IRI reversed the biomarkers related to oxidative stress and TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (Table 3). As a result, it was observed that SA alleviated renal function disorder through anti-inflammatory and antioxidative effects. Therefore, it can be an appealing target in the development of methods for treating oxidative stress and new pharmacological treatments in AKI. Renal I/R-induced oxidative stress and inflammatory reactions are associated with disrupted kidney function as they lead to a significant increase in serum BUN, Cr and AST levels [27–29]. In this study, the high increase in BUN, Cr and AST levels, which shows glomerular dysfunction mediated by renal I/R, suggested that I/R injury caused a significant disruption in glomerular function (Table 4). In addition to these traditional serum biochemical markers of renal injury, kidney KIM-1 and NGAL are accepted as more precision and trustworthy biomarkers [30, 31]. KIM-1 has been defined as the first non-myeloid phosphatidylserine receptor that gives a phagocytic phenotype to damaged epithelium cells both *in vivo* and *in*

*vitro*. NGAL is a critical component of congenital immunity against bacterial infection, and it is expressed by hepatocytes, immune cells, and renal tubule cells in case of various diseases [32–34]. In the mice model, Hua et al. [35] investigated that the nephrotoxicity of acetaminophen (300 mg/kg) caused a significant increase in serum BUN, Cr and NGAL, KIM-1 mRNA levels, in addition to a moderate change in the renal morphology of the mice. In our study, a significant increase was observed in the renal tissue KIM-1 and NGAL, serum BUN, Cr and AST levels of I/R group. SA treatment decreased tissue KIM-1 and NGAL, serum BUN, Cr and AST levels depending of the dosage, which showed that SA was significantly protective of renal function in rats following I/R injury (Tables 4 and 5). High levels of I/R-induced ROS and proinflammatory cytokines can cause lead to cell death and lipid peroxidation [36]. Activation of caspase-3 indicates irreversible cell apoptosis [37, 38]. Bcl-2 is an anti-apoptotic protein [39]. By causing both apoptosis and necrosis of renal tubule cells, it leads to the release of damage-related molecular model molecules and the spread of inflammation [40]. In our immunohistochemical results, it was observed that in I/R injury, Caspase-3 expression increased, while Bcl-2 expression decreased. However, SA treatment decreased Caspase-3 expression and increased Bcl-2 expression (Figure 3). This suggested that SA had an anti-apoptotic effect. As a result, in this study, the renoprotection mechanism of SA was shown *in vivo* in renal I/R injury. We believe that SA has positive effects on inflammation, oxidative stress, and apoptosis resulting from renal I/R injury. Administering SA before renal ischemia could reduce the severity of I/R injury with its antioxidant capacity and free radical cleaning activity. Besides, as can be understood from histological and immunohistochemical evaluations, we demonstrated that SA treatment significantly decreased kidney injury induced by I/R. More research is needed in order to reveal more benefits of SA in renal I/R injury.

## Conclusion

I/R-induced renal injury results from a dynamic process that includes inflammation, oxidative stress, apoptosis and some mediators in complicated interaction. The development of oxidative stress and lipid peroxidation seems to be the leading factors that accelerate inflammation and cell death during I/R. In this study, it was demonstrated the renoprotection mechanism of SA *in vivo* in renal I/R injury. It was shown that SA decreased renal glomerular and tubule dysfunction in the kidneys of rats exposed to I/R injury. Besides, the antioxidant, anti-

inflammatory, and anti-apoptotic features of SA downregulated renal oxidative stress, inflammation, and apoptosis. Administering SA before renal ischemia may have reduced the severity of I/R injury with its antioxidant capacity and free radical cleaning activity. In addition, the histopathological and immunohistochemical evaluation of the kidneys confirms the protective effect of SA. A better understanding of the pathophysiology underlying the functional defects occurring in ischemic acute renal failure and the therapeutic approaches will require us to keep the complexity of the disease in mind. Moreover, more research on SA both *in vivo* and *in vitro* is needed before clinical applications.

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