

## Protective effect of naringin in rat model of renal ischemia reperfusion injury

*Efecto protector de la naringina en modelo de rata con lesión por reperfusión de isquemia renal*

Mutlu Deger<sup>1</sup>, Nebil Akdogan<sup>1</sup>, Volkan Izol<sup>1</sup>, Halil Mahir Kaplan<sup>2</sup>, Perçin Pazarci<sup>3</sup>, Ibrahim Atilla Aridogan<sup>1</sup>

### ABSTRACT

**Objective:** We aimed to research that naringin whether protects from renal ischemia/reperfusion induced renal damage in rats. **Methods:** Twenty-four Wistar albino female rats randomly were divided into three groups: 1) control group, in which the rats were only performed right nephrectomy; 2) a second group received right nephrectomy and left kidney ischemia (1 h) and reperfusion (24 h) group ischemia/reperfusion (I/R); 3) a third group received 50 mg/kg naringin orally once a day for two weeks before ischemia/reperfusion (I/R/N). Expression of cyclooxygenase-2 (COX-2), cytosolic phospholipase A<sub>2</sub> (cPLA2), inducible nitric oxide synthase (iNOS), caspase-3, B-cell lymphoma-2 (Bcl-2), Bcl-2 associated x protein (Bax), serum creatinine (Cr), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 6 (IL-6) were measured by using enzyme-linked immunosorbent assay (ELISA). **Results:** Naringin-treated rats that performed renal ischemia/reperfusion demonstrated significant decrease in Cr, IL-6 and TNF- $\alpha$  levels when compared

to the only renal ischemia/reperfusion performed rats. While renal ischemia/reperfusion caused a decrease of bcl-2 ( $1.72 \pm 0.20$  pg/ml) levels, while an increase of COX-2 ( $11882 \pm 642$  pg/ml), cPLA2 ( $2448 \pm 139$  pg/ml), iNOS ( $4331 \pm 438$  IU/ml), cleaved caspase-3 ( $7.33 \pm 0.76$  ng/ml) and Bax ( $2.33 \pm 0.44$  ng/ml) levels. The treatment of naringin reversed these kidney effects ( $7.47 \pm 60.35$  pg/ml;  $9299 \pm 327$  pg/ml;  $2001 \pm 78$  pg/ml;  $3112 \pm 220$  IU/ml;  $3.38 \pm 0.54$  ng/ml;  $2.33 \pm 0.44$  ng/ml, respectively) ( $p < 0.05$ ). **Conclusion:** This study showed that naringin treatment attenuated renal damage induced by ischemia/reperfusion in rats.

**KEYWORDS:** renal ischaemia reperfusion injury; naringin; inflammation; apoptosis

### RESUMEN

**Objetivo:** Nuestro objetivo fue investigar si la naringina protege del daño en los riñones provocado por isquemia-reperfusión renal en ratas. **Material y métodos:** De forma aleatoria, dividimos 24 ratas albinas Wistar hembras en tres grupos: 1)

*Correspondencia:*  
Dr. Mutlu Deger  
ORCID: 0000-0002-8357-5744  
drmutludeger@gmail.com

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- 1) Department of Urology, Faculty of Medicine, Cukurova University, Adana, Turkey
- 2) Department of Pharmacology, Faculty of Medicine, Cukurova University, Adana, Turkey
- 3) Department of Medical Biology, Faculty of Medicine, Cukurova University, Adana, Turkey

### *Ethical approval:*

This study was approved by the Animal Care Committee and Ethics Committee of Cukurova University. All of the procedures were performed according to accepted standards of Guide for the Care and Use of Laboratory Animals.

grupo control, en el que solo se les realizó a las ratas una nefrectomía derecha; 2) un segundo grupo isquemia-reperfusión, con nefrectomía derecha e isquemia de riñón izquierdo (1 h) y perfusión (24 h); 3) un tercer grupo al que se le administró 50 mg/kg de naringina por vía oral una vez al día durante dos semanas antes de la isquemia-reperfusión. Por medio de un ensayo inmunoabsorbente ligado a enzimas (ELISA), se midieron las siguientes expresiones: ciclooxigenasa-2 (COX-2), fosfolipasa citosólica A2 (cPLA2), óxido nítrico sintetasa inducible (ONSi), caspasa-3, linfoma de células B2 (Bcl-2), proteína X asociada a Bcl-2 (Bax), creatinina sérica (Cr), factor de necrosis tumoral alfa (FNT- $\alpha$ ) e interleucina 6 (IL-6). **Resultados:** Las ratas tratadas con naringina por isquemia-reperfusión renal mostraron un descenso significativo en los niveles de Cr, IL-6 y FNT- $\alpha$  en comparación con las ratas a las que se les indujo isquemia-reperfusión renal pero que no se les suministró naringina. La isquemia-reperfusión renal provocó un descenso de los niveles de Bcl-2 ( $1,72 \pm 0,20$  pg/ml) y un ascenso en los niveles de COX-2 ( $11882 \pm 642$  pg/ml), cPLA2 ( $2448 \pm 139$  pg/ml), ONSi ( $4331 \pm 438$  UI/ml), caspasa-3 escindida ( $7,33 \pm 0,76$  ng/ml) y Bax ( $2,33 \pm 0,44$  ng/ml). El tratamiento con naringina disminuyó estos efectos en el riñón ( $7,47 \pm 60,35$  pg/ml;  $9299 \pm 327$  pg/ml;  $2001 \pm 78$  pg/ml;  $3112 \pm 220$  UI/ml;  $3,38 \pm 0,54$  ng/ml;  $2,33 \pm 0,44$  ng/ml, respectivamente) ( $p < 0,05$ ). **Conclusión:** En este estudio se demostró que el tratamiento con naringina atenuó el daño renal producido por isquemia-reperfusión en ratas.

**PALABRAS CLAVE:** lesión por perfusión de isquemia renal; naringina; inflamación; apoptosis

## INTRODUCTION

Renal transplantation, partial nephrectomy, revascularization of renal artery, trauma, and hydronephrosis can cause ischemic renal injury which is the common cause of acute renal failure. Also, renal ischemia may result in chronic renal failure<sup>(1-2)</sup> Although restoration of blood flow saves kidney from damage, reperfusion-induced nephrotoxicity may occur in kidney. Previous studies have shown that NO (nitric oxide)

and reactive oxygen species (ROS) play a role in ischemic injury.<sup>(3-4)</sup> These ROS (excessive production) can damage most of the cellular components.<sup>(5)</sup> Abundant amounts of oxygen ( $O_2$ ) which is present in the reperfused ischemic tissue induces excessive free oxygen radicals which reduce antioxidant defense mechanisms.<sup>(6)</sup> In addition, inflammation as well as free oxygen radicals have been shown to be responsible for the severity of ischemia/reperfusion (I/R) injury.<sup>(7)</sup> Inflammation with I/R injury is considered "aseptic inflammation".<sup>(8)</sup> Aseptic inflammation, like septic inflammation, is accompanied by an accumulation of neutrophils and macrophages and an increase in proinflammatory mediators.<sup>(9)</sup> It also increases the activity of inducible nitric oxide synthase (iNOS) and causes NO formation.<sup>(10)</sup> In the light of previous studies antioxidant and anti-inflammatory activity may be beneficial in pre-reperfusion and post-reperfusion periods of ischemic tissue.

Naringin is a flavonoid that is anti-inflammatory, antiviral, anticancer, antimutagenic, antiallergic, analgesic, hypotensive activity, blood cholesterol-lowering effect, and has anti-inflammatory activity at doses of 100 mg/kg.<sup>(11)</sup> In a study, naringin was shown to inhibit the NF- $\kappa$ B / COX-2-caspase-1 pathway.<sup>(12)</sup> However, it has been reported that the enzyme iNOS reduces activity by decreasing NO production.<sup>(13)</sup> For this purpose, we aimed to reveal the possible protective effect of naringin against ischemia-reperfusion induced renal injury according to evidence of biochemical and enzyme analyses.

## MATERIALS AND METHODS

Twenty-four Wistar albino female rats weighing 250-300 g were obtained from Cukurova University Animals Research Center. They were placed in a controlled room with controlled temperature ( $21 \pm 2^\circ\text{C}$ ) and humidity ( $60 \pm 5\%$ ), in which a 12:12 hour light-dark cycle was maintained. The rats were fed with standard commercial pellets and water ad libitum. This study was approved by the Animal Care Committee and Ethics Committee of Çukurova University. All of the procedures were performed according to accepted standards of Guide for the Care and Use of Laboratory Animals. The rats were randomly divided into three groups: (1) control group (C), in which

the rats were only performed right nephrectomy (n=8); (2) right nephrectomy and left kidney ischemia (1 h) and reperfusion (24 h) group (I/R) (n=8); (3) I/R/N group received 50 mg/kg naringin orally once a day for two weeks before I/R (n=8). Intraperitoneal (IP) ketamine (75 mg/kg) and xylazine (8 mg/kg) were preferred as anesthesia method for rats. After right nephrectomy through the right dorsolateral incisions were done in all rats, the left renal artery and vein were isolated for 30 minutes without further surgical intervention to allow recirculation in rats in the I/R groups. Later, ischemia was induced by clamping the left renal vessels for 1 hour. Left kidney reperfusion was achieved during 24 h reperfusion. After the bleeding control, the skin and subcutaneous tissues were sutured and the surgical procedure was terminated. All rats were decapitated 24 hours after surgery. Blood samples were taken for biochemical analysis in the tail veins of each rat just before decapitation. After decapitation, the left kidneys were quickly isolated and stored at -80°C for Quantitative Analysis by ELISA.

### Quantitative Analysis

#### Tissue Homogenization

Kidney samples were homogenized by modified RIPA Buffer and by using ultrasonication on ice. Then tissue homogenates were centrifuged (10,000 rpm +4°C) for 10 minutes, and pellets were discarded, and supernatants were saved for quantitative analyses (ELISA).

### Total Protein Analyses

We used the Bradford method for the determination of total protein levels and the standardization of samples of homogenization.

### Quantitative Analyses of Homogenizates

Expression of cyclooxygenase-2 (COX-2), cytosolic phospholipase A<sub>2</sub> (cPLA2), inducible nitric oxide synthase (iNOS), caspase-3, B-cell lymphoma-2 (BCL-2), Bcl-2 associated x protein (Bax), serum creatinine (Cr), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) were measured by using enzyme-linked immunosorbent assay (ELISA) (Shanghai Sunred Biological Technology Co. Ltd) kits according to the manufacturer's instructions.

### STATISTICS

Data from samples were shown as means ± S.E.M., and differences between groups were tested by analysis of variance (ANOVA) corrected for Bonferroni multiple comparisons. P values less than 0.05 were considered to be significant.

## RESULTS

### Effect of naringin on the serum parameters

The serum parameters are summarized in **Table 1**. The serum levels of Cr, IL6, and TNFα were significantly higher in the I/R group when compared to the C group. Naringin treatment reduced levels of Cr, IL6 and TNFα when compared to I/R group, respectively.

**Table 1.** The serum levels of Cr, IL6 and TNFα

	Cr, mg/dl	IL6, pg/ml	TNFα, pg/ml
Control	0.52 ± 0.03	107.6 ± 4,5	54.7 ± 1.4
I/R	2.85 ± 0.57*	131.4 ± 5.8*	83.1 ± 3.6*
I/R+Naringin	1.03 ± 0.02#	106.9 ± 2.6#	61.7 ± 3.0#

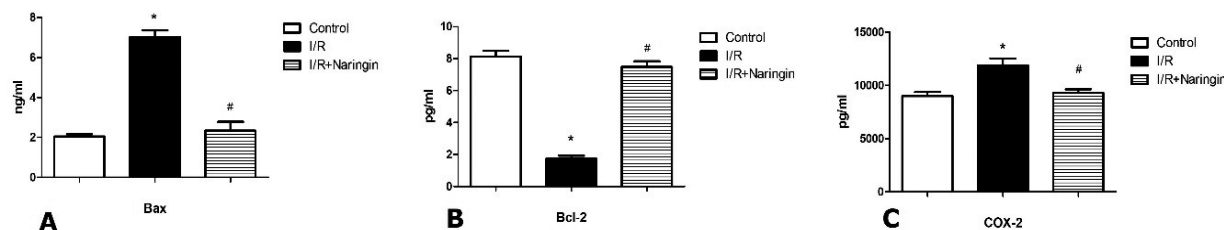
\*: For control p < 0.05. # For I/R p < 0.05

### Effect of naringin on inflammatory and apoptotic mediator in kidney

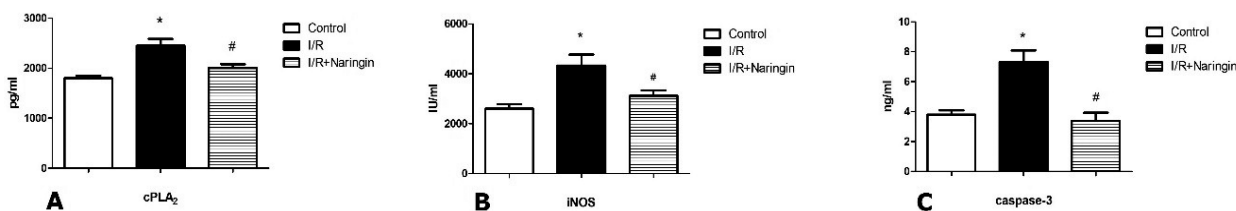
While renal I/R caused a decrease of bcl-2 (1.72 ± 0.20 pg/ml) levels, and increased COX-2 (11882 ± 642 pg/ml), cPLA2 (2448 ± 139 pg/ml), iNOS (4331 ± 438 IU/ml), cleaved caspase-3

(7.33 ± 0.76 ng/ml) and Bax (2.33 ± 0.44 ng/ml) levels, the treatment of naringin reversed these kidney effects (7.47 ± 60.35 pg/ml; 9299 ± 327 pg/ml; 2001 ± 78 pg/ml; 3112 ± 220 IU/ml; 3.38 ± 0.54 ng/ml; 2.33 ± 0.44 ng/ml, respectively) (p < 0.05). (**Figure 1-2**)

**Figure 1.** **1A.** Effect of naringin on I/R induced Bax expression (n=8). **1B.** Effect of naringin on I/R induced Bcl-2 expression (n=8). **1C** Effect of naringin on I/R induced COX expression (n=8). Statistical analysis: ANOVA. Post hoc: Bonferroni. (\*: for control  $p < 0.05$ , #: for I/R  $p < 0.05$ )



**Figure 2.** **2A.** Effect of naringin on I/R induced cPLA<sub>2</sub> expression (n=8). **2B.** Effect of naringin on I/R induced iNOS expression (n=8). **2C.** Effect of naringin on I/R induced cleaved caspase-3 (n=8). Statistical analysis: ANOVA. Post hoc: Bonferroni. (\*: for control  $p < 0.05$ , #: for I/R  $p < 0.05$ .)



## DISCUSSION

In this study, we evaluated inflammatory and apoptotic mediators together. Studies have shown that renal ischemia causes an increase in COX-2, cPLA<sub>2</sub> and iNOS enzymes, which are inflammatory mediators.<sup>(10, 14)</sup> Renal ischemia increased pro-apoptotic enzymes, which are cleaved caspase-3 and Bax while decreased antiapoptotic enzymes, which is bcl-2. This study showed that the use of naringin decreases renal I/R injury. In this study, while one kidney was nephrectomized, the artery of the other kidney remained clamped for one hour to create I/R. Because the peak serum Cr following ischemia occurs at 24-hour of reperfusion, reperfusion was evaluated after 24 hours in our study.<sup>(15)</sup>

Inflammation has an important role in nephrotoxicity.<sup>(16)</sup> The COX-2 enzyme, one of the inflammatory mediators of nephrotoxicity, and the prostaglandins synthesized by this enzyme also play a role. Studies have shown that selective COX-2 inhibitors attenuate severity of nephrotoxicity.<sup>(17)</sup> In a study, naringin was shown to inhibit the NF- $\kappa$ B/COX-2-caspase-1 pathway.<sup>(12)</sup> The expression of inducible iNOS, particularly in inflammatory cells, can have detrimental

effects, including direct effects of NO on various proteins and enzymes. It is also associated with the generation of NO, reactive oxygen species (ROS), peroxynitrite and other reactive species.<sup>(18)</sup> In a study by Gutiérrez-Venegas, they found that naringin attenuated inflammatory events by inhibiting the expression of iNOS, reducing NO production.<sup>(13)</sup> Studies have also shown that reperfusion induces oxidative stress which increases intracellular calcium level and increased calcium level leads to elevation of the activity of phospholipase A2 enzyme.<sup>(19)</sup> Oxidative stress is induced by excessive ROS and free superoxide radicals. ROS both plays role as a mediator and in the damage of cellular components. While it acts as a second messenger in the signal transduction pathways cause damage cell in excessive production.<sup>(20)</sup> In excessive production of ROS antioxidant defense system of cell fails. In a study showed that oxygen species also modulates NF- $\kappa$ B, p53 and AP-1.<sup>(21)</sup> H<sub>2</sub>O<sub>2</sub> which is a member of ROS plays a role in various physiological stimuli.<sup>(21)</sup> Oxidative stress plays a pivotal role in apoptosis. Apoptosis is essential for cellular function, survival and shaping of organs. It is also known as programmed cell death. Studies show that antioxidants block or

delay apoptosis. Bcl-2, a member of apoptotic pathway, is an anti-apoptotic mediator and this mediator prevents apoptosis by an anti-oxidative mechanism.<sup>(22)</sup> Studies have shown that naringin has great antioxidant capacity.<sup>(23)</sup>

In a study, Amini *et al.* aimed the effects of co-administration of trimetazidine and naringin on renal I/R injury in rat models. Administration of the trimetazidine, naringin, and their combination decreased the plasma level of Bax mRNA expression, microRNA-10a, and caspase-3, but increased Bcl-2 mRNA expression in the kidney tissue. In addition, antioxidant activity, renal blood flow and Cr clearance were improved. They concluded that trimetazidine, naringin, and their combination might be beneficial as potent therapeutic factors against renal I/R injury.<sup>(24)</sup> In a study by Singh *et al.*, investigating the protective effect of naringin against renal I/R injury in a rat model, they found that naringin alleviated renal dysfunction, reduced morphological changes, high thiobarbituric acid reactive substances levels, and restored depleted renal antioxidant enzymes.<sup>(25)</sup> Differently in both studies, in our study, naringin was shown to decrease renal I/R damage with inflammatory and apoptotic mediators such as bcl-2 COX-2 cPLA2, iNOS, cleaved caspase-3 and Bax.

## CONCLUSION

In the light of our findings it seems that naringin protects I/R induced nephrotoxicity by decreasing inflammation and apoptosis.

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