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The Protective Effect of Adalimumab on Renal Injury in a Model of Abdominal Aorta Cross-Clamping*

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Abstract

Background. Adalimumab (ADA) is a potent inhibitor of tumor necrosis factor (TNF- α). ADA treatment suppresses proinflammatory cytokines, leading to a decrease or inhibition of the inflammatory process.

Objectives. The aim of this study was to investigate the possible protective effects of ADA on oxidative stress and cellular damage on rat kidney tissue after ischemia/reperfusion (I/R).

Material and Methods. A total of 30 male Wistar albino rats were divided into three groups: control, I/R, and I/R plus ADA (I/R + ADA); each group comprised 10 animals. The control group underwent laparotomy without I/R injury. After undergoing laparotomy, I/R groups underwent two hours of infrarenal abdominal aortic cross ligation, which was followed by two hours of reperfusion. ADA (50 mg/kg) was administered as a single dose, intraperitoneally, to the I/R + ADA group, 5 days before I/R.

Results. The I/R group's TNF- α (1150.9 ± 145.6 pg/mg protein), IL-1 β (287.0 ± 32.4 pg/mg protein) and IL-6 (1085.6 ± 56.7 pg/mg protein) levels were significantly higher than those of the control (916.1 ± 88.7 pg/mg protein, $p = 0.003$; 187.5 ± 37.2 pg/mg protein, $p < 0.001$; 881.4 ± 57.1 pg/mg protein, $p < 0.001$, respectively) and I/R + ADA groups (864.2 ± 169.4 pg/mg protein, $p = 0.003$; 241.4 ± 33.4 pg/mg protein, $p = 0.010$; 987.7 ± 66.5 pg/mg protein, $p = 0.004$, respectively). To date, a few histopathological changes have been reported regarding renal I/R injury in rats due to ADA treatment whereas I/R caused severe histopathological injury to kidney tissue.

Conclusions. ADA treatment significantly attenuated the severity of kidney I/R injury, inhibiting I/R-induced oxidative stress and renal damage. Because of its anti-inflammatory and antioxidant effects, ADA pretreatment may have protective effects on experimental kidney injury (*Adv Clin Exp Med* 2016, 25, 2, 219–226).

Key words: ischemia, reperfusion injury, adalimumab, carbonic anhydrases, tumor necrosis factor alpha.

Renal ischemia/reperfusion (I/R) occurs in various clinical situations, such as hypotension, transplantation, renal artery stenosis, embolic disease, and cardiovascular surgery. It is a major cause of acute kidney injury [1]. Not only ischemia, but also reperfusion, can lead to tissue damage. Infiltration of neutrophils and macrophages occurs after reperfusion of the kidney [2]. Oxidative stress and

the generation of reactive oxygen species (ROS) during I/R increase the production of proinflammatory cytokines like tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), and IL-1 β [3, 4]. These cytokines accelerate apoptosis and cellular damage. ROS are potent oxidizing and reducing agents, which activate neutrophil and lipid peroxidation, leading to cell membrane damage. Factors

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important in I/R include inflammatory cell aggregation, upregulation of cytokines, and T-cell-mediated immune reactions [5].

Adalimumab (ADA) is a potent inhibitor of TNF- α . It suppresses TNF- α , IL-1 β , and IL-6, leading to reduction or inhibition of the inflammatory process [6, 7]. Infliximab, which is another blocker of TNF- α , has been demonstrated to be protective against renal I/R injury [8]. However, ADA has not been investigated in I/R injury. TNF- α is an important cytokine leading to ROS production. It has been reported that the inhibition of TNF- α decreases the production of ROS and the release of proinflammatory cytokines [9]. Carbonic anhydrase II (CA II) is a zinc metalloenzyme that is responsible for catalyzing the reversible hydration reaction of carbon dioxide to form carbonic acid [10]. It is found in various tissues, especially in the proximal tubules and the collecting duct of the kidney [11]. A high level of CA II has been found to be associated with renal cell carcinoma and the production of carbonate radicals [12, 13].

The aims of this study were to determine whether ADA ameliorates I/R-induced kidney tissue injury by suppressing cell apoptosis via TNF- α inhibition and whether this inhibition alters the activity of CA II in kidney during I/R injury.

Material and Methods

Animals

A total of 30 Wistar albino male rats aged 12–15 weeks (weight range 250–300 gr) were used in this study. They were randomly divided into 3 groups: The I/R group with $n = 10$, the I/R + ADA group with $n = 10$ and the control group with $n = 10$. This study was performed following the Guide for the Care and Use of Laboratory Animals (NIH, 1985) and was approved by the local ethical committee of our university (approval numbers: 2012/18).

Experimental Design

A saline solution only was given to the rats of both the control and the I/R groups. A midline laparotomy and an infrarenal abdominal aortic (IAA) cross without occlusion was performed for the control group. After the laparotomy and an IAA clamping for 120 min, reperfusion was allowed for 120 min in the I/R group. After the dilution of ADA (Humira 40 mg/0.8 mL; Abbott, Abbott Park, IL, USA) in saline it was given as a single dose injection of 50 mg/kg to the I/R + ADA group [14]. After 5 days of ADA administration, ischemia for 120 min and reperfusion for 120 min were applied to the I/R + ADA group.

Aortic Occlusion and I/R

Previous studies were used as a model for the design of I/R in this study [15]. Ketamine hydrochloride (50 mg/kg) was used intramuscularly to anesthetize the rats. The rats were put under a heating lamp in a supine position before the application of the surgical procedures. The abdomen was shaved and the skin was prepared aseptically. A midline laparotomy was then performed and the abdominal aorta was clamped using an atraumatic microvascular clamp. The occlusion of the aorta was proven after the loss of the distal arterial pulsation. The incision was closed and covered with plastic wrap to maintain body temperature and prevent fluid loss. After 120 min of ischemia, the clamp was removed and reperfusion of the lower extremities was maintained for 120 min. After this period, all of the rats were euthanized under anesthesia. Kidney samples were then taken from all the groups for biochemical and histopathological examination.

Biochemical Parameters

After blood samples (10 mL) were obtained from all the rats, they were put into tubes to be prepared for the biochemical tests. The samples were allowed to stay for 15 min at 25°C before being centrifuged at 3,000 rpm for 10 min. Commercial kits (ARCHITECT c16000, Abbott Laboratories, IL, USA) were used to analyze the biochemical parameters such as urea, creatinine, alanine aminotransferase and aspartate aminotransferase

Tissue Homogenates

The tissue samples were put in a phosphate-buffered saline (PBS) for homogenization at pH 7.4 before being centrifuged at 10,000 g for 20 min. They were then homogenized for 2 min in a cold phosphate buffer to provide a 10% homogenate. After that, 100 mg of wet tissue was taken for per mm. After aliquots of the supernatant were obtained, they were put into tubes to be frozen at -80°C . The concentration of the cytokines TNF- α , IL-1 β , and IL-6 were measured within one month.

Measurement of Protein

Benzethonium chloride was used as a denaturing agent in a turbidimetric procedure that was applied for the tissue homogenate protein assay. A turbidimetric method at 404 nm (ARCHITECT c16000, Abbott Laboratories, IL, USA) was used to quantify the proteins in the suspension

Tissue TNF- α

TNF- α level was measured by a rat TNF- α enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, Vienna, Austria). The absorbance was measured with the ELISA reader using a wave length 450 nm. The coefficients of variation of intra-assay and inter-assay were in sequence $< 5\%$ and $< 10\%$. TNF- α assay had a limit of detection (LOD) of 11 pg/mL. The obtained values were divided by the protein levels, and the results were recorded as pg/mg protein.

Tissue IL-1 β

IL-1 β level was measured also by a rat IL-1 β ELISA kit (eBioscience, Vienna, Austria). The absorbance was measured with the ELISA reader using a wave length 450 nm. The intra-assay and inter-assay coefficients of variation were in sequence $< 10\%$ and $< 10\%$. IL-1 β assay had a LOD of 4 pg/mL. The obtained values were divided by the protein levels, and the results were recorded as pg/mg protein.

Tissue IL-6

IL-6 level was measured by rat IL-6 ELISA kit (Invitrogen, CA, USA). The absorbance was measured with the ELISA reader using a wave length 450 nm. The intra-assay and inter-assay coefficients of variation were in sequence 3.5% and 6.3%. IL-6 assay had a LOD of < 5 pg/mL. The obtained values were divided by the protein levels, and the results were recorded as pg/mg protein.

Immunohistological Evaluation

Sections of 3–4 μm thickness were cut for the immunohistochemical staining. They were put in xylene for 20 min before applying alcohol series (50–100%) and then stood for 10 min in a hydrogen peroxide solution. After that the section were washed with PBS. They were put in a citrate buffer solution before being heated 4–5 min by 800-Watt power. The sections were then allowed to stand for 20 min in a secondary blocker substance. Each slide was put in different dilutions containing primary antibody (TNF- α 1 $\mu\text{g}/\text{mL}$; CA II 1/250/500) for 75 min before staining them by anti-CA II (cod: ab124687, Abcam plc., Cambridge, UK) and anti-TNF- α antibody (cod: ab66579, Abcam plc.). While Diaminobenzidine solution was used as a chromogen, a counterstain Mayer's hematoxylin was used for 3–5 min. PBS was used as a negative tissue control. After being covered with appropriate covering materials all the preparations

were photographed. They were subdivided into four categories depending on the percentage of immunopositivity of the tissue as (+) for mild, (++) for moderate, (+++) for severe, and (++++ for very severe. Sections of 4–5 μm thickness were cut for the hematoxylin and eosin staining to be used in the histopathological examination. The regions suitable for histopathological examination were photographed. Two histologists blindly evaluated all the preparations. A thorough evaluation was made to the results of the statistical comparisons that were obtained during, between and within group evaluations.

Statistical Analyses

The results are given as the mean \pm SD. The Kruskal–Wallis test was applied for the group's comparison. A Bonferroni adjusted Mann–Whitney U test was applied for the comparison of the two groups. P -value < 0.05 was considered to be statistically significant.

Results

Biochemical Parameters

Creatinine level of I/R group was significantly higher (0.4 ± 0.05 mg/dL) than the control group (0.5 ± 0.05 mg/dL, $p = 0.002$) and I/R + ADA group (0.5 ± 0.07 mg/dL, $p = 0.012$). The TNF- α level of I/R group (1150.9 ± 145.6 pg/mg protein) was significantly higher than the control group (916.1 ± 88.7 pg/mg protein, $p < 0.001$) and I/R + ADA group (864.2 ± 169.4 pg/mg protein, $p = 0.006$). IL-1 β level of I/R group (287.0 ± 32.4 pg/mg protein) was significantly higher than the control group (187.5 ± 37.2 pg/mg protein, $p < 0.001$) and I/R + ADA group (241.4 ± 33.4 pg/mg protein, $p = 0.010$). IL-6 level of I/R group (1085.6 ± 56.7 pg/mg protein) was significantly higher than the control group (881.4 ± 57.1 pg/mg protein, $p < 0.001$) and I/R + ADA group (987.7 ± 66.5 pg/mg protein, $p = 0.004$) groups. The results are shown in Table 1.

Histological Parameters

Results of Hematoxylin and Eosin Staining

The kidneys of the control group showed no pathological deformities damaging the integrity of the tissue or cellular structures, including the tubules and glomeruli. The morphological struc-

Table 1. All the biochemical result of three groups

	Control	I/R	I/R + ADA
AST (IU/L)	23.3 ± 7.5	65.3 ± 11.5 ^{W,*}	46.7 ± 8.5 [*]
ALT (IU/L)	41.1 ± 11.7	59.2 ± 17.5	51.4 ± 8.8
Creatinine (mg/dL)	0.4 ± 0.05	0.5 ± 0.05 ^Y	0.5 ± 0.07 ^M
Urea (mg/dL)	39.8 ± 4.6	22.2 ± 9.3 ^F	42.0 ± 13.8
TNF-α pg/mg protein	916.1 ± 88.7	1150.9 ± 145.6 ^{F,x}	864.2 ± 169.4
IL-1β pg/mg protein	187.5 ± 37.2	287.0 ± 32.4 ^{*,β}	241.4 ± 33.4 ^α
IL-6 pg/mg protein	881.4 ± 57.1	1085.6 ± 56.7 ^{*,+}	987.7 ± 66.5 ^ε

I/R – ischemia/reperfusion; ADA – adalimumab; TNF-α – tumor necrosis factor-alpha; IL-1β – interleukin-1 beta; IL-6 – interleukin 6; for AST: *p < 0.001 vs. control group; ^Wp < 0.001 vs. I/R + ADA group; for creatinine: ^Yp = 0.002, ^Mp = 0.012 vs. control group; for urea: ^Fp = 0.001 vs. I/R + ADA group; for TNF-α: ^Fp = 0.003 vs. control group; ^xp = 0.003 vs. I/R + ADA group; for IL-1β: *p < 0.001, ^αp = 0.010 vs. control group; ^βp = 0.010 vs. I/R + ADA group; for IL-6: *p < 0.001; ^εp = 0.005 vs. control group; ⁺p = 0.004 vs. I/R + ADA group.

tures of the control-group cells showed a normal histological appearance. The epithelial cell structures of the blood vessels and the proximal tubules in the cortex-medulla border had a normal histological appearance. There was no histopathological degeneration observed in the connective tissues of the cortex-medulla border zone other than a few lymphocytic cells. Mesenchymal cells of Bowman's capsule and the filtration space had a normal histological appearance (Fig. 1A).

Pathological deformations of the epithelial structure of the proximal and distal tubules were observed in the I/R group. Intense degenerative structures, dilatation, and fluid accumulation in the lumen related to edema were observed, in particular in the epithelium of the proximal tubules surrounding the glomeruli (Fig. 1B). Although there was an increase in the connective tissue and edema in border area of the cortex and medulla, there was a decrease in leukocytes. The lumens of the tubules were much expanded due to shedding and deformation of the epithelium of the distal tubule. Bowman's capsule and visceral cells were edematous, and the filtration space was wider than that of the control group. The deformations of the proxi-

mal tubule cells were eosinophilic (Fig. 1B). There were lower tissue and cellular deformity in the histopathological examination of I/R + ADA group than I/R group (Fig. 1C). The cells of the proximal tubules showed less degeneration, but their nuclei stained mildly basophilic. The decreased dilatation of the distal tubules was in the presence of intermittent edema, but it was larger than that of the control group. Regardless of the mild increase in the connective tissue in the cortex-medulla border zone, the numbers of lymphocytes were substantially reduced. Although there was little edema of the tubular epithelial cells or intense shedding in the I/R group, these characteristics were not completely absent, especially in the area of outer cortex, which exhibited extensive cellular deformation (Fig. 1C).

Results of Immunoperoxidase Staining

The CA II activity of I/R group was significantly higher than the control group (p < 0.001) and I/R + ADA group (0.5 ± 0.07 mg/dL, p = 0.009). TNF-α activity of I/R group was significantly higher than the control group (p = 0.003) and I/R

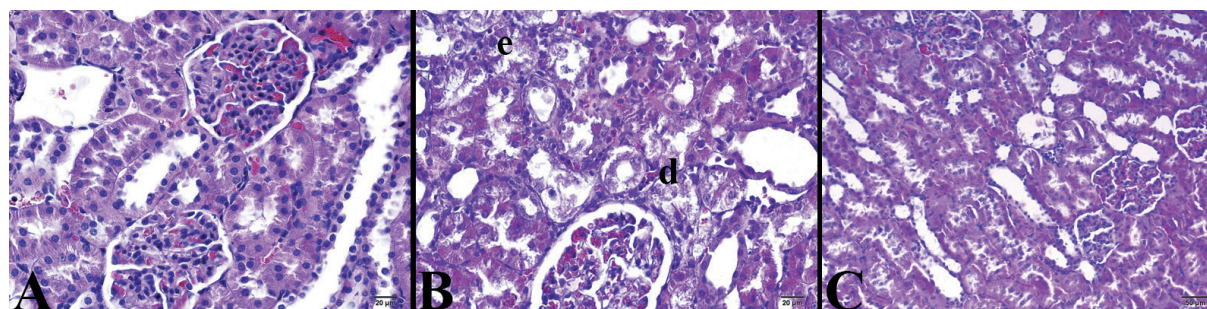


Fig. 1. A light microscopic examination of kidney tissue. A – control group, B – I/R group, C – I/R + ADA group, d – degenerative cell, e – edema

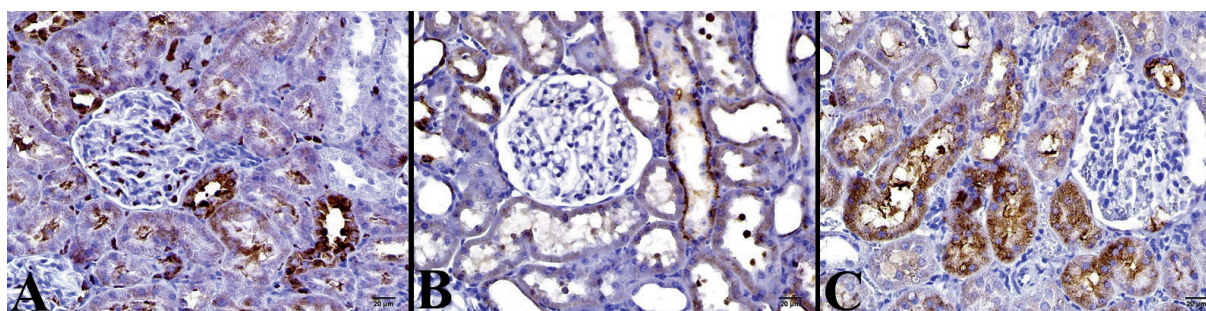


Fig. 2. A light microscopic examination of kidney tissue stained using immunoperoxidase method. A – control group, B – I/R group, C – I/R + ADA group, anti-carbonic anhydrase II antibody

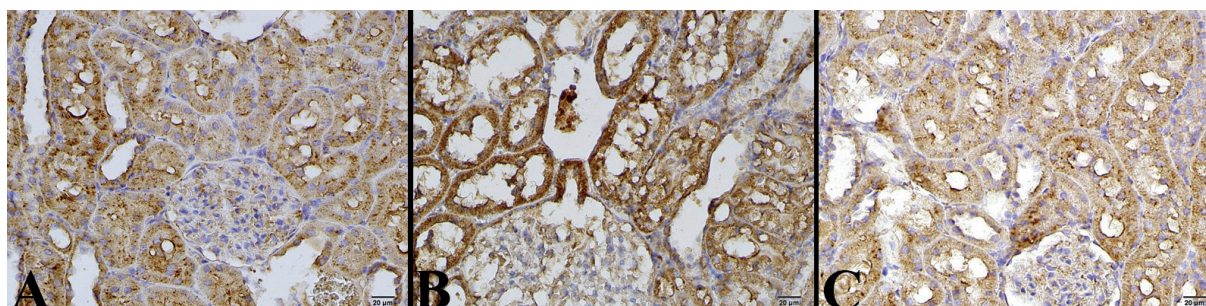


Fig. 3. A light microscopic examination of kidney tissue stained using immunoperoxidase method. A – control group, B – I/R group, C – I/R + ADA group, anti-TNF- α antibody

Table 2. Histopathologic examination of renal tissue

Groups	Cell loss (mean \pm SD)	Edema (mean \pm SD)	Tubular deformation (mean \pm SD)	Lymphocyte accumulation (mean \pm SD)	Anti-CA II (mean \pm SD)	Anti-TNF- α (mean \pm SD)
Control	0 \pm 0	0 \pm 0	0 \pm 0	0.6 \pm 0.5	1.8 \pm 0.3	2.2 \pm 0.5
I/R	3.2 \pm 0.4 ^{*,§}	2.9 \pm 0.3 ^{*,β}	3.1 \pm 0.3 ^{*,α}	3.0 \pm 0.5 ^{*,π}	3.4 \pm 0.5 ^{*,x}	3.2 \pm 0.4 ^{*,δ,$+$}
I/R+ADA	2.1 \pm 0.5 [*]	2.1 \pm 0.7 [*]	2.3 \pm 0.6 [*]	2.1 \pm 0.3 [*]	2.7 \pm 0.5 ^R	2.3 \pm 0.5

CA – carbonic anhydrase; TNF- α – tumor necrosis factor alpha; I/R – ischemia/reperfusion; ADA – adalimumab. For cell loss: * $p < 0.001$ vs. control group; § $p < 0.001$ vs. I/R + ADA group; for edema: * $p < 0.001$ vs. control group; $^{\beta}$ $p = 0.008$ vs. I/R + ADA group; for tubular deformation: * $p < 0.001$ vs. control group; $^{\alpha}$ $p = 0.006$ vs. I/R + ADA group; for lymphocyte accumulation: * $p < 0.001$ vs. control group; $^{\pi}$ $p = 0.002$ vs. I/R + ADA group; for CA II: * $p < 0.001$, R $p = 0.10$ vs. control group; x $p = 0.009$ vs. I/R + ADA group; for TNF- α : $^{\delta}$ $p = 0.003$ vs. control group; $^+$ $p = 0.003$ vs. I/R + ADA group.

+ ADA group ($p = 0.003$). The results of CA II are shown in Fig. 2. The results of TNF- α results are shown in Fig. 3. The results of histopathological evaluation are shown in Table 2.

Discussion

In several studies, renal dysfunction induced by I/R was characterized by renal tubular deformities, such as tubular dilation, obstruction, and necrosis, suggesting a tubular origin of these functional defects [16, 17]. Many mechanisms are included in renal I/R injury, including the accumulation of ROS infiltration of inflammatory cells and the

release of proinflammatory mediators, such as cytokines and chemokines [18, 19]. The release of ROS from activated leucocytes during I/R is a major contributor to the peroxidation of lipid membranes and free-radical-induced DNA damage in the kidney [20]. Studies have shown that TNF- α , IL-1 β , and IL-6 are elevated during I/R and that they are responsible for activation of the apoptosis pathway [21]. Our study indicated that the trigger of renal I/R injury is the production of proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-6. It further showed the ability of ADA pretreatment to prevent the release of these cytokines. In contrast to our findings, it has been clearly demonstrated that renal I/R injury causes a significant in-

crease in TNF- α , IL-1 β , and IL-6 levels in tissue samples in the I/R group. In the current study, these cytokines were suppressed more in the ADA treatment group than in the I/R groups. ADA may prevent reperfusion injury by suppressing the release of TNF- α , IL-1 β , and IL-6. The histopathological examination of the ADA treatment group revealed less damage than in the I/R group. Based on the immunohistochemical analysis, TNF- α activity was low in the I/R + ADA group. As an antioxidant and an inhibitor of TNF- α , ADA suppresses the release of cytokines during I/R injury and decreases renal damage.

The kidney maintains acid-base balance by two main mechanisms: firstly by reabsorption of bicarbonate from the proximal tubule and secondly by excretion of protons (H⁺) and ammonium from the distal nephron [22]. CA is a metalloenzyme that is responsible for catalyzing the reversible hydration reaction of carbon dioxide to bicarbonate. In humans, CA has 12 catalytically active and highly homologous isoforms. CA II is found in various tissues, including the kidney [23]. Overexpression of CA II not only increases Cl⁻/HCO₃⁻ exchange activity, leading to elevation of intracellular bicarbonate [24, 25], but also converts excess bicarbonate to carbonate radicals [26, 27]. A recent study suggested carbonate radicals to be the major mediator of various types of oxidative damage [28]. Several studies have shown that the effect of nitrogen dioxide and carbonate radicals on carbohydrates and proteins could lead to formation of nitration and nitrosation products inside the endothelial cells during reperfusion injury [29, 30]. A previous study has demonstrated peroxymonocarbonate, a product of the spontaneous reaction of bicarbonate with H₂O₂ not only to be an intermediate substance for superoxide dismutase and peroxidase enzymes but also to be a precursor for carbonate radical anions [31].

In the same way, the addition of bicarbonate to H₂O₂ and the scavenger enzyme system has been reported to increase DNA damage. Also, a previous study suggested that extra sodium bicarbonate may be harmful to myocardial cells because carbon dioxide released from sodium bicarbonate diffuses

into cells faster than does HCO₃⁻ (paradoxical acidosis) [32]. In our study, the CA II level of the I/R group was higher than that of the control and ADA treatment groups.

In addition, rapidly growing cancer cells need bicarbonate for growth and metastasis [33]. The expression of CA II in tumor endothelial cells has been observed in various cancers, including esophageal, renal, and lung cancers [34]. Therefore, CA inhibitors may be used to combat tumor angiogenesis. ADA treatment may decrease bicarbonate levels via inhibition of CA II enzyme activities, leading to the production of fewer peroxy carbonate radicals. TNF- α has been shown to increase CA II overexpression in patients with pancreatic cancer [35]. ADA may protect against kidney tumors by inhibition of CA II overexpression.

As a potent TNF- α inhibitor ADA may suppress the expression of CA II. The results of the present study has shown ADA which reduces the release of TNF- α to be protective against kidney I/R injury as a result of its anti-inflammatory and antioxidant characteristics. Studies of ADA treatment in rats are limited. It was reported that the highest serum level of ADA could be achieved after of 5 days of subcutaneous administration [36]. In this study we administered ADA intraperitoneally 5 days before giving I/R and demonstrated that it was protective in I/R. In addition, the serum urea level in the I/R, control, and ADA groups was low. Low levels of urea may be related to the inhibition of the arginase I enzyme, a member of the urea cycle. Arginase I overexpression decreases the expression of the inflammatory cytokines TNF- α and IL-6 [37]. Increased TNF- α and IL-6 may be shown to lower the expression of arginase I. Further studies are needed in this regard.

In conclusion, the findings of the present study have demonstrated that ADA pretreatment may prevent or attenuate renal injury in rats. The possible mechanism of action of ADA may include anti-inflammatory activity and reduced CA II inhibition. ADA may decrease proinflammatory cytokine levels by inhibition of TNF- α , IL-1 β , and IL-6, and may decrease carboxyl radicals *via* inhibition of CA II enzyme activity.

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