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# Effects of kefir on ischemia-reperfusion injury

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**Abstract. – OBJECTIVE: We aimed to investigate the effect of kefir on Ischemia-Reperfusion (I/R) injury on rats.**

**MATERIALS AND METHODS: 24 male Sprague-Dawley rats between 250-350 g were selected. Rats were divided into three groups, and there were eight rats in each group. Rats were fed for 60 days. All of the rats were fed with the same diet for the first 30 days. In the second thirty days, kefir [10 cc/kg/day body weight (2 x $10^9$ cfu/kg/day)] was added to the diet of the study group by gavage method. In all groups, lung and kidney tissues were removed after the procedure and rats were sacrificed. The biochemical and histopathological changes were observed in the lung and kidney within the samples. Serum urea, creatinine and tumor necrosis factor (TNF-α) were determined.**

**RESULTS: Kefir + I/R groups was compared with I/R groups, a significant decrease ($p < 0.05$) was seen in Lipid peroxidation (MDA) levels of lung and renal tissues. Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GSH-Px) activities of lung and kidney tissues decreased in I/R groups ($p < 0.05$). The enzyme activities in Kefir + I/R groups of renal tissues were significantly ($p < 0.05$) higher than I/R, not significantly different in lung tissues ($p < 0.05$). Kefir reduced the levels of serum urea, creatinine and TNF-α significantly.**

**CONCLUSIONS: This would be useful in this model against ischemia/reperfusion, and shows the protective effect of kefir in tissue and serum functions.**

*Key Words:*
Kefir, Ischemia-reperfusion injury, Lung, Kidney.

## Introduction

Kefir grains had been found for the first time by the tribes living in the mountainous regions of the North Caucasus. Kefir is a creamy looking and acidic drink which is fermented naturally from milk. Kefir consists of two microorganisms; lactic acid bacteria and yeast[1,2]. As a result of metabolic activity of yeast and lactic acid bacteria in kefir, lactic acid, ethyl alcohol and carbon dioxide is formed[3]. There are a variety of useful biological activities of kefir (commercially available in Danem Ltd. Sti., Isparta, Turkey). Acetic acid bacteria and yeasts in kefir microflora has antibiotic activity. Together with these, as shown in studies in animals, kefir has antidiabetic, antibacterial and antifungal effects. Kefir is shown to have strong antioxidant effect[4].

Ischemia is reversible or irreversible cell/tissue damage caused by an insufficient blood flow perfusing organ or tissue[5]. During ischemia, adenine nucleotide degradation in hypoxic tissues is increased. As a result, precursor of reactive oxygen species (ROS) increases intracellular accumulation of hypoxanthine. After ischemia, by restoration of blood flow in that area (reperfusion) and re-introduction of molecular oxygen, intracellular ROS is formed quickly[6].

Performing reperfusion may lead to more damage than ischemia in tissues. Basically, in post-ischemic reperfused tissue, inflammatory and metabolic damage characterized by edema and dysfunction begins as a result of disruption of cellular integrity. Events often are not confined to those tissues. Various organs such as lungs, kidneys, liver and heart, and cells those may be in every organ such as endothelium and epithelium are affected by many activated systems and toxic mediators. After reperfusion injury, clinical complications due to the failure of distant organs may even lead to death[7,8].

By the help of several drugs and agents, protection of tissues against reperfusion injury which is formed by providing blood after is-

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chemia, is targeted. In this study, we tried to demonstrate the benefits of kefir that is available easily and also the beneficial effects of it on ischemia reperfusion model.

## Materials and Methods

The study began after obtaining written permission from Canakkale Onsekiz Mart University Ethics Committee of Experimental Animals. All animal care and use were in accordance with the European Convention for Animal Care. 24 male Sprague-Dawley rats between 250-350 g were selected. Rats were divided into three groups, and there were eight rats in each group. The dosage to reveal the beneficial effects of kefir was determined as 10 cc/kg/day body weight ($2 \times 10^9$ cfu/kg/day) based on preliminary studies with various doses ($3\times10^8$, $9\times10^9$, $1.8\times10^{10}$ cfu/kg bw)[9,10]. Rats were fed for 60 days. All of the rats were fed with the same diet for the first 30 days. In the second thirty days, kefir (1 ml/100 g) was added to the diet of the study group by gavage method. No rats showed an abnormal condition before the operation.

### Study Groups

Twenty-four rats were divided into three groups, randomly and in equal numbers (n = 8). In the control group, laparotomy and dissection of infrarenal abdominal aorta (IAA) was performed, but occlusion was not performed (Sham group). In the group of aortic ischemia reperfusion (A/R), dissection of abdominal aorta (AA) was performed, 180 minutes of ischemia was performed by placing cross-clamp to AA, and 60 minutes of reperfusion was performed after removing cross-clamp (Control group). In the group of A/R + kefir, 180 minutes of ischemia was performed by placing cross-clamp to AA, and 60 minutes of reperfusion was performed after removing the cross-clamp (Operations Group).

### Operative Procedures and Techniques

Ketamine (50 mg/kg) and xylazine (5 mg/kg) were used intraperitoneally for premedication of rats. Anesthesia was maintained with intermittent injections of ketamine, without intubation or mechanical ventilation. Temperature probes were inserted into the rectum. The procedures were performed to the rats in the supine position. After preparing sterile operative field, laparotomy was made with a standard midline incision. After pulling the intestines aside, the retroperitoneum was reached, and by opening the retroperitoneum abdominal aorta was reached. During the surgical procedure, cross-clamp was placed just below the renal arteries. Ischemia time was kept 180 minutes, and then cross-clamp was taken and the organs were allowed to be reperfused for 60 minutes. During this process, the body temperature was kept between 36.5 and 37.5°C. After the procedure, the abdominal wall was closed by suturing with 5/0 prolene. 12 hours after the procedure, the animals were given phenobarbital (20 mmg/kg) and sacrificed. The kidneys and lungs were carefully dissected and were fixed in formalin for 7 days.

### Histopathological Scoring

Lung and kidney tissues were fixed in 10% formalin, embedded in paraffin, and sectioned (6 m). Paraffin sections were stained with hematoxylin and eosin. The lung injuries scored that were interalveolar oedema, congestion, and leukocytes infiltration. Alveolar oedema and congestion were scored on a scale from 0 to 3 where 0 = absence of pathology (< 5% of maximum pathology), 1 = mild (< 10%), 2 = moderate (15-20%), and 3 = severe (20-25%). Leukocyte infiltration was evaluated to determinate the severity of inflammation resulting from contusion. Each section was divided into 12 subsections, and leukocytic infiltration was examined in each of the subsections at a magnification of 400x with the following scale; 0: no extravascular leukocytes; 1: < 10 leukocytes; 2: 10-45 leukocytes; 3: > 45 leukocytes. The average of the numbers was used for comparison[11,12].

The kidney injury included tubular necrosis, cast formation, loss of brush border, tubular dilatation and immune cell infiltration. Scoring for each category was as follows: 0, no change; 1, < 10%; 2, 11-25%; 3, 25-45%; 4, 46-75%; and 5, > 76% area changes. Scores for all the categories were added for the final injury scoring[13].

## Biochemical Analyses

### Serum Measurements of Urea and Creatinine

Serum samples were separated by centrifuging at 4000 rpm for 10 min at 4ºC within 1 h after collection, and were stored in a –86ºC freezer before being used for biochemical analysis. Urea and creatinine values from each sample were determined

with highly sensitive ELISA spectrophotometry (Thermo Scientific Multiskan GO Microplate Spectrophotometer, Saunt Louis, MO, USA).

### TNF-α Measurement

The Rat TNF-α ELISA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of rat TNF-α in cell lysate and tissue lysate. A specific antibody for rat TNF-α is placed on a 96-well plate in this assay. After pipetting all samples and standarts into the wells, TNF-α is attached to the wells by means of immobilized antibody. Biotinylated anti-rat TNF-α antibody is added to the cleaned wells. HRP-conjugated streptavidin is added by pipette to the wells after removal of unconnected biotinylated antibody. The wells are washedagain. TMB substrate solution is added to the wells and depending on the amount of TNF-α bound, a color change is monitored. The color changes from blue to yellow by the Stop Solution and the color density is measured at 450 nm. From each sample, TNF-α was measured in duplicate with a highly sensitive ELISA kit (Sigma Aldrich®-RAB0480, Saint Louis, MO, USA) produced only for rats, according to the tecnical bulletin.

### Biochemical Estimations of Kidney and Lung Tissues

After macroscopic analyses, rat tissues were kept at -80°C. For biochemical investigation, malondialdehyde (MDA) levels, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH) activities from each supernatant were measured in duplicate with highly sensitive ELISA spectrophotometry, respectively. The protein concentrations were indicated by the Lowry method using total protein kit (Sigma Aldrich, Total protein kit-TP0300-1KT, Saint Louis, MO, USA). All the data was defined as the mean ± standard deviation (SD) results based on per mg of protein.

### Determination of Lipid Peroxidation Level

Lipid peroxidation levels in kidney and lung tissues were determined by estimating MDA levels using the thiobarbituric acid reactive subtance (TBARS) test (Cell Biolabs, Inc. STA-330 MDA Assay Kit, San Diego, CA, USA). The rat tissues were immediately excised and treated with cold saline. The tissues were weighed and homogenized in 10 mL of 100 mg/mL phosphate buffer solution (PBS). To prevent further oxidation, 0.01 mL butylated hydroxytoluene (BHT) solutions were added to 1 mL of sample volume. The homogenate (0.1 mL) was added to a solution containing 0.1 mL of sodium dodecyl sulfate (SDS) lysis solution, mixed and incubated for 5 min in room temperature. TBA reagent was prepared with TBA acid diluent according to the kit assay procedure and added to reaction mixture. The mixture was incubated at 98°C for 1 h and cooled. 5 mL of n-butanol/pyridine (15:1) was added to the mixture and all contentwas vortexed for 1 min and centrifuged for 30 min at 4000 rpm. The supernatant absorbance was measured at 532 nm. A standard curve was generated using MDA standarts. All samples were measured in duplicate. The results were expressed as nmol MDA per milligram protein (nmol/mg protein).

### Catalase Activity

Decomposition of $H_2O_2$ in the presence of CAT was followed at 540 nm (Cell Biolabs, Inc. STA-341 Catalase Activity Assay Kit, San Diego, CA, USA). CAT activity was defined as the amount of enzyme required to decompose 1 nanomole of $H_2O_2$ per minute, at 25°C and pH 7.8. 1 mM of EDTA for per gram of tissue was added homogenized rat kidney and lung tissues to prevent oxidation and centrifuged at 10.000 xg for 15 minutes at 4°C. The supernatant was removed for procedure. Hydrogen peroxide was mixed assay diluent to achieve hydrogen peroxide working solution (HPWS-12 mM). HPWS was added 20 µl of each homogenates and CAT standarts and incubated exactly 1 min. Reaction was stopped by adding 0.05 mL of catalase quencher into each well and mixed. 5 µl of each reaction well was transferred to fresh wells. 0.25 mL of choromogenic working solution, attained according to the kit procedure, was added each well of plate. The plate was incubated 60 minutes and and read 520 nm. Results were expressed as millimole per minute per milligram protein (µmol $min^{-1}$/mg protein-U/mg protein).

### Superoxide Dismutase Activity

Superoxide radicals generated by xanthine and xanthine oxidase and constitute formazan dye after reacting with nitro blue tetrazolium[14]. Superoxide dismutase activity is measured at 560 nm. 150 µmol/L NBT, 0.3 mmol/L xanthine, 0.6 mmol/L EDTA, 400 mmol/L $Na_2CO_3$, 1 g/L bovine serum albumin (BSA) and 2 M $(NH_4)_2SO_4$ were mixed as reactive solution. 0.1 mL of homogenized tissues were added 2.85 mL of reaction mixture. 0.05 mL of xanthine oxidase was added, mixed quickly by

inverting to each sample and incubated 20 min at room temperature. After incubation 0.1 mL of stop solution copper chloride was added all tubes and the absorbance of samples was read at 560 nm against blank.

SOD activites (inhibition rate %) were calculated using following equation: SOD activity = [(Absorbance Blank {B} – Absorbance Sample {S})] / B × 100.

Results were expressed millimole per minute per milligram protein (U/mg protein).

### Glutathione Peroxidase (GSH-Px) Activity

Glutathione Peroxidase Activity Assay Kit (MedSanTek, Istanbul, Turkey) is a quantitative assay for measuring the total glutathione content with in a sample (GSH/GSSG). Oxidized glutathione (GSSG) is reduced by Glutathione Reductase to reduced monomeric glutathione (GSH) in the presence of NADPH. NADPH is oxidized to $NADP^+$ in company with decrease in absorbance at 340 nm (A340). The proportion of decrease in the A340 shows amount of the glutathione peroxidase in the sample. One unit of Glutathione Peroxidase is defined as the amount of enzyme that will cause the oxidation of 1 nmole of NADPH to $NADP^+$ per minute at 25ºC. Glutathione peroxidase activity in unknown sample was calculated using extinction coefficient of NADPH. The results were expressed millimole per minute per milligram protein (U/mg protein).

### Statistical Analysis

The statistical analyses were determined by one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (version 19.0, SPSS Inc., Chicago, IL, USA) software. The Tukey's test option was used for acquiring the differences among the groups and the results were considered to be significant at $p < 0.05$. All data was expressed as mean ± standard deviation (SD) in each group.

## Results

According to the histopathological results, while normal lung and kidney tissues were observed in control group, the IR and K + I/R groups showed hemorrhage, oedema, and leukocyte infiltration. The histopathological scoring demonstrated that the scoring of the K + I/R group was significantly positive compared to the IR group (Table I and Figure 1). In kefir treated rats, lung tissues had normal architecture of alveolar spaces when it was compared to the I/R group (Figure 2A). Necrotic cells were slightly decreased in the kidney tissues K + I/R group as compared with IR group (Figure 2B).

Lipid peroxidation levels were found to be higher in I/R groups compared to intact groups ($p < 0.05$). Also it was determined that lung and renal SOD, CAT and GSH activities ($p < 0.05$) decreased in I/R groups. K + I/R groups were compared with I/R groups, a significant decrease ($p < 0.05$) was seen in MDA levels of lung and renal tissues. CAT, SOD and GSH activities in K + I/R groups of renal tissues were significantly ($p < 0.05$) higher than I/R, not significantly different in lung tissues ($p < 0.05$) (Table II).

Serum levels of urea, creatinine and TNF-α were significantly different in each group ($p < 0.05$) (Table III). When K + I/R groups were compared with I/R groups, the urea, creatinine and TNF-α levels were low in K + I/R groups than I/R groups (Figure 3).

## Discussion

Lung injury is known to occur after temporary occlusion of the aorta and following reperfusion of lower extremities. Reperfusion injury in the lungs is frequently observed after cardiopulmonary bypass, pulmonary thromboendarterectomy and lung transplantation. This damage in the

**Table I.** Comparison of alveolar oedema/congestion scores and leukocyte infiltration of the lung tissue in each group.

| Groups | Oedema/congestion scores | Leukocyte infiltration |
|---|---|---|
| Control | 1.40 ± 0.09 | 1.21 ± 0.4 |
| IR | 2.83 ± 0.7[a] | 2.62 ± 1.01[a] |
| K-IR | 1.87 ± 0.9[b] | 2.01 ± 0.6[b] |

Datas are presented as mean ± SD; [a]$p < 0.05$ compared with Control and K + I/R groups; [b]$p < 0.05$ compared with Control group.

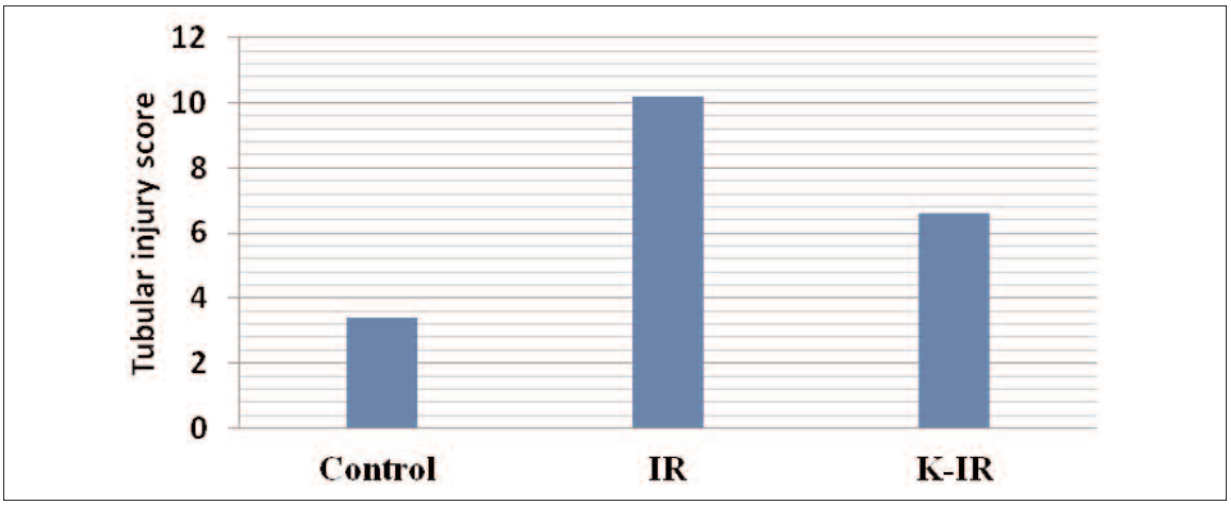


**Figure 1.** Kidney tubular injury scores. Datas are presented as mean ± SD. *$p < 0.05$ compared with Control and Control + K+I/R groups; **$p < 0.05$ compared with Control group.

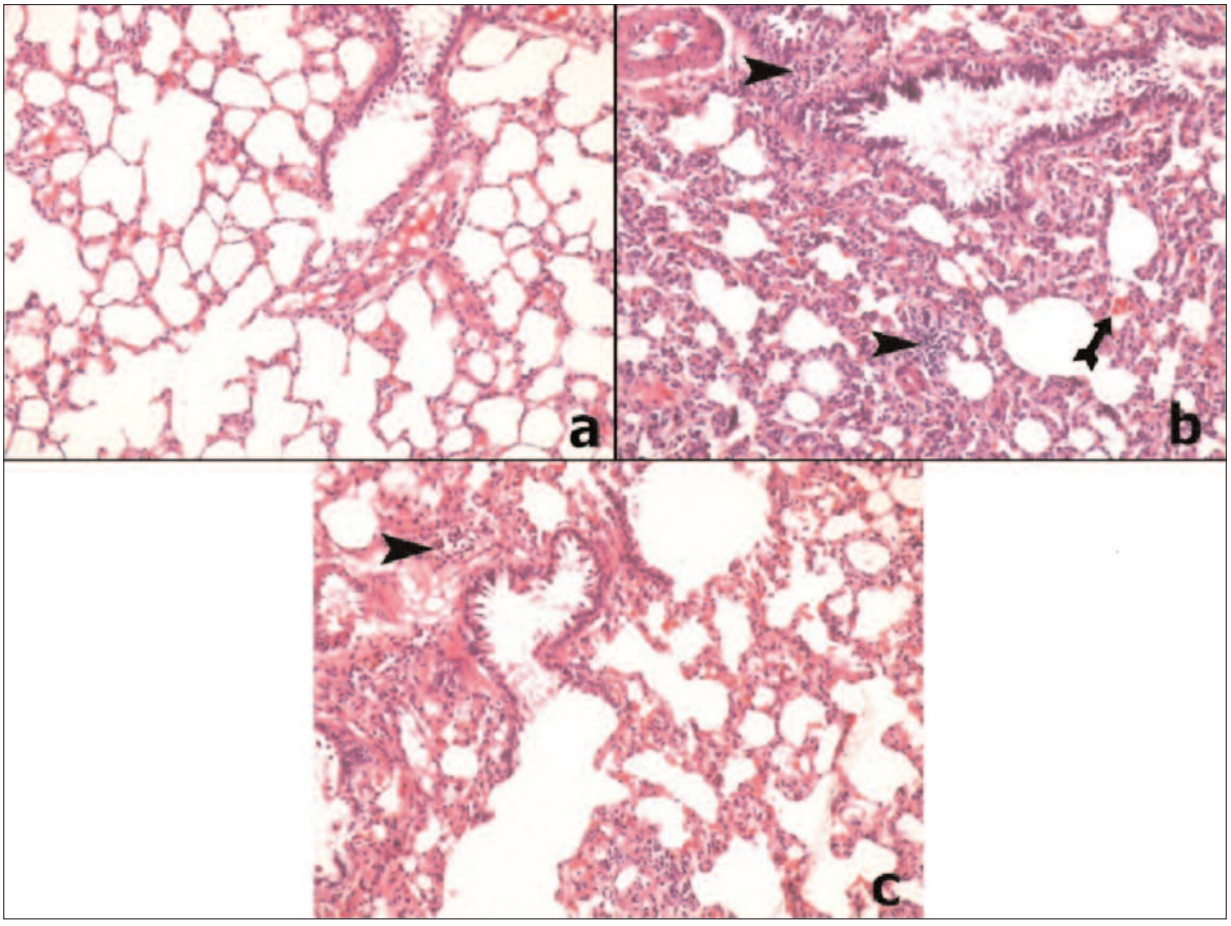


**Figure 2 A.** ***a,*** In the control group, the histology of lung tissues was normal in appearance. ***b,*** I/R group: haemorrhage and infiltration of inflammatory cells around the alveoli. ***c,*** Kefir + I/R group: Kefir treated rats showing normal architecture of alveolar spaces. *Arrows:* Haemorrhages areas; *Arrowheads:* Inflammatory cells (Hematoxylin-Eosin stain, ×200).

**Table II.** Effects of kefir on changes in catalase (CAT), lipid peroxidation levels (MDA), enzymatic activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH) of kidney and lung tissues

| Groups | SOD (U/mg protein) | CAT (U/mg protein) | MDA (nmol/mg protein) | GSH (U/mg protein) |
|---|---|---|---|---|
| **Kidney** | | | | |
| Intact | 9.16 ± 0.35 | 11.04 ± 0.30 | 1.65 ± 0.03 | 11.79 ± 0.99 |
| I/R | 5.74 ± 0.51[a] | 8.80 ± 0.10[a] | 2.13 ± 0.17[a] | 6.41 ± 0.63[a] |
| K + I/R | 7.38 ± 0.68 | 10.10 ± 0.42 | 1.80 ± 0.05b | 9.39 ± 0.65 |
| **Lung** | | | | |
| Intact | 13.60 ± 0.16 | 1.08 ± 0.01 | 0.50 ± 0.04 | 12.56 ± 2.4 |
| I/R | 6.22 ± 0.35[a] | 0.99 ± 0.02[b] | 1.96 ± 0.10[a] | 3.93 ± 0.80[a] |
| K + I/R | 10.59 ± 0.27 | 1.04 ± 0.01[b] | 0.79 ± 0.11[b] | 7.10 ± 1.24 |

*Note:* Results were given as mean ± standard deviation. Means in the same column by the letter "a" are significantly different in each group to the One-way ANOVA-Tukey's test. [a]$p < 0.05$. The means shown with the letter "b" are not significantly different with intact or I/R groups to the One-way ANOVA-Tukey test, [b]$p < 0.05$.

lung is mediated by lymphocytes, pulmonary arterial endothelial cells, alveolar macrophages and pulmonary alveolar type II cells[15].

Prevention of damage caused by polymorphonuclear neutrophil leuckocyte (PMNL) may be provided by clearance of free oxygen radicals formed in the environment by activation of the antioxidant system. Until today, various agents such as superoxide dismutase, allopurinol, catalase, mannitol, vitamin C, alpha-tocopherol, L-carnitine, pentoxifylline were tried in the treatment to eliminate the adverse effects of free oxygen radicals formed after ischemia-reperfusion, and they have been shown to be effective[16].

Ischemia reperfusion injury in kidneys is crucial for kidneys, and is a serious cause of morbidity and mortality for patients. Acute renal failure due to ischemia is characterized by a decrease in glomerular filtration rate, tubular necrosis and an increase in renal vascular resistance[17]. Serum creatinine level is one of the markers of kidney function. After IR injury, as an indicator of damage and dysfunction in renal proximal tubule cells, the level of serum creatinine rises[18]. In preventing reperfusion injury occurring in the kidney, many agents such as allupurinol, glutathione, superoxide dismutase, alphatocopherol were used for clearance of free oxygen radicals[19].

Probiotics are alive microorganisms composed of bacteria (most notably lactic acid bacteria) and yeast. Probiotics are nutrients which are used in treatment of diseases. Kefir is a probiotic, and it is a dairy product which is whitish yellow in color, sour and sparkling. Kefir contains high levels of lactic acid bacteria, and they give kefir powerful antioxidant properties[20]. Lipid peroxidation is the reaction of collapse of cell membrane fatty acids to various products by free oxygen radicals. Lipid peroxidation is increased with the increased amount of free radicals in the environment. Kefir prevents lipid peroxidation more powerfully and effectively than vitamin E which is known as a strong antioxidant[21].

Lipid peroxidation is terminated by transformation of radicals to MDA. The increase of MDA levels in many tissues, results in an increase in reactive oxygen species (ROS). Because MDA is active both within and outside of the cells, it affects particularly phospholipids in the structure of membrane and disrupts the integrity of the membrane structure[22].

**Table III.** Effects of kefir on changes in serum measurements of Urea, Creatinine and TNF-α levels.

| Groups | Urea (mg/dL) | Creatinine (mg/dL) | TNF-α (pg/mL) |
|---|---|---|---|
| Intact | 61.33 ± 5.45 | 0.45 ± 0.03 | 14.76 ± 1.67 |
| I/R | 137.86 ± 9.93[a] | 1.57 ± 0.11[a] | 29.25 ± 1.56[a] |
| K + I/R | 92.22 ± 5.03 | 0.81 ± 0.08 | 19.18 ± 1.01 |

*Note:* Results were given as mean ± standard deviation. Means in the same column by the same letter are significantly different in each group to the One-way A.

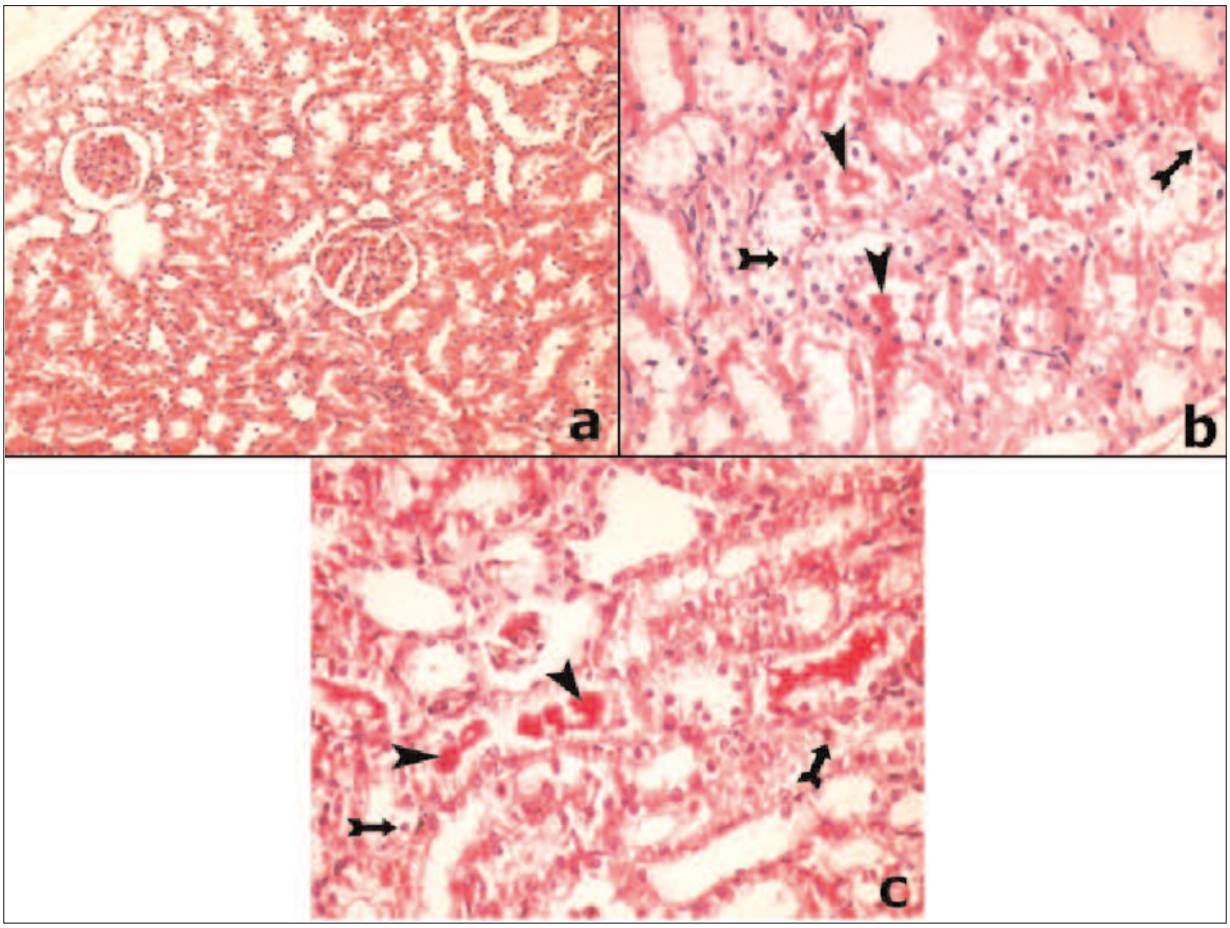


**Figure 2 B.** ***a,*** In control group, the histology of kidney tissues was normal in appearance. ***b,*** I/R group; necrotic cells, tubule injuries were remarkably higher in the kidney tissues of the IR group. ***c,*** Necrotic cells were slightly decreased in the kidney tissues Kefir + I/R group as compared with I/R group. Arrows: necrotic cells; Arrowheads indicate injured tubules, debris and cast formation (Hematoxylin-Eosin stain, ×200).

Ischemia/reperfusion injury after increasing ROS biochemical activities of antioxidant systems is insufficient cause. In ischemic tissue, antioxidant enzymes such as SOD, CAT and GSH-Px become inhibited quickly due to ROS, and the cells become susceptible to ROS in the period after reperfusion of cells. Depending on the increase in the amount of ROS, lipid peroxidation, and thus the amount of MDA, the end product of lipid peroxidation, is also increased[23].

Glutathione has two forms; oxidized and reduced. Reduced glutathione (GSH) has a protective effect against oxidative damage. As a result of tissue damage, oxidized glutathione (GSSG) levels increase. Thus, increase in the amount of oxidized glutathione causes decrease in the activity of glutathione peroxidase enzyme[24].

The effects of kefir against ROS have been shown in several studies in the literature. Güven et al[25] observed that kefir increased the levels of glutathione and glutathione peroxidase in a model of oxidative damage created in mice. In another study conducted on kefir[26], the effects of kefir on the levels of glutathione and MDA were investigated. It has been suggested that it has a protective effect. In the study of Paller[27], in the reperfusion period after ischemia, it was observed that the level of tissue glutathione decreases due to high oxidative stress. In the ischemia/reperfusion injury of the liver, it has been determined that kefir shows protective effect by increasing SOD[28]. In our study, in ischemia/reperfusion model created on rats (180 min of ischemia and 60 min reperfusion), antioxidant enzyme activities were diminished, glutathione levels were lower, and lipid peroxidation end product, MDA levels were increased significantly in tissues with I/R injury as compared to

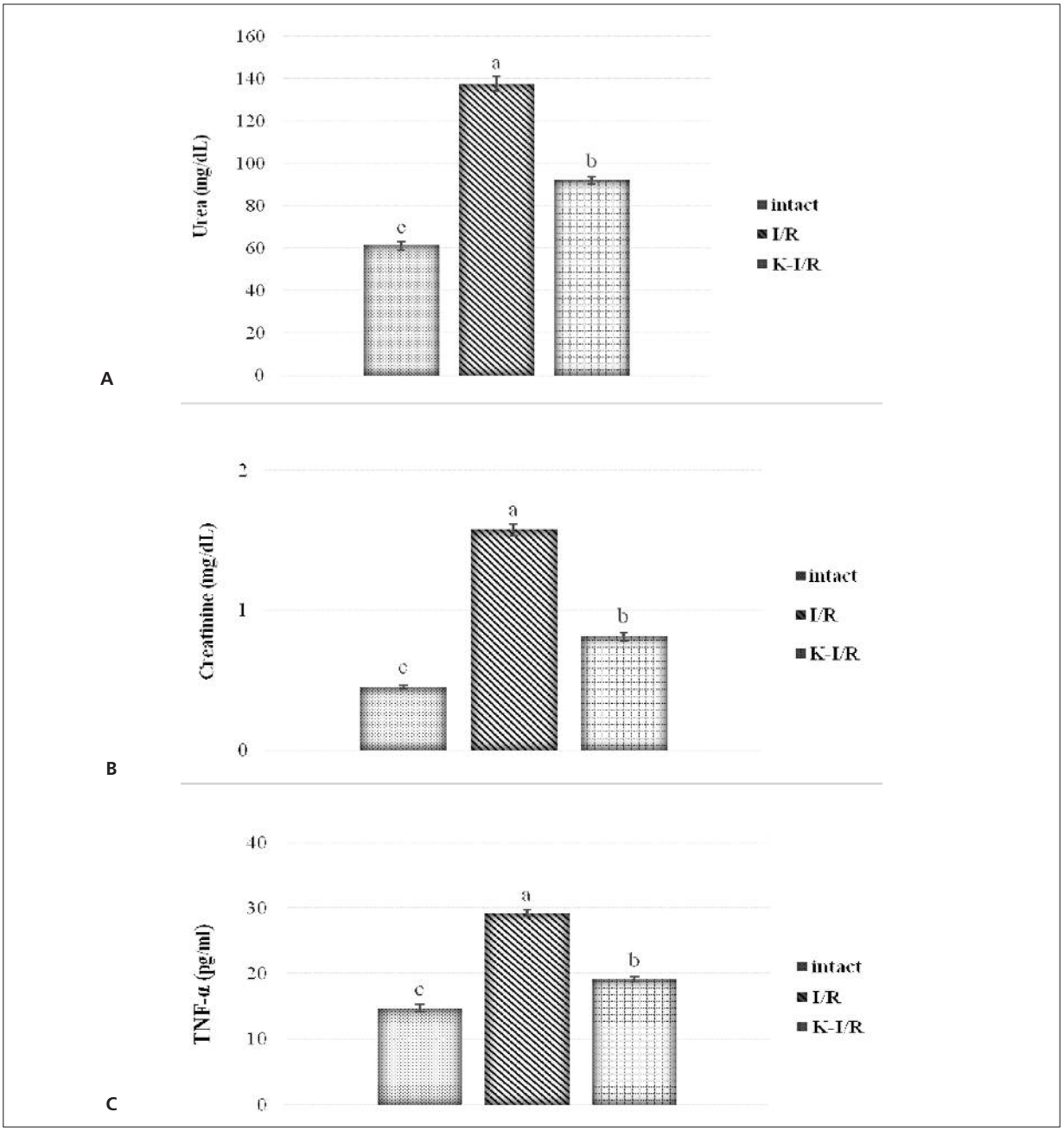


**Figure 3.** I/R: Ischemia/Reperfusion; K + I/R: Kefir + Ischemia/Reperfusion. ***A,*** The effects of kefir on changes in serum activities of Urea. ***B,*** The effects of kefir on changes in serum measurements of creatinine. ***C,*** The effects of kefir on changes in serum levels of TNF-α. Means shown the letter "b" is significantly different in each groups $p < 0.05$.

the control group. In the kefir group, antioxidant enzyme activities were higher, and MDA levels were lower than I/R group. In our study, when the amount of tissue antioxidant enzymes and MDA levels were evaluated, it was seen that the results were compatible with previous studies.

In a study conducted on mice, it was determined that kefir reduces hepatocyte damage, inflammation and proinflammatory cytokines and increases antioxidant activity[29]. Tumor necrosis factor (TNF-α) is a cytokine that responds to tissue injury, and it is a proinflammatory acute phase protein released by a variety of macrophages with high activity. Xing et al[28] found that various probiotics reduced the level of serum TNF-α in rats with hepatic ischemia reper-

fusion injury. In our study, in a similar manner, the quantitative increase in the amount of TNF-α in the group of tissue I/R proves the correctness of the study. As compared with the kefir group, it was observed that TNF-α levels were significantly lower than I/R group. In I/R group, in accordance with the histopathological examination, there was an increase in necrosis.

In order to evaluate the renal function, evaluation of serum urea and creatinine levels is quite important. In our study, urea and creatinine levels indicative of renal glomerular function abnormalities were significantly increased, but these levels were significantly lower than the I/R group.

## Conclusions

According to the findings of this I/R model, kefir reduces tissue MDA levels, increases antioxidant enzyme activities and glutathione levels, and reduces the levels of serum urea, creatinine and TNF-α significantly. This would be useful in this model against ischemia/reperfusion, and shows the protective effect of kefir in tissue and serum functions.

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### Conflict of Interest

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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