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RESEARCH ARTICLE

COMPARISON OF EFFECTIVENESS ASCORBIC ACID AND MAGNESIUM ON RENAL ISCHEMIA-REPERFUSION MODEL IN RATS

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ABSTRACT

Purpose: This study aims to determine the effectiveness of ascorbic acid and magnesium administration separately or combined before ischemia in the rat renal ischemia and reperfusion damage of the model.
Material and Methods: Thirty-five Wistar rats were randomized into five groups of seven animals each. Group 1 only underwent laparotomy, followed for 285min until sacrification. Group 2 45min ischemia; 240min reperfusions, no drugs were applied. Group 3 250mg/kg ascorbic acid was applied 60min before ischemia-reperfusion (IR), Group 4 200mg/kg magnesium sulfate was applied 60min before IR, Group5 both drugs were applied at the same doses 60min before IR. Malondialdehyde (MDA) and glutathione (GSH) levels were determined in the renal tissues received, serum blood urea nitrogen (BUN) and creatinine levels were measured in blood samples. Hematoxylin and eosin (H&E) and periodic acid schiff (PAS) paintings for histological examinations. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) immunohistochemical staining for apoptotic cell examinations.
Results: Oxidative stress parameter MDA value was found significantly lower in Group3 (p:0.034) and Group5 (p<0.001) compared to Group2. Endogen antioxidant parameter GSH value of Group 4 (p<0.001) and Group 5 (p<0,001) were significantly higher than the other groups. TUNEL counts of the Group5 was significantly lower than the counts Group2 (p:0,005).
Conclusion: It was concluded that before ischemia, ascorbic acid and magnesium sulfate combination reduce the kidney damage in the rat renal IR model.

Keywords: ascorbic acid, ıschemia-reperfusion ınjury, magnesium, rat

INTRODUCTION

Ischemia is a reduction or cessation in blood flow to a tissue or organ, while reperfusion is defined as the renewal of blood flow to the tissue or organ after ischemia. Reperfusion may cause more damage to tissue than ischemic injury (1). This situation is called

reperfusion injury. One of the organs primarily exposed to IR injury is the kidneys and acute kidney injury is defined as a critical clinical situation related to high morbidity and mortality. Kidney IR injury may occur in a variety of clinical conditions like kidney transplantation, partial nephrectomy, cardiopulmonary bypass, sepsis, urologic interventions, and hydronephrosis (2). Among the reasons for the kidneys being sensitive to IR injury are the complicated microvascular network structure and high energy requirements (3).

In the formation of IR injury, local and systemic responses play roles characterized by the production of reactive oxygen radicals, complement activation, leukocyte-endothelial cell adhesion, transendothelial leukocyte migration, platelet-leukocyte aggregation, increased microvascular permeability, and endothelium-linked increase permeability (4). The production of increased reactive oxygen radicals causes dysfunction of the antioxidant system. This dysfunction causes tubular cell injury and apoptosis. The immune response begins as a result of the release of inflammatory cytokines. The activated complement system forms another injury path (5).

Ischemic preconditioning, defined as the adaptation of tissue through protective mechanisms against stress, was identified as the best-known mechanism to protect against IR injury (6). Among methods used for ischemic preconditioning, the most commonly chosen are distant ischemic preconditioning and pharmacologic conditioning (7).

Many studies have researched the protective efficacy of different agents applied for pharmacologic conditioning for renal IR injury. There are studies showing agents like ascorbic acid (8-14), magnesium sulfate (2,15-18), vitamin E (9), hydrocortisone (9), L-arginine (10), atorvastatin (19), tadalafil (20), monoaminoxidase inhibitors (21), dexmedetomidine (22) and mannitol (23) reduce IR injury.

High-dose ascorbic acid inhibits nicotinamide adenine dinucleotide phosphate (NADP)-oxidase and inducible nitric oxide synthase (NOS) activation to reduce and repair microcirculation disorders linked to oxidative injury; thus, preclinical studies have shown that it may reduce the effects of IR injury (9,11). Varieties of mechanisms have been proposed for how ascorbic acid intervenes in the oxidative-derived interaction between inflammatory and endothelial cells. P-selectin may affect aqueous-phase oxygen radicals, while ascorbic acid was proposed to neutralize these radicals and reduce the upregulation of this critical adhesion molecule (10). At the same time, water-soluble antioxidants cut aqueous-phase oxygen radicals before affecting lipoprotein lipids and thus, were stated to prevent the initiation of lipid peroxidation (10).

Magnesium is an electrolyte found in large amounts in both intracellular and extracellular areas. At the same time, it is a co-factor included in more than 300 enzyme systems regulating biochemical reactions in the body including protein synthesis, muscle and nerve conduction, neuromuscular signal conduction, blood sugar control, and blood pressure regulation. It is an ion channel regulator contributing to the preservation of cellular ion balance. It plays an important role in the active transport of calcium and potassium ions along cell membranes (16).

The intracellular calcium increase occurring with stimulation of L-type calcium channels increases the inflammatory response; and was identified to cause disruption of mitochondrial functions, necrosis, or apoptosis in the cell (2). The L-type calcium channel blockage formed by the L-type calcium channel blocker of magnesium was stated to be protective against IR injury (2). Magnesium is also a well-known NMDA receptor antagonist (15). It directly inhibits lipid peroxidation and was shown to reduce endothelial and neuronal reperfusion injury (18).

To date, there are many studies investigating the effectiveness of ascorbic acid and magnesium on IR damage in separate applications. Our study aimed to investigate the effectiveness of the combined application of ascorbic acid and magnesium on IR damage.

MATERIAL AND METHODS

Ethical Considerations

This study investigating the efficacy of ascorbic acid and magnesium in a renal IR model in rats was completed in Dokuz Eylul University Multidisciplinary Experimental Animal Laboratory with ethical approval from Dokuz Eylul University Animal Experiments Local Ethics Committee (Date: 31.10.2018, Decision No: 47/2018). The study included 35 adult male Wistar rats with weights from 250-300 gr. Subjects obtained from Dokuz Eylul University Faculty of Medicine (DEUFM) Experimental Animals Laboratory were fed with standard rat feed and water ad libidum. Rats were housed in standard laboratory conditions (12-hour day-12-hour night lighting; lights on at 07:00 am, 22-24 °C room temperature, 50-60% humidity).

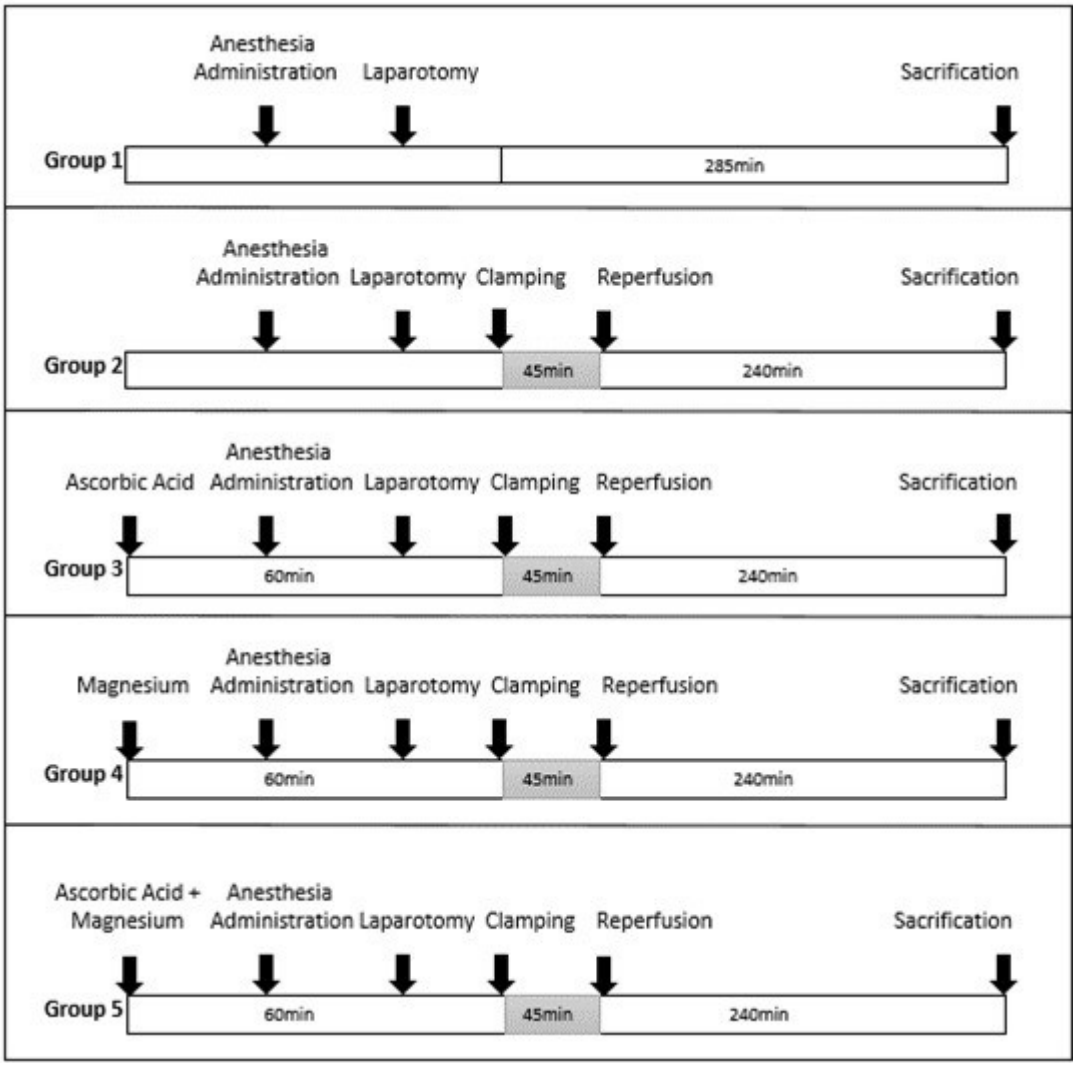

Figure 1. The schematic appearance of the experimental model

During experimental procedures, the care of laboratory animals abided by international guidelines.

Anesthesia

Subjects had anesthetized with 50 mg/kg ketamine (Ketalarflc., Pfizer Pharma GMBH, Germany) and 7 mg/kg xylazine hydrochloride (Alfazyne 2%, Alfasan International, Holland) i.p. When necessary, ketamine (25 mg/kg) was administered to keep anesthesia depth fixed by examining reflex responses (pedal reflex, palpebral and corneal reflexes when given painful stimulation to the foot with tweezers).

Experiment Groups and Protocol

The duration of ischemia in the kidneys is usually limited to 30 to 60 minutes. It has been suggested that renal ischemia lasting more than 60 minutes causes acute tubular necrosis and renal failure. If the duration of renal ischemia is shorter than 30 minutes, rapid proliferation of tubular epithelial cells can repair damaged renal tubules and may be accompanied by improvement in renal function. The effects of renal IR injury begin at the earliest at 4 hours and reach their peak at 24 hours. In this study, an ischemia time of 45 minutes and a reperfusion time of 4 hours were selected based on information obtained from the literature.

Sham Group (Sham, Group 1, n=7): Right and left renal pedicles opened after laparotomy. Rats were left under anesthesia for 285 min without any other intervention.

Ischemia-Reperfusion Group (IR, Group 2, n=7): Right and left renal pedicles opened after laparotomy, both pedicles placed in atraumatic clamp for 45 min ischemia then clamps opened for 240 min reperfusion.

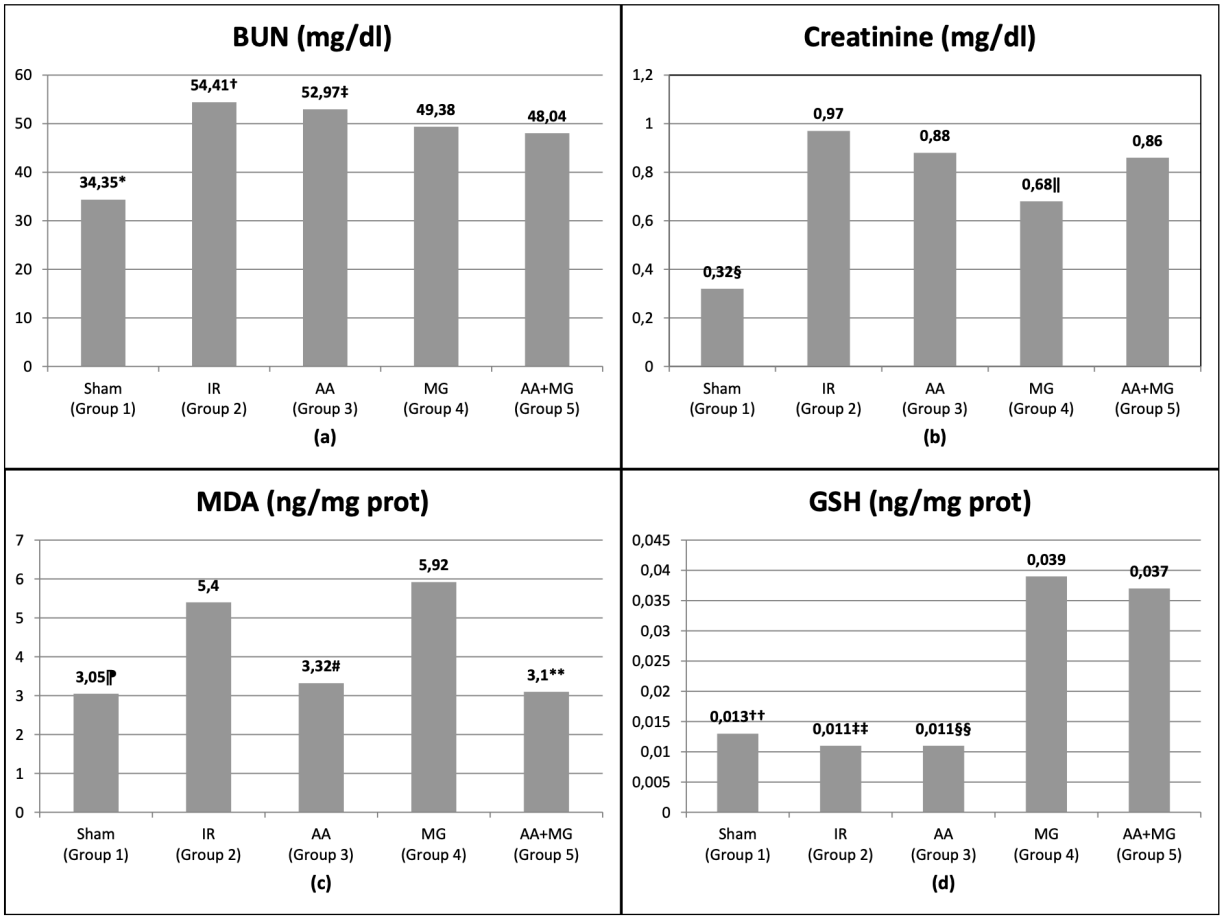

Figure 2. Comparisons between groups used the Kruskal-Wallis analysis, while in-group comparisons used the Mann-Whitney U test. (a) *significant difference compared to Group 2 (p: 0.009), Group3 (p: 0.006) and Group4 (p: 0.025); †significant difference compared to Group 5 (p: 0.021); ‡ significant difference compared to Group5 (p: 0.035). (b) § significant difference compared to Group 2, Group 3, Group 4 and Group 5(p: 0.002); ‖ significant difference compared to Group 2 (0.002) and Group 3 (0.035). (c) ⁋ significant difference compared to Group2 (p: 0.008) and Group 4 (p: 0.001); # significant difference compared to Group 2 (p: 0.034) and Group4 (p: 0.003); ** significant difference compared to Group 2(p: 0.014) and Group 4 (p: 0.001). (d) †† significant difference compared to Group 4 (p<0.001) and Group 5(p<0.001); ‡‡ significant difference compared to Group 4 (p<0.001) and Group 5(p<0.001); §§ significant difference compared to Group 4 (p<0.001) and Group 5(p<0.001).

Ascorbic Acid Group (AA, Group 3, n=7): Before laparotomy, 250 mg/kg ascorbic acid diluted with 0.9% NaCl to total volume 1 mL was administered through i.p route. Right and left renal pedicles opened after laparotomy (1 hour after medication administration), both pedicles placed in atraumatic clamp for 45 min ischemia then clamps opened for 240 min reperfusion.

Magnesium Group (MG, Group 4, n=7): Before laparotomy, 200 mg/kg magnesium sulfate diluted with 0.9% NaCl to total volume 1 mL was administered through i.p route. Right and left renal pedicles opened after laparotomy (1 hour after medication administration), both pedicles placed in atraumatic clamp for 45 min ischemia then clamps opened for 240 min reperfusion.

Ascorbic Acid + Magnesium Group (AA+MG, Group 5, n=7): Before laparotomy, 250 mg/kg ascorbic acid and 200 mg/kg magnesium sulfate diluted with 0.9% NaCl to total volume 1 mL was administered through i.p. route. Right and left renal pedicles opened after laparotomy (1 hour after medication administration), both pedicles placed in atraumatic clamp for 45 min ischemia then clamps opened for 240 min reperfusion.

Ascorbic acid dose (8-14) and magnesium dose (2,15-18) in this study were determined based on previous studies in the literature.

Table 1. Scoringmethod to be used in the histomorphological evaluation

Score	Indication
0	No change
1	Slight brush edge loss and no signs of necrosis
2	Medium brush edge loss and no signs of necrosis
3	Severe brush edge loss and no signs of necrosis

The detailed schematic appearance of the experimental model applied to the groups is presented in Figure 1.

Experimental Study Model

All rats were pinned to the operation table in the supine position after anesthesia and an abdominal midline incision was used. The right and left kidneys were opened and pedicles dissected. Rats were heated with a heating lamp on the operation table during the study to protect against hypothermia and body temperature was held to 37-37.5 ºC by measuring with a rectal probe. To prevent dehydration, a subcutaneous physiologic serum solution was administered with a 3 mL/kg dose per hour. During the waiting period, the abdomen was closed with damp sterile buffers and surgical forceps. Left and right renal total ischemia were induced by compressing the renal pedicles with an atraumatic clamp. Sufficient occlusion was observed with the formation of renal paleness. After the ischemia duration ended, clamps were free and reperfusion was allowed.

Rats in all groups had anesthesia administered and after laparotomy, right and left nephrectomy was performed for histopathologic investigation, all blood was taken for biochemical tests with a cardiac puncture. At the same time, the study was ended with this method, and rats were sacrificed by exsanguination.

Biochemical Assessment

To evaluate oxidative status, oxidative stress parameter MDA and an endogen antioxidant GSH were used. The right and left kidney samples were collected and stored at -80 °C. Homogenization was performed with tissue lyzer (For 0.1 g tissue to 1000 µl PBS). After centrifugation, the supernatants were collected for the assay. The protein concentrations of the samples were defined with BCA kit (Thermo Fisher Scientific, Inc.). MDA and GSH values were determined by using Elabscience MDA and GSH ELISA kits (Elabscience Biotech, China) according to the kit protocols. The results were given as MDA (ng/mg prot) and GSH (ng/mg prot). Assessment of these biochemical parameter kits was completed in DEUFM Medical Biochemistry Department. Serum blood urea nitrogen (BUN) (mg/dL) and creatinine (mg/dL) values in blood samples were determined in DEUFM Hospital Central Laboratory.

Histomorphologic Assessment of Renal Tissue

After the sacrifice of subjects, kidneys were sliced horizontally from the renal pelvis and placed in 10% formol solution for tissue fixation. After 48-hours of fixation, tissues had routine tissue monitoring procedures completed and were submerged in paraffin blocks. Tissues submerged in paraffin blocks had sections of 5 µm thickness taken and histologic immunohistologic staining was performed. For histologic investigations H&E and PAS staining were used, while TUNEL immunohistochemical staining was performed for apoptotic cell investigations.

H&E staining was used to assess general tissue morphology. PAS staining was used to investigate glomerular, tubular, interstitial, and vascular lesions. Five different standard randomized areas were chosen from the cortical region and investigated at x20 magnification with a light microscope. A total of 15 images were taken, with three images for each standard area. The scoring to be used in the assessment is specified in Table 1.

Preparates with TUNEL immunohistochemical staining were investigated. For the quantitative assessment of apoptotic cells, each section had 10 random areas with x20 magnification with nearly 200 cells counted. All histomorphologic analyses were performed by two histologists blind to the experimental groups.

Exclusion

It was planned that rats requiring resuscitation would be excluded from the study.

Table 2. Histomorphological Evaluation Score (values presented as mean ± SD)

	Group 1 Sham (n=7)	Group 2 IR (n=7)	Group 3 AA (n=7)	Group 4 MG (n=7)	Group 5 AA+MG (n=7)	p
Historical Damage Score	**0.20 ± 0.069***	**2.20 ± 0.106†**	**1.14 ± 0.117**	**1.05 ± 0.112**	**1.14 ± 0.124**	**<0.001**

* Significant difference compared to Group 2 (p<0.001), Group 3 (p<0.001), Group 4 (p<0.001) and Group 5 (p<0.001);† significant difference compared to Group 3 (p<0.001), Group 4 (p<0.001) and Group5 (p<0.001).

Statistical Assessment

The statistical assessment used the Statistical Package of Social Sciences 15 (SPSS 15.0, Chicago, IL, USA) program. Comparisons between groups used the Kruskal-Wallis analysis, while in-group comparisons used the Mann-Whitney U test. Values are presented as mean± standard deviation (mean ± SD). Values of p<0.05 were accepted as statistically significant.

RESULTS

This study completed in Dokuz Eylül University Multidisciplinary Experimental Animals Laboratory included a total of 35 male Wistar rats with a mean weight of 281.65 g (±15.77). There was no rat requiring resuscitation, and all rats completed the study.

Biochemical Results

The biochemical assessments for rats in all groups are presented in Figure 2.

Comparison of BUN in Group 1, Group 2, Group 3, Group 4, and Group 5 found the differences were statistically significant (p:0.003). The BUN values in Group 1 were determined to be significantly lower than Group 2 (p:0.009), Group 3 (p:0.006), and Group 4 (p:0.025). This result is interpreted as showing that IR injury was induced; hence, experimental conditions were provided. The BUN value in the Group 5 was found to be significantly low compared to both Group 2 (p:0.021) and Group 3 (p:0.035), leading to consideration that ascorbic acid and magnesium together were successful in lowering BUN values.

When assessed in terms of creatinine values, the differences between the Group 1, Group 2, Group 3, Group 4, and Group 5 were statistically significant (p<0.001). The creatinine value in Group 1 was determined to be significantly low compared to the other four experimental groups (p:0.002). This result may be interpreted as showing that IR injury was induced and experimental conditions provided, similar to the results obtained for BUN. In Group 4, creatinine values were lower compared to both Group 2 (p:0.002) and Group 3 (p:0.035). This may be interpreted as showing that magnesium may reduce the elevation in creatinine, at least at the dose and duration used in the experiment.

In terms of MDA values, the differences between the groups were found to be statistically significant when Group 1, Group 2, Group 3, Group 4, and Group 5 were compared (p<0.001). The MDA values in Group 3 were statistically significantly lower compared to Group 2 (p:0.034) and Group 4 (p:0.003). Similarly, the MDA values in Group 5 were statistically significantly low compared to Group 2 (p:0.014) and Group 4 (p<0.001). Both groups administered ascorbic acid had a fall in MDA values compared to the sham group leading to the consideration that ascorbic acid along is sufficient to lower MDA levels.

In terms of glutathione values, when Group 1, Group 2, Group 3, Group 4, and Group 5 are compared, there were statistically significant differences found between the groups (p<0.001). The significant differences identified between Group 4 and Group 5 compared with the other groups (p<0.001) are interpreted as showing that magnesium alone is beneficial to elevate GSH levels.,

Histomorphologic Finding

Histologic investigations analyzed tissue morphology, glomerular, tubular, interstitial, and vascular lesions with H&E and PAS staining. These assessments were performed and interpreted by two different experts blind to the groups. Both expert opinions were compatible with rats in all groups. Administration of ascorbic acid and magnesium separately and together was identified to have a preventive effect on IR injury in all samples.

The scores obtained as a result of the histomorphological evaluation of renal tissue are presented in Table 2.

Table 3. TUNEL count results (values presented as mean ± SD)

	Group 1 Sham (n=7)	Group 2 IR (n=7)	Group 3 AA (n=7)	Group 4 MG (n=7)	Group 5 AA+MG (n=7)	p
Apoptotic Cell Count	**29.28 ± 4.49***	**96.85 ± 12.4†**	**65.14 ± 29.72**	**57.28 ± 11.04**	**63.28 ± 20.53**	**<0.001**

*significant difference compared to Group 2 (p: 0.002), Group 3 (p: 0.04), Group 4 (p: 0.002) and Group 5 (p: 0.007); † significant difference compared to Group 3 (p: 0.002), Group 4 (p: 0.002) and Group 5 (p: 0.005).

When renal sections belonging to the Group 1 are evaluated, the fibrous capsule was intact outside the cortex, and the renal corpuscles in the cortex had normal structure. The proximal tubules, distal tubules, and collector tubules were observed to have normal structure. The characteristic structure and features of epithelial cells were preserved with normal structure observed for glomeruli.

When renal sections from Group 2 are investigated, the widening of cortical veins was observed. In the cortical region, there was more mononuclear cell infiltration in the peritubular area, brush-like edge loss in proximal tubule cells, tubular atrophy, and tubular dilatation. Especially in the corticomedullar junction, peritubular erythrocyte extravasation was observed.

When sections from Group 3 are investigated, mononuclear cell infiltration in the cortical peritubular area, degeneration observed in tubule cells, and erythrocyte extravasation in the cortex was clearly reduced compared to Group 2. Additionally, the brush-like edge loss observed in tubules, tubular atrophy, and tubular dilatation was observed at lower rates in the ascorbic acid.

When sections belonging to Group 4 are investigated, a clear reduction was observed in mononuclear cell infiltration in the cortical peritubular area, degeneration observed in tubule cells, and erythrocyte extravasation in the cortex compared to the IR group. Additionally, the brush-like edge loss observed in tubules, tubular atrophy, and tubular dilatation was observed at lower rates in the magnesium group.

When sections from Group 5 are investigated, the variations forming as a result of separate administrations were not encountered in the preparate structure with different features. The administration together was not observed to be superior to administration separately.

PAS staining observed villus loss in the Group 2 group. Especially in medullar cells, vacuolization and PAS+ intense areas were observed. Though there were clear improvements in Group 3, Group 4 and Group 5 villi did not recover, and the presence of PAS+ accumulation in the medulla was preserved. Occasional reductions were observed in vacuolization in medullar cells.

According to the TUNEL immunohistochemical staining results obtained from renal tissue investigation, the mean apoptotic cell counts are presented in Table 3.

When mean apoptotic cell counts according to TUNEL staining are compared, there were statistically significant differences between Group 1, Group 2, Group 3, Group 4, and Group 5 ($p<0.001$). The mean cell counts in Group 1 were determined to be significantly low compared to Group 2 (p:0.002), Group 3 (p:0.04), Group 4 (p:0.002), and Group 5 (p:0.007). This result is interpreted to show that IR injury was induced and experimental conditions created.

The mean apoptotic cell counts in Group 3 (p:0.02), Group 4 (p:0.002), and Group 5 (p:0.005) groups were identified to be significantly low compared to Group 2. This leads to the consideration that the ascorbic acid and magnesium administration reduced apoptotic cell counts by similar degrees.

DISCUSSION

In this experimental study evaluating the efficacy of ascorbic acid and magnesium administered separately and together before ischemia in a rat renal IR model, both agents were identified to positively change biochemical parameters, histomorphologic and immunohistologic outcomes.

Renal IR injury may be induced experimentally with two different methods. One of these is unilateral nephrectomy and clamping of the contralateral renal artery or renal pedicle and the other is bilateral clamping of the renal pedicle or renal arteries. The most well-defined method identified uses temporary closure of only the renal artery or the renal artery and vein together. Results obtained from studies suggest

that clamping of the renal artery and veins together is the ideal method to induce ischemia injury (24,25). In this study, bilateral renal ischemia was induced with atraumatic microvascular clamps. Observation of paleness in the kidney when perfusion is stopped and disappearance of arterial pulse confirm ischemia, similarly when blood perfusion is begun again after opening the clamps at the end of the ischemia duration, physical examination determined arterial pulse in the renal pedicles and change in a pale color. The ischemia-reperfusion duration is stated to be as important as the renal ischemia method. Reference scans identified that when different durations are used to induce IR injury, variations may occur according to organ and the duration. The critical ischemia duration is linked to the organ; with more than 5 min ischemia of the brain causing notable degrees of neuron death and infarctus, while this duration is reported as 15-20 minutes for the liver and kidneys(11). The ischemia duration in kidneys is generally limited to 30 to 60 minutes. A renal ischemia duration of more than sixty minutes is proposed to cause acute tubular necrosis and renal failure. If the renal ischemia duration is shorter than 30 minutes, the rapid proliferation of tubular epithelial cells may repair injured renal tubules and may be accompanied by improvement in renal functions (26-28). Different data were obtained about the duration necessary to cause injury to the kidney with ischemia (29-36). In this study, a 45-min ischemia duration was applied in light of the information obtained from the literature.

Williams et al. researched renal IR injury effects with reperfusion lasting 0, 0.5, 1, 2, 4, 6, 9, and 24 hours and 1 week after 45 minutes of ischemia in blood and tissue samples (28). According to these researchers, the effects of renal IR injury begin in the 4th hour at the earliest and peak in the 24th hour. Cochrane et al. stated that renal injury after reperfusion was observed in the 4th hour at the earliest (37). In accordance with this data, a 4-hour reperfusion duration was chosen in the experimental model in our study.

The histomorphologic, immunohistologic, and biochemical data obtained in our study displayed a significant difference in the IR group compared to the sham group showing the chosen ischemia and reperfusion durations were sufficient to induce injury and confirm the IR model was correctly applied.

In the literature, there are studies of protective pharmacologic agents used separately and together for rat renal IR injury. However, the agents researched to prevent renal IR injury and the studies have no definite standards. Partial positive effects on IR injury were researched for vitamin E(9), hydrocortisone(9), L-arginine(10), atorvastatin(19), tadalafil(20), monoaminoxidase inhibitors(21), dexmedetomidine(22), and mannitol(23); but there was no study encountered administering ascorbic acid and magnesium together to prevent renal IR injury.

Determination of the excessive production of reactive oxygen species (ROS) and the role of antioxidant enzymes in the pathogenesis of mitochondrial injury induced as a result of IR brought antioxidant and free radical scavenger treatments to the agenda. With the aim of preventing or treating renal IR injury, one of the methods applied to ascorbic acid was shown to have positive effects on IR injury in many previous studies. Seo et al. in studies assessing a hepatic ischemia-reperfusion injury in rats administered 30 mg/kg, 100 mg/kg, 300 mg/kg, and 1000 mg/kg doses to four different groups 5 minutes before ischemia (14). While antioxidant efficacy was shown at low doses, the administration of high doses showed prooxidant effects. In this study, the ascorbic acid dose was chosen as 250 mg/kg.

Mohamed et al. performed a study researching the protective role of vitamin C and L-arginine in a rat renal IR model (10). In this study with 30 min ischemia and 30 min reperfusion duration planned, 3 groups were created with one administered 500 mg/kg i.p. vitamin C 24 hours before ischemia, one group administered 400 mg/kg i.p. L-arginine for 3 days before ischemia and the final group administered both together. The groups administered vitamin C and both medications had BUN, creatinine, and MDA measurements which showed a protective effect against renal IR injury. Ji et al. studied the same model to identify the acceptable durations as renal ischemia markers (38). With this aim, they determined serum BUN and creatinine values in blood samples taken at 0, 6, 12, 24, 48, and 72 hours and concluded the increase in the 24th hour was significant. Shokeir et al. in a study examining BUN and creatinine values for renal injury in the 2nd, 24th, and 48th hours, identified that the BUN and creatinine reached the highest values atthe 24th hour and had fallen at the 48th hour (6). In our study, a significant fall was not identified in BUN and creatinine values in the groups administered ascorbic acid which was concluded to be due to this study not waiting for the 24-hour duration as in these studies.

The results for BUN and creatinine in Figure 2 demonstrate statistically significant changes across groups. However, to assess the clinical importance of these findings, it is critical to compare these levels to baseline healthy renal function and consider their implications in the context of IR injury.

BUN and creatinine are key markers for renal function, with elevated levels often indicating impaired kidney function due to reduced filtration capacity. In this study, BUN and creatinine levels were significantly elevated in the IR group compared to the sham group, confirming the establishment of renal ischemia-reperfusion injury. Importantly, the reductions observed in BUN and creatinine levels in the groups treated with magnesium sulfate and ascorbic acid—especially when used in combination—indicate a potential protective effect against IR injury.

Clinically, even modest reductions in BUN and creatinine levels can be meaningful as they suggest a lower degree of renal injury and better preservation of renal function. Previous studies have shown that BUN and creatinine levels are highly sensitive indicators of renal damage and recovery post-IR injury. For instance, Ji et al. observed significant elevations in these markers at 24 hours post-ischemia, correlating with peak renal damage (38). Similarly, Shokeir et al. noted that BUN and creatinine peaked at 24 hours and began to decline by 48 hours, aligning with partial recovery of renal function (6). Although the reperfusion time in this study was limited to 4 hours, the significant reductions in BUN and creatinine in treated groups indicate early signs of renal protection.

The reduction in creatinine levels in the magnesium group suggests that magnesium may play a role in mitigating renal IR injury by stabilizing mitochondrial function and reducing calcium overload, mechanisms that are known to contribute to acute renal failure during IR injury (16,39). Furthermore, the combination of ascorbic acid and magnesium appeared to enhance the protective effects, possibly through synergistic antioxidant and anti-inflammatory actions. This combination may optimize the mitigation of oxidative stress and apoptosis, key contributors to IR-induced renal damage.

These findings align with those of Akan et al., who demonstrated that magnesium sulfate significantly reduced BUN and creatinine levels in a diabetic rat renal IR model (2). Additionally, the observed reductions are consistent with the hypothesis that magnesium can act as a cofactor for enzymatic processes, including glutathione synthesis, contributing to antioxidant defense mechanisms (45). In conclusion, the changes in BUN and creatinine observed in this study are both statistically and biologically significant, providing evidence of the potential renal protective effects of ascorbic acid and magnesium, either alone or in combination, in the early stages of renal IR injury.

Ergin et al. in studies researching the effect of ascorbic acid in an IR injury model in rats administered 100 mg/kg bolus ascorbic acid 15 minutes before ischemia and then 50 mg/kg/hr dose as an infusion in the 2-hour reperfusion period (8). They showed ascorbic acid had a reducing effect on IR injury in tissue MDA levels. Similarly, in our study, an increase in MDA levels was identified in Group 2 in MDA results from renal tissue samples and this data is interpreted as showing IR injury increased lipid peroxidation. The administered ascorbic acid lowered MDA levels, while magnesium administration was not identified to have any effect. In the group with ascorbic acid and magnesium administered together, the reduction in MDA levels was similar to Group 3 confirming that the MDA reduction was linked to ascorbic acid. A study by Korkmaz et al. with the same ischemia duration as this study identified a significant fall in GSH values in the IR group after 3 hours of reperfusion (13). Contrary to the similar ischemia duration, Zhu et al. applied 48 hours of reperfusion and stated the GSH values fell in the IR group (12). Contrary to different reperfusion durations, contrary to these studies where GSH values were identified to fall, in the IR group in our study a fall in GSH values was not observed. A possible reason for this is that the oxidative stress induced in the model was within the compensatory capacity of GSH. Azari et al. created four groups in a study researching the effects of hydrocortisone, vitamin C, and vitamin E against renal IR damage (9). They administered 50 mg/kg vitamin C intravenous, 20 mg/kg vitamin E intramuscular, 50 mg/kg hydrocortisone intravenous and the final group had all three agents administered immediately before reperfusion. The histologic assessment used a score formulated according to histomorphologic data based on tubular degeneration, necrosis, and degree of inflammation in tissue, and the group with combination treatment was shown to have IR injury significantly reduced in renal tissue. In our study, sections stained with H&E and PAS were assessed

by two expert histologists. After histomorphologic investigation, ascorbic acid and magnesium were shown to have a preventive effect against IR injury.

Previous studies have demonstrated that magnesium impacts ion channels (inhibits calcium ions) and inhibit NMDA glutamate receptors, thereby preventing sustained stimulation of these receptors. Also magnesium inhibits enzyme activity, regulates substance metabolism, and regulates excitability of cholinergic neurons by acting on hormone receptors. It has similar effects to adrenocortical hormones, and presents obvious anti inflammatory and immunoregulatory effects (39,40). MDA and GSH levels are critical biomarkers for assessing oxidative stress and the antioxidant defense system. In this study, the observed increase in MDA levels in the IR group confirms the occurrence of lipid peroxidation, a hallmark of oxidative stress in renal ischemia-reperfusion injury. Conversely, the administration of ascorbic acid and magnesium sulfate, both separately and in combination, resulted in significant reductions in MDA levels and increases in GSH levels, highlighting their potential as protective agents.

The relationship between increased MDA and GSH levels is pivotal in understanding the oxidative stress mechanisms at play. MDA is a byproduct of lipid peroxidation, and its reduction indicates decreased oxidative damage to cellular membranes. On the other hand, GSH is a critical endogenous antioxidant, and its elevated levels in the groups treated with magnesium alone or in combination with ascorbic acid suggest enhanced antioxidant defense. Magnesium may contribute to GSH synthesis by acting as a cofactor for glutathione synthetase, as reported in the literature (45). This finding aligns with previous studies demonstrating magnesium's role in improving oxidative stress parameters via direct and indirect mechanisms (16,39).

The combined administration of ascorbic acid and magnesium led to the most pronounced reductions in MDA and increases in GSH, suggesting potential additive or synergistic effects. While ascorbic acid primarily acts as a direct scavenger of ROS, neutralizing free radicals and preventing lipid peroxidation, magnesium contributes by stabilizing cellular ion balance and enhancing enzymatic antioxidant defenses. The dual mechanisms may provide complementary protection against oxidative stress, resulting in a more effective mitigation of renal IR injury. These findings are consistent with previous studies indicating that combining antioxidants and ion channel modulators can produce synergistic protective effects in oxidative stress models (2,39).

Future research should focus on delineating the precise molecular pathways underlying this synergistic interaction, including the modulation of signaling pathways like nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates the expression of antioxidant enzymes. Nonetheless, the current data strongly support the hypothesis that the combination of ascorbic acid and magnesium sulfate offers enhanced protection against renal IR injury through complementary antioxidant mechanisms.

The 200 mg/kg magnesium dose chosen in this study was selected from doses recommended in the literature (2,8,39). A study by Akan et al. researching the protective effects of magnesium sulfate in a diabetic rat renal IR injury model administered 20 mg/kg i.p. magnesium sulfate 5 minutes before ischemia and used BUN, creatinine values, and histomorphologic scoring for identification of bilateral renal IR injury (2). The data from this study, where magnesium was identified to induce significant variations in all three parameters, were assessed as showing reduced acute renal injury. In our study, magnesium was identified to cause a significant fall in creatinine values compared to Group 2. Results from H&E, PAS, and TUNEL immune staining indicated a significant reduction was caused in IR injury. The TUNEL test ensuring in situ recognition of DNA cleavage for identification of apoptotic cells in renal tissue in rats is a marker accepted by the majority of histologists (42). Yoon Kyung et al. proposed TUNEL staining showed apoptosis to assess mean apoptotic cell counts in an induced renal IR model (43). However, Yang et al. compared by calculating the apoptotic index instead of mean counts when assessing apoptotic cells in a renal IR model (44). In this study, the assessment was performed taking the mean counts proposed by Yoon Kyung et al. and a clear increase in TUNEL positive cells was observed in Group 2 compared to Group 1. Administration of ascorbic acid and magnesium had a significant reduction in apoptotic cell counts compared to Group 2. This significant reduction identified in apoptosis indicates that the important pathway in IR injury of apoptosis may be prevented by administering magnesium and/or ascorbic acid. Pundir et al. in studies researching the effects of NMDA receptor agonists and antagonists on acute renal injury in rats with bilateral renal IR injury model induced and

administered 600 mg/kg i.p. magnesium sulfate 1 hour before ischemia and for the previous 4 days (total 5 days). The researchers identified significant reductions in creatinine and GSH values in the study and showed magnesium reduced acute renal injury. In our study, creatinine values in Group 4 were significantly reduced compared to Group 2, and kidneys were protected from IR injury. In our study, increases in GSH levels were identified in Group 4 and Group 5, with no change in GSH in the group administered ascorbic acid. The increase in GSH in Group 4 and Group 5 leads to the consideration that GSH increased linked to magnesium and this may be linked to magnesium being a cofactor for GSH synthase, a key enzyme for GSH synthesis (45).

CONCLUSION

In this study, we evaluated the effects of 250 mg/kg ascorbic acid and 200 mg/kg magnesium sulfate on the IR model induced in rats by tissue MDA and GSH, serum BUN and creatinine measurements, and histomorphologic and immunohistologic investigation results.

Administration of 250 mg/kg ascorbic acid and 200 mg/kg magnesium separately and together before ischemia in a rat renal IR model was identified to reduce IR injury.

The administration of ascorbic acid and magnesium sulfate before ischemia to prevent IR injury is considered a simple, noninvasive, and reliable method.

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