European Review for Medical and Pharmacological Sciences 2016; 20: 1642-1655

Effect of magnesium sulfate on renal ischemia-reperfusion injury in streptozotocin-induced diabetic rats

M. AKAN[1], S. OZBILGIN[1], N. BOZTAS[1], A. CELIK[2], S. OZKARDESLER[1], B.U. ERGUR[3], E. GUNELI[2], A.R. SISMAN[4], P. AKOKAY[3], R. MESERI[5]

[1]Department of Anesthesiology and Reanimation, Faculty of Medicine, Dokuz Eylül University, Izmir, Turkey
[2]Department of Laboratory Animal Science, Institute of Health Sciences, Dokuz Eylül University, Izmir, Turkey
[3]Department Dept of Histology and Embryology, Faculty of Medicine, Dokuz Eylül University, Izmir, Turkey
[4]Department of Biochemistry, Faculty of Medicine, Dokuz Eylül University, Izmir, Turkey
[5]Department of Nutrition and Dietetics, Faculty of Health Sciences, Ege University, Izmir, Turkey

Abstract. – OBJECTIVE: Ischemia/reperfusion (I/R) injury is a major cause of acute organ dysfunction and I/R related acute renal failure is a common clinical problem. Diabetes mellitus is defined as a risk factor for the development of acute renal injury as diabetic nephropathy compromises the renal tolerance to ischemia. The aim of this study was to investigate the protective effect of magnesium sulfate in a diabetic rat renal I/R injury model.

MATERIALS AND METHODS: Diabetes mellitus was induced using streptozotocin. Thirty-five rats were divided into five groups: Group I: Nondiabetic sham group; Group II: Diabetic sham group; Group III: Diabetic I/R group; Group IV: Diabetic I/R + prophylactic (preischemic) $MgSO_4$; and Group V: Diabetic I/R + therapeutic (following reperfusion) $MgSO_4$ group. $MgSO_4$ was administered 200 mg/kg intraperitoneally. Renal I/R (45 min ischemia + 4 h reperfusion) was induced in both kidneys. Histomorphological, immunohistochemical (caspase-3 and iNOS) and biochemical (BUN, Creatinine) methods were performed to assess the blood and tissue samples.

RESULTS: Histomorphological injury scores and immunostaining intensities (for both caspase-3 and iNOS) were significantly lower in the MgSO4 administered groups (prophylactic and therapeutic) than in the Diabetic IR group. There were no significant differences in biochemical parameters (BUN, Cr) between the $MgSO_4$ administered groups and the Diabetic IR group.

CONCLUSIONS: In the present study, it was demonstrated by histomorphological and immunohistochemical methods that magnesium sulfate administration before ischemia or following reperfusion significantly reduced renal I/R injury in a diabetic rat model.

Key Words:
Acute kidney injury, Experimental diabetes mellitus, Reperfusion injury, Magnesium sulfate.

Introduction

Ischemia/reperfusion (I/R) injury is a major cause of acute organ dysfunction and as the kidneys are highly sensitive to ischemia, I/R injury related acute renal failure is a common clinical problem with high mortality and morbidity[1-3]. Renal I/R injury can be observed in clinical situations, such as renal transplantation, partial nephrectomy, suprarenal aortic surgery, cardiopulmonary bypass, cardiopulmonary resuscitation and other urologic conditions[3-5]. The pathogenesis of renal I/R injury is mediated by multiple mechanisms including oxidative stress, systemic and local inflammatory responses, endothelial dysfunction, apoptosis and necrosis[6,7]. Caspase becomes activated in ischemic tissues as a result of intracellular calcium (Ca^{2+}) accumulation and is an indicator of cell death[8,9]. Inducible nitric oxide synthase (iNOS) is a protein regulated by hypoxia. Nitric oxide (NO) generated by iNOS is involved in many pathological states, including renal I/R, and leads to oxidative damage of critical cellular macromolecules[10-12].

Corresponding Author: Mert Akan, MD; e-mail: mertakan@hotmail.com

Diabetes mellitus (DM) is a common chronic disease progressing with hyperglycemia, dyslipidemia, and metabolic disorders and has several major complications including macrovascular and microvascular lesions[13]. Diabetic microvascular lesions compromise the renal tolerance to I/R; therefore, DM is defined as a risk factor for the development of acute renal injury. It was demonstrated that short ischemia period (for 30 min) in nondiabetic rats resulted in reversible acute renal failure but caused a progressive injury with end-stage renal failure in diabetic rats[12,14]. However, the mechanisms underlying the enhanced susceptibility of the kidney to I/R in DM have not been thoroughly established.

Several different methods have been developed to prevent I/R injury. Pharmacological conditioning is one of the most commonly preferred methods used to reduce the effects of I/R injury[15]. Various studies with different drugs for reducing renal I/R injury have been conducted. It has been reported that agents, including magnesium sulfate, N-acetylcysteine, activated protein C, captopril, insulin and dexmedetomidine, reduce renal I/R injury[16-21]. Magnesium sulfate ($MgSO_4$) has a potent anti-inflammatory effect and is a powerful antioxidant. It inhibits the endotoxin-dependent inflammatory molecule upregulation[22]. Magnesium is a L-type calcium channel blocker and L-type calcium channel blocking may be effective in preventing I/R injury by decreasing the calcium overload related to tissue damage[23]. There are limited experimental studies on the protective effect of $MgSO_4$ on renal I/R injury[17,20,24] but no study related to the effect of $MgSO_4$ on I/R injury in diabetic kidneys was found in our PubMed and EMBASE search.

The aim of this study was to evaluate the effects of $MgSO_4$ administration before ischemia (prophylactic) or after reperfusion (therapeutic) by using biochemical (BUN, Cr), histomorphological and immunohistochemical methods in a diabetic rat renal I/R injury model.

Materials and Methods

Animals

After approval of the Institutional Animal Experimentation Ethics Committee (Document Date: 16.06.2015, Protocol No: 27/2015), the study was conducted at the Multidisciplinary Laboratory of Animal Experiments in our institution. In total, 35 adult female Wistar albino rats weighing 216-249 g were used in this study. The animals were housed in a light controlled room (12 h light/dark cycle, 22 ± 1°C) and allowed free access to standard pellet diet and water. All experimental procedures and animal care methods were performed in accordance with local Animal Experimentation Ethics Committee rules and national guidelines.

Induction of Diabetes

Freshly prepared streptozotocin (STZ) was used to induce diabetes by a single intraperitoneal (i.p.) injection (45 mg/kg, [STZ, Sigma Chemical Co., St. Louis, MO, USA])[25]. Streptozotocin was prepared in a 0.1 M phosphate-citrate buffer (pH: 4.5) and an equal volume of buffer was injected i.p. into the non diabetic sham group. The induction of diabetes was controlled on the third day following STZ administration. Blood samples were taken from the tail vein and rats with random blood glucose > 250 mg/dL (13.9 mmol/L) on glucometer (Accu-Chek; Roche Diagnostics, Mannheim, Baden-Wurttemberg, Germany) were accepted as diabetic and included in the study. The rats were observed for 18 days (two weeks following diabetes inducement) in the laboratory and weight changes with blood glucose measurements (basal, third, 13th, 18th days) were recorded. The study was conducted on the 18th day of observation.

Experimental Design and Surgical Procedure

Rats were randomly divided into five groups: *Group I (sham, n=7),* nondiabetic sham operated group; *Group II (Diabetic sham, n=7),* diabetic sham operated group; *Group III (Diabetic IR*, n=7), diabetic I/R group; *Group IV (Diabetic IR + Mg-P, n=7),* diabetic I/R + prophylactic preischemic administration (200 mg/kg, i.p., 5 min before ischemia) of $MgSO_4$ (Magnesium sulfate, 1500 mg/ 10 ml amp, Biofarma, Turkey); *Group V (Diabetic IR + Mg-T),* diabetic I/R + therapeutic postischemic administration (200 mg/kg, i.p., 5 min after reperfusion) of $MgSO_4$.

The rats were anesthetized with ketamine (50 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.) and in order to maintain anesthetic depth supplemental ketamine (25 mg/kg, i.p.) was administered considering reflex responses. Following anesthesia, all rats were secured to the operation table in supine position and warmed with a heating lamp to maintain a rectal

body temperature of 37-37.5°C throughout the procedure. Laparotomy was performed with a midline abdominal incision and bilateral renal pedicles were carefully exposed. To prevent hypovolemia, isotonic saline solution (3 ml/kg, i.p.) was administered hourly and the abdomen was closed with a moist sterile pad during the reperfusion period. In the sham groups (Group I + Group II) bilateral renal pedicles were exposed without any intervention after laparotomy and rats were kept under anesthesia for an additional 285 min (ischemia + reperfusion duration) to standardize the anesthesia duration for all groups. In Groups III + IV + V, for I/R injury model, bilateral renal pedicle occlusion was performed with atraumatic microvascular clamps for 45 minutes. Adequate occlusion was confirmed by the lack of pulsation in renal pedicles and presence of pallor in the kidneys. This sustained ischemia model using same clamps was confirmed in our previous studies by using a laser current meter (Laser Flo BPM2, Vasamedic, St Paul, MN, USA)[9,26]. At the end of the ischemic period, the clamps were removed to start the reperfusion phase for 4 hours. In Group IV (IR + Mg-P), $MgSO_4$ (200 mg/kg, i.p.) was administered 5 min before renal ischemia (prophylactic) and then renal I/R (45 min ischemia + 4 h reperfusion) was induced in both kidneys. Different from Group IV, $MgSO_4$ (200 mg/kg, i.p.) was administered 5 min after reperfusion (therapeutic) in Group V (IR + Mg-T). At the end of reperfusion, the animals were anesthetized, blood samples were drawn from the right atrium for the measurement of biochemical parameters (BUN, Cr) and left kidneys were excised, then the rats were sacrificed by a cardiac puncture exsanguination. The kidneys were fixed in 10% buffered formaldehyde and embedded in paraffin wax for histomorphological examination.

Rats in need of resuscitation were excluded from the study.

Histomorphological Evaluation

Each kidney tissue was fixed with 10% formaldehyde. All histomorphological analyses described below were performed by two histologists blind to experimental groups. Kidney tissues were processed by routine histological methods and embedded in paraffin blocks. Paraffin blocks were placed in a rotary microtome (Leica RM 2135, Leica Instruments, Nussloch, Germany) with disposable metal microtome blades (Type S35, Feather Company, Osaka, Japan) to obtain serial coronal sections of 4-5 μm thickness. Three chosen transverse sections from each sample were stained with hematoxylin-eosin. Then the sections were examined under light microscopy (Olympus BX-51, Olympus, Tokyo, Japan) in terms of structural changes in proximal tubules (tubular atrophy, loss of tubular brush border, vacuolization, tubular dilatation, cast formation), mononuclear cell (MNC) infiltration, capillary dilatation, interstitial structural changes, renal corpuscle morphology, and necrotic/apoptotic cells. Histomorphological injury scoring was carried out using a semiquantitative method based on a scale of 0 to 4 as follows: 0 = None, 1 = 1-25%, 2 = 26-50%, 3 = 51-75% and 4 = 76-100%[21].

Immunohistochemical Methods (iNOS and Active Caspase-3)

Active caspase-3 and iNOS immunohistochemistry were applied to the paraffin sections. After deparaffinization and rehydration, sections were treated with 10 mM citrate buffer (Cat No.AP-9003-125 Labvision, Fremont, CA, USA) for five minutes. Then, sections were incubated in a solution of 3% H_2O_2 for 5 min to inhibit endogenous peroxidase activity. They were then incubated with blocking solution (Invitrogen, Histostain -Plus Kit Broad Spectrum, 85-9043). Then the sections were incubated overnight at 4°C with the specific primary antibody; anti-iNOS monoclonal (anti-iNOS mouse monoclonal Ab, Genetex, Irvine, CA, USA) and active caspase-3 (AB3623, Millipore, Temecula, CA, USA, and Polyclonal antibody) antibody. On the next day, sections were incubated with biotinylated IgG secondary antibodies and then with streptavidin for 30 min each prepared according to kit instructions (Invitrogen Plus Broad Spectrum, 85-9043). Sections were finally stained with DAB (3,3'-diaminobenzidine substrate) and counter-stained with mayer hematoxylin and examined using a light microscope (Olympus BX51, Olympus Optical Co. Ltd, Tokyo, Japan).

Semi-quantification of Immunostaining Data

A grading system was used to score the quantity of iNOS and active caspase-3 positive staining in the sections. The score was defined as the following: 0 = no immunoreactivity; 1 = remarkably a little positive staining; 2 = positive staining was moderate; 3 = strong positive staining

was evenly distributed in the whole image. To maintain consistency of scoring, each section was graded by two histologists blind to experimental groups.

Measurement of Biochemical Parameters

Serum creatinine and blood urea nitrogen (BUN) were used as indicators of impaired renal function. The blood samples collected 4 hours after reperfusion were placed in the refrigerator at 4°C for 20 min and centrifuged (6000 rpm for 3 min) to separate serum. These biochemical parameters (BUN, CR) were analyzed photometrically with an autoanalyzer (Beckman Olympus AU 5800, Brea, CA, USA) in our hospital central biochemistry laboratory.

Statistical Analysis

SPSS 15.0 (Statistical Package for the Social Sciences ver. 15, Chicago, IL, USA) was used for statistical analyses. A Kruskal-Wallis analysis of variance was performed to assess the differences among all groups. For univariate analysis, Mann-Whitney *U*-test was used for comparison of two independent groups. Wilcoxon Signed Rank was conducted in order to determine weight and blood glucose level changes over time within groups. All data are presented as mean ± standard deviation (mean ± SD) and median (minimum-maximum). A value of $p < 0.05$ was considered statistically significant.

Results

A total of 35 rats were included in the study. One diabetic rat died in the observation period before the experiment. One rat in the Diabetic IR group (Group III) died during the reperfusion period and was excluded from the study. Also, histomorphological evaluation could not be performed on one rat in the Mg prophylaxis group (Group IV) due to a technical problem. Thus, 33 biochemical and 32 histomorphological variables were analyzed for the study.

There was no significant difference between diabetic and nondiabetic rats in terms of basal weights ($p = 0.508$) and basal blood glucose levels ($p = 0.639$). Blood glucose levels increased significantly within observation period both in nondiabetic and diabetic groups, the latter being more distinct (resp., $p = 0.018$ and $p < 0.001$). In the nondiabetic group, mean weight increased significantly in the observation period, whereas in the diabetic group there was even an insignificant decrease (resp., $p = 0.018$ and $p = 0.207$) (Table I).

The histomorphological injury scores of all groups are presented in Table II. As a general histomorphological evaluation, total cell injury (TCI) scores of the Nondiabetic sham group were significantly lower than those in the Diabetic sham and Diabetic IR groups (resp., $p = 0.001$ and $p = 0.001$). Diabetic IR group TCI scores were significantly higher than the Diabetic sham group ($p = 0.019$). The TCI scores in Diabetic IR+Mg-P and Diabetic IR+Mg-T groups were significantly lower than the Diabetic IR group (resp., $p = 0.011$ and $p = 0.006$). There was no significant difference between the TCI scores of Diabetic IR+Mg-P and Diabetic IR+Mg-T groups ($p = 0.859$).

Mononuclear Cell Infiltration

The MNC infiltration scores of the Nondiabetic sham group were significantly lower than those in the Diabetic sham and Diabetic IR groups (resp., $p = 0.001$ and $p = 0.001$). The difference between the MNC scores of Diabetic

Table I. Blood glucose levels and weights of the rats.

	Blood Glucose (mg/dL)			Weight (g)		
	Basal	Day 18	p*	Basal	Day 18	p*
Nondiabetic rats (n = 7)	100.86 ± 4.56 (102.0)	154.86 ± 22.26 (154.0)	0.018	233.43 ± 9.03 (237.0)	257.29 ± 8.34 (260.0)	0.018
Diabetic rats (n = 27)	99.74 ± 7.24 (99.0)	408.04 ± 88.90 (402.0)	< 0.001	236.22 ± 8.28 (237.0)	230.59 ± 20.09 (230.0)	0.207
p**	0.639			0.508		

Data are presented as mean±SD and median; (*)Blood glucose and weight changes with time in nondiabetic and diabetic rats; Wilcoxon Signed Rank test; (**)Comparison of basal glucose and weight values for nondiabetic and diabetic rats; Mann-Whitney U test.

Table II. Histomorphological injury scores in groups.

Groups	MNC infiltration	Capillary vasodilatation	Proximal tubules injury	Total cell injury score
Group I (Nondiabetic sham)	0.0	0.14±0.38	0.0	0.0
(n = 7)	0.0	0.0	0.0	0.0
	0-0	0-1	0.0	0.0
Group II (Diabetic sham)	1.83 ± 0.75	1.33 ± 0.52	1.50 ± 0.55	1.50 ± 0.55
(n = 6)	2.0	1.0	1.5	1.5
	1-3	1-2	1-2	1-2
Group III (Diabetic IR)	2.33 ± 0.52	2.17 ± 0.75	2.33 ± 0.52	2.5 ± 0.55
(n = 6)	2.0	2.0	2	2.5
	2-3	1-3	2-3	2-3
Group IV (Diabetic IR+Mg-P)	1.50 ± 0.55	1.33 ± 0.52	1.50 ± 0.55	1.33 ± 0.52
(n = 6)	1.5	1.0	1.5	1.0
	1-2	1-2	1-2	1-2
Group V (Diabetic IR+Mg-T)	0.57 ± 0.53	1.29 ± 0.76	1.14 ± 0.69	1.29 ± 0.49
(n = 7)	1.0	1	1	1.0
	0-1	0-2	0-2	1-2
***p** values**				
$p_{1,2}$	0.001	0.003	0.001	0.001
$P_{1,3}$	0.001	0.002	0.001	0.001
$p_{1,4}$	0.001	0.003	0.001	0.001
$p_{1,5}$	0.023	0.008	0.003	0.001
$p_{2,3}$	0.206	0.057	0.030	0.019
$p_{2,4}$	0.423	0.999	0.999	0.575
$p_{2,5}$	0.010	0.999	0.335	0.447
$p_{3,4}$	0.030	0.057	0.030	0.011
$p_{3,5}$	0.002	0.067	0.009	0.006
$p_{4,5}$	0.018	0.999	0.335	0.859

Group I; Nondiabetic sham, Group II; Diabetic sham, Group III (Diabetic IR); Renal ischemia/reperfusion in diabetic rats, Group IV (Diabet IR+Mg-P); Renal ischemia/reperfusion diabetics rats with prophylactic Mg, Group V (Diabetic IR+Mg-T) Renal ischemia/reperfusion in diabetic rats with therapeutic Mg. Data are presented as mean ± SD, median, minimum and maximum. *Mann-Whitney U test. $p_{1,2}$: Comparison of Nondiabetic sham and Diabetic sham; $p_{1,3}$: Comparison of Nondiabetic sham and Diabetic IR; $p_{1,4}$: Comparison of Nondiabetic sham and Diabetic IR + Mg-P; $p_{1,5}$: Comparison of Nondiabetic sham and Diabetic IR+ Mg-T; $p_{2,3}$: Comparison of Diabetic sham and Diabetic IR; $p_{2,4}$: Comparison of Diabetic sham and Diabetic IR + Mg-P; $p_{2,5}$: Comparison of Diabetic sham and Diabetic IR + Mg-T; $p_{3,4}$: Comparison of Diabetic IR and Diabetic IR + Mg-P; $p_{3,5}$: Comparison of Diabetic IR and Diabetic IR + Mg-T; $p_{4,5}$: Comparison of Diabetic IR + Mg-P and Diabetic IR + Mg-T.

sham and Diabetic IR groups was not statistically significant ($p = 0.206$). The MNC infiltration scores in Diabetic IR+Mg-P and Diabetic IR+Mg-T groups were significantly lower than the Diabetic IR group (resp., $p = 0.030$ and $p = 0.002$). Also, Diabetic IR+Mg-T group scores were significantly lower than the Diabetic IR+Mg-P group ($p = 0.018$).

Capillary Vasodilatation

The capillary vasodilatation scores of the Nondiabetic sham group were significantly lower than those in the Diabetic sham and Diabetic IR groups (resp., $p = 0.003$ and $p = 0.002$). The difference between the capillary vasodilatation scores of Diabetic sham and Diabetic IR groups was not statistically significant ($p = 0.057$); however, the scores in Diabetic IR group were slightly higher. There were no significant differences between the capillary vasodilatation scores of Diabetic IR and Diabetic IR+Mg-P; Diabetic IR and Diabetic IR+Mg-T; and Diabetic IR+Mg-P and Diabetic IR+Mg-T groups (resp., $p = 0.057$, $p = 0.067$ and $p = 0.999$).

Proximal Tubules Injury

The proximal tubules injury (PTI) scores of the Nondiabetic sham group were significantly lower than those in the Diabetic sham and Diabetic IR groups (resp., $p = 0.001$ and $p = 0.001$). The PTI scores in the Diabetic IR group were significantly higher than the Diabetic sham group ($p = 0.030$). The scores in Diabetic IR+Mg-P and Diabetic IR+Mg-T groups were significantly lower than the Diabetic IR group (resp., $p = 0.030$ and $p = 0.009$). There was no significant difference be-

tween PTI scores of Diabetic IR+Mg-P and Diabetic IR+Mg-T groups ($p = 0.335$).

Histomorphological Examination

Nondiabetic sham group kidney sections showed a normal structural organization with normal proximal, distal tubules and glomerulus in the cortex. The brush border and the basal membranes were intact. There were no loss and irregularity noticed (Figure 1-A). In the Diabetic sham group, infiltration of MNCs, brush border loss in the proximal tubule cells, tubular atrophy,

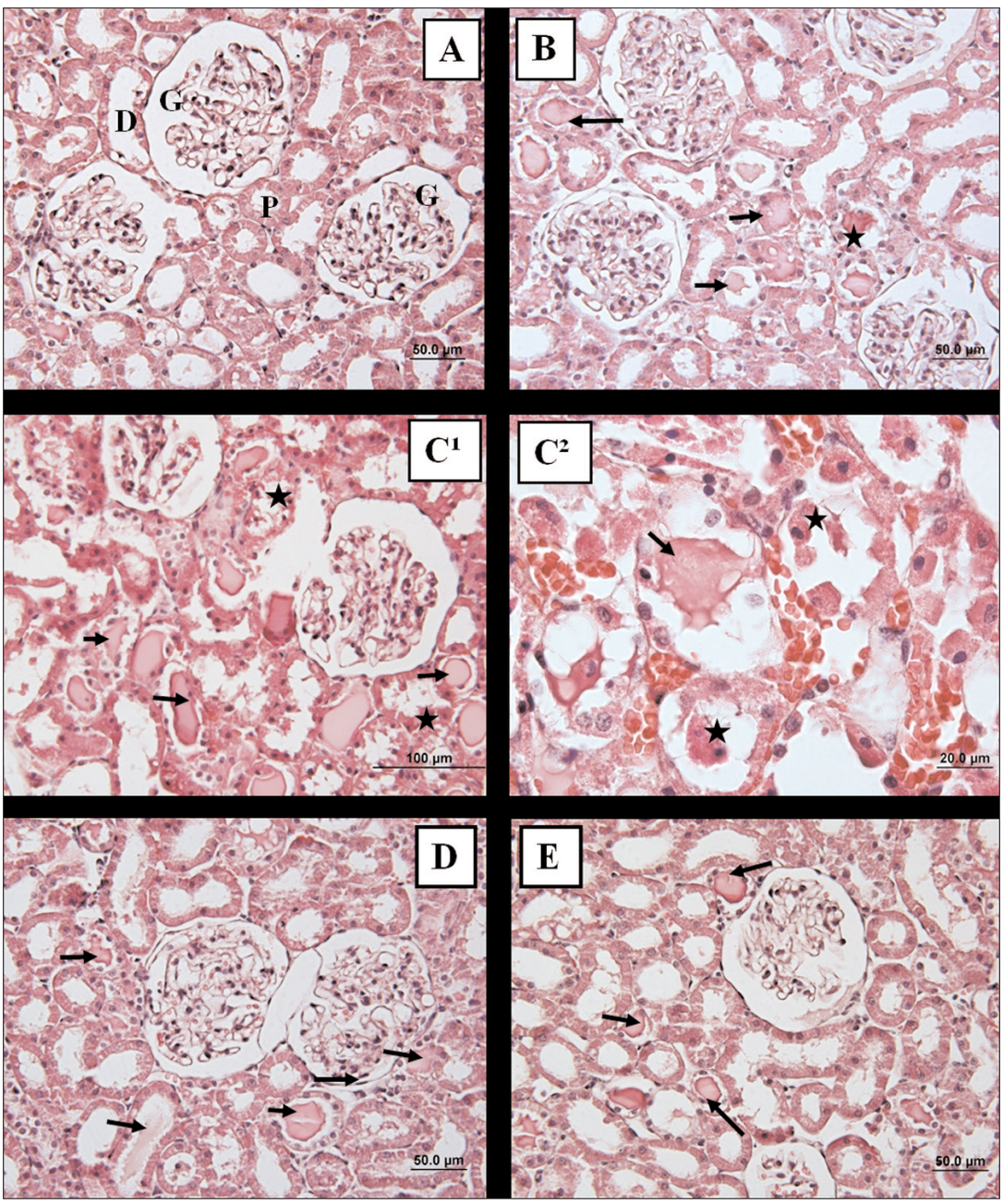

Figure 1. Representative light-microscopic images of hematoxylin-eosin stained kidney sections belong to; ***(A)*** Nondiabetic Sham group (×40), ***(B)*** Diabetic Sham group (×40), ***(C^{1,2})*** Diabetic ischemia-reperfusion (IR) group (resp., ×20 and ×100), ***(D)*** Diabetic IR+Mg-P group (×40) and ***(E)*** Diabetic IR+Mg-T group (×40). Hyalin substance deposition (the accumulation of proteinaceous material in tubules) is marked with a *black arrow* (→) and debris in the lumen of the proximal tubule is marked with a *black star* (★). In micrographs proximal tubules, distal tubules and renal glomerulus are shown with (P), (D), (G), respectively.

and tubular dilatation were observed in some areas. In addition, proteinaceous material deposition, rarely cell debris in tubule lumens and erythrocyte extravasations were observed in some tubules (Figure 1-B). In the Diabetic IR group, MNC infiltrations, erythrocyte extravasations, tubular atrophy, tubular dilatation, proteinaceous material deposition and cell debris in the tubule lumens were more prominent when compared with the Diabetic sham group (Figure 1-C[1,2]). In the Diabetic IR+Mg-P group, the histomorphologic changes, especially in MNC infiltration, tubule cells degeneration, proteinaceous material deposition and erythrocyte extravasations, decreased when compared with the Diabetic IR group (Figure 1-D). In the Diabetic IR+Mg-T group, similar histomorphological changes were observed as in the Diabetic IR+Mg-P group (Figure 1-E). In a histomorphologic comparison of Diabetic IR+Mg-T and Diabetic IR+Mg-P groups, MNC infiltration, the brush border loss in proximal tubule cells, proteinaceous material deposition, cast formation, and cell debris in the lumen of tubules were observed less in Diabetic IR+Mg-T group.

Immunohistochemical Evaluation (Active Caspase-3 and iNOS)

The immunohistochemical staining intensity scores of all groups are presented in Table III. In the Nondiabetic sham group, caspase-3 and iNOS immunostaining intensities were significantly lower than those in the Diabetic sham group (resp., $p = 0.011$ and $p = 0.009$). Immunostaining intensities of caspase-3 and iNOS were significantly lower in the Nondiabetic

Table III. Immunostaining intensity scores (i-NOS and active caspase-3) in groups.

Groups	Caspase	i-NOS
Group I (Nondiabetic Sham)	0.71 ± 0.49	0.57 ± 0.53
(n=7)	1.0	1.0
	0-1	0-1
Group II (Diabetic Sham)	1.67 ± 0.52	1.67 ± 0.52
(n = 6)	2.0	2.0
	1-2	1-2
Group III (Diabetic IR)	2.33 ± 0.52	2.33 ± 0.52
(n = 6)	2.0	2.0
	2-3	2-3
Group IV (Diabetic IR+Mg-P)	1.33 ± 0.52	1.5 ± 0.55
(n = 6)	1.0	1.5
	1-2	1-2
Group V (Diabetic IR+Mg-T)	1.43 ± 0.53	1.43 ± 0.53
(n = 7)	1.0	1.0
	1-2	1-2
***p** values**		
$p_{1,2}$	0.011	0.009
$p_{1,3}$	0.002	0.002
$p1,4$	0.054	0.018
$p_{1,5}$	0.030	0.018
$p_{2,3}$	0.056	0.056
$p_{2,4}$	0.269	0.575
$p_{2,5}$	0.409	0.409
$p_{3,4}$	0.014	0.030
$p_{3,5}$	0.018	0.018
$p_{4,5}$	0.014	0.030

Group I; Nondiabetic Sham, Group II; Diabetic Sham, Group III (Diabetic IR); Renal ischemia/reperfusion in diabetic rats, Group IV (Diabet IR+Mg-P); Renal ischemia/reperfusion diabetics rats with prophylactic Mg, Group V (Diabetic IR+Mg-T) Renal ischemia/reperfusion in diabetic rats with therapeutic Mg. Data are presented as mean ± SD, median, minimum and maximum. *Mann-Whitney U test. $p_{1,2}$: Comparison of Nondiabetic Sham and Diabetic sham; $p_{1,3}$: Comparison of Nondiabetic Sham and Diabetic IR; $p_{1,4}$: Comparison of Nondiabetic Sham and Diabetic IR + Mg-P; $p_{1,5}$: Comparison of Nondiabetic Sham and Diabetic IR+ Mg-T; $p_{2,3}$: Comparison of Diabetic sham and Diabetic IR; $p_{2,4}$: Comparison of Diabetic Sham and Diabetic IR + Mg-P; $p_{2,5}$: Comparison of Diabetic Sham and Diabetic IR + Mg-T; $p_{3,4}$: Comparison of Diabetic IR and Diabetic IR + Mg-P; $p_{3,5}$: Comparison of Diabetic IR and Diabetic IR + Mg-T; $p_{4,5}$: Comparison of Diabetic IR + Mg-P and Diabetic IR + Mg-T.

sham group compared with the Diabetic IR group (resp., $p = 0.002$ and $p = 0.002$). There were no significant differences in immunostaining intensities between the Diabetic sham and Diabetic IR groups (for both, $p = 0.056$). In the Diabetic IR+Mg-P group, caspase-3 and iNOS immunostaining intensities were significantly lower than in the Diabetic IR group (resp., $p = 0.014$ and $p = 0.030$). Similarly, immunostaining intensities of the Diabetic IR+Mg-T group were significantly lower than in the Diabetic IR group (for both, $p = 0.018$). Caspase-3 staining intensity of the Diabetic IR+Mg-P group was significantly lower than in the Diabetic IR+Mg-T group ($p = 0.014$); however, iNOS staining intensity of the Diabetic IR+Mg-P group was significantly higher than in the Diabetic IR+Mg-T group ($p = 0.030$).

Nondiabetic sham kidneys were nearly negative for active caspase-3 and iNOS immunostaining in renal tubular structures other than very small numbers of immunpositive proximal tubule epithelial cells (Figure 2-A, Figure 3-A). In contrast, active caspase-3 and iNOS were expressed highly in the Diabetic sham and Diabetic IR groups in several proximal and distal tubules; however, immunostaining intensities were more prominent in the Diabetic IR group (Figure 2-B, $C^{1,2}$; Figure 3-B, $C^{1,2}$). Compared with the Diabetic IR group, the Diabetic IR+Mg-P and Diabetic IR+Mg-T groups showed less immunostaining intensities for both active caspase-3 and iNOS antibodies (Figure 2-D, E; Figure 3-D, E).

Biochemical Parameters

The biochemical evaluation of all groups is presented in Table IV. The BUN values of the Nondiabetic sham group were significantly lower than those of the Diabetic sham and Diabetic IR groups (resp., $p = 0.032$ and $p = 0.010$). The Nondiabetic sham group had significantly lower Cr values than the Diabetic IR group ($p = 0.022$), but there was no significant difference between the Nondiabetic sham and Diabetic sham groups ($p = 0.774$). In means of BUN and Cr levels, there were significant differences between the Diabetic sham and Diabetic IR groups (resp., $p = 0.016$ and $p = 0.004$). The Diabetic IR+Mg-P and Diabetic IR+Mg-T groups had no significant differences compared to the Diabetic IR group in means of BUN and Cr values (resp. for BUN; $p = 0.999$, $p = 0.999$, for Cr; $p = 0.283$, $p = 0.391$).

Discussion

In this experimental study, the effects of magnesium sulfate administration before ischemia (prophylactic) or following reperfusion (therapeutic) were investigated in a diabetic rat renal I/R model and both methods were significantly found to reduce I/R injury according to histomorphological and immunohistochemical results. However, this protective effect of $MgSO_4$ was not significantly determined by using biochemical parameters (BUN, Cr).

Renal I/R injury is a major cause of acute renal failure and may occur during several clinical conditions[3-5]. Diabetes mellitus is defined as a risk factor for the development of acute renal injury, as diabetic nephropathy compromises the renal tolerance to I/R injury. Glomerular lesions, infiltration of inflammatory cells, tubular atrophy, and interstitial fibrosis are the common histopathologic features of the diabetic nephropathy. Ischemia/reperfusion injury in a diabetic kidney results in a severe inflammatory response[14,16]. Increased susceptibility to renal I/R injury has been reported in several studies with diabetic rat models[27,28]. As DM is a common disease with an increasing prevalence, preventing renal I/R injury in diabetic patients by investigating possible protective and therapeutic strategies is clinically important.

In our experimental diabetes model, a single dose of STZ (45 mg/kg, i.p.) was used to induce DM in rats and a random blood glucose level of > 250 mg/dL (13.9 mmol/L) was accepted as diabetic[19,25,26,29]. There were no significant differences between diabetic and nondiabetic rats in terms of basal weights and basal blood glucose levels. Although different observation durations following the induction of diabetes were reported in the rat models[12,19,27], the rats were observed for 15 days following STZ administration to develop diabetic nephropathy[14,16]. In our study; all histomorphological injury scores, immunostaining intensities (for both caspase-3 and iNOS) and BUN values were significantly higher in the Diabetic sham group than in the Nondiabetic sham group. These results suggest that diabetic nephropathy was successfully induced in diabetic rats.

Renal IR injury is a complex inflammatory phenomenon that begins during ischemia and progresses with the reperfusion of ischaemic renal tissue that initiates a complex series of cellular events. Both inflammation and apoptosis develop in renal IR injury. Inflammatory cell infil-

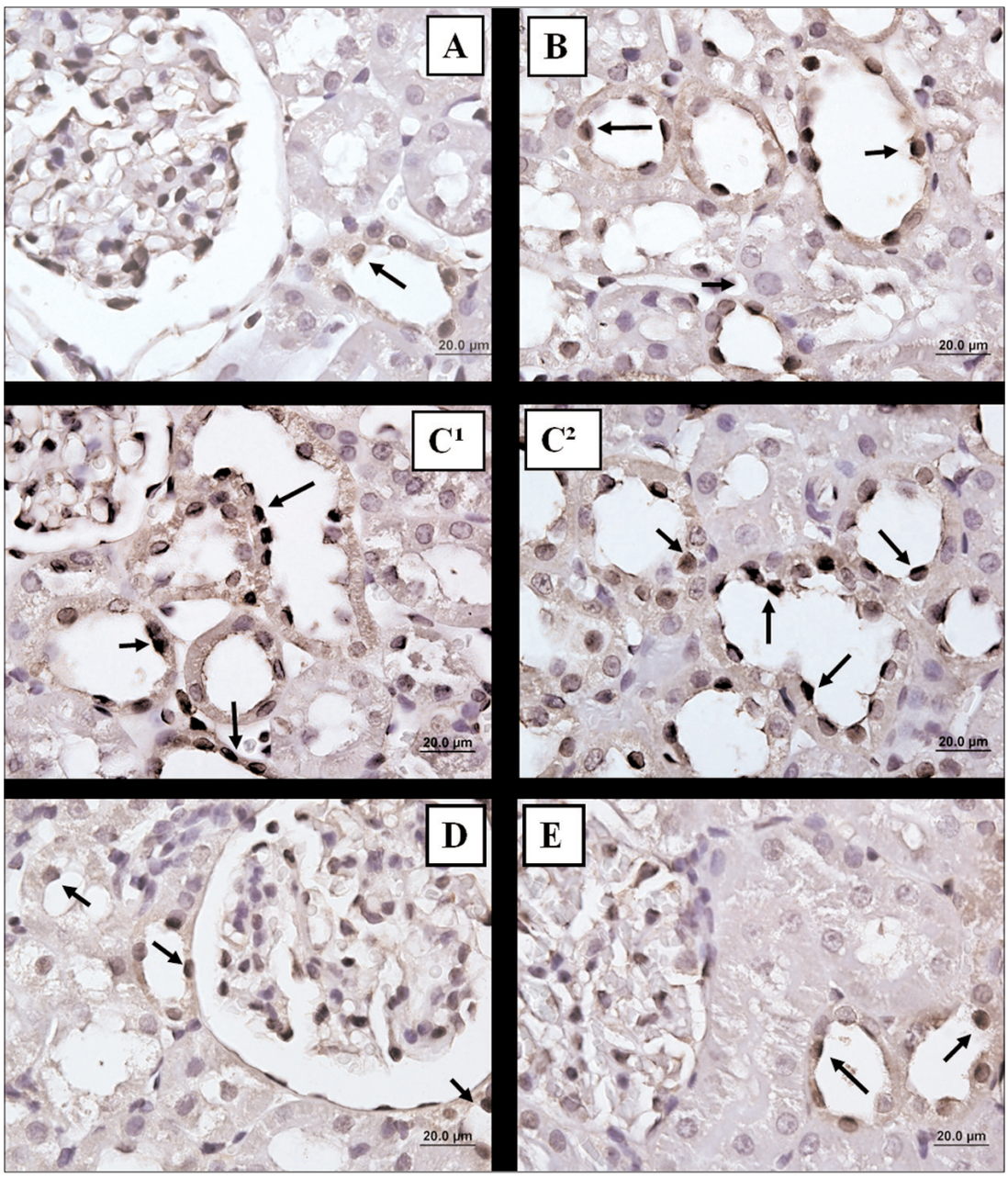

Figure 2. ***A,*** Representative light-microscopic images of anti-active caspase-3 immunohistochemically stained proximal and distal tubule cells in the kidney sections; ***(A)*** Nondiabetic Sham group, ***(B)*** Diabetic Sham group, ***(C¹,²)*** Diabetic ischemia-reperfusion (Diabetic IR) group, ***(D)*** Diabetic IR + Mg-P group and ***(E)*** Diabetic IR + Mg-T group. The active caspase-3 immune positive cells were observed in different quantities for all groups in the proximal and distal tubule epithelial cells. The positive immunostained cells are marked with *black arrow* (→) in sections ***A, B, C, D*** and ***E*** (Original magnification ×100).

tration, increased microvascular permeability, interstitial edema, parenchymal cell dysfunction, interstitial/tubular structural changes, apoptosis and acute tubular necrosis have been shown histopathologically in various experimental studies of renal I/R injury[26,30-32]. Besides, apoptosis and ischemic cell injury could be demonstrated by different immunohistochemical methods including caspase-3 and iNOS immunoreactivity[8-12]. Caspase-3 and iNOS become activated in ischemic tissues and are indicators of cell death. These changes can occur during the initial 24 h follow-

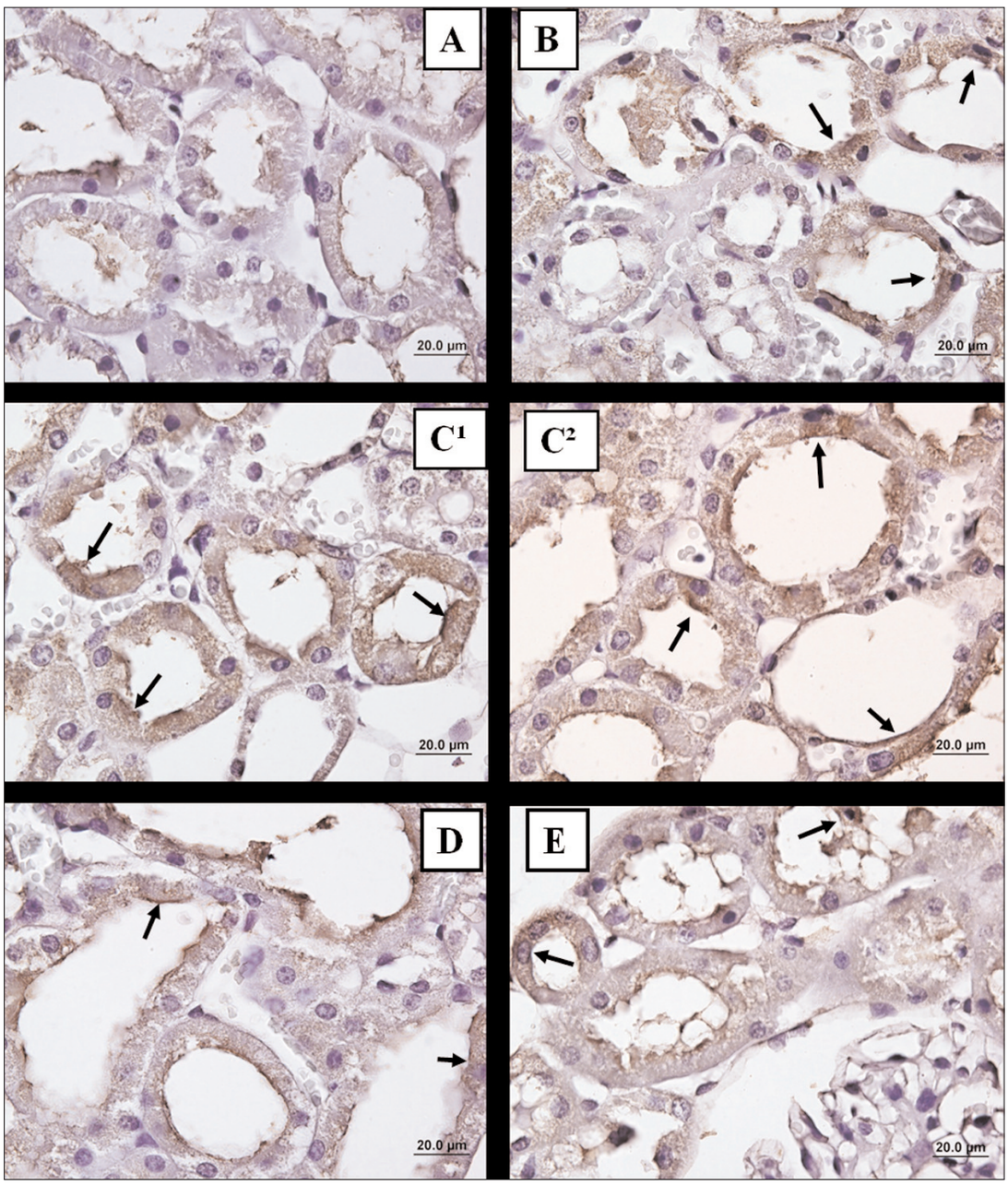

Figure 3. Representative light-microscopic images of anti-iNOS immunohistochemically stained proximal and distal tubule cells in the kidney sections in; ***(A)*** Nondiabetic Sham group, ***(B)*** Diabetic Sham group, ***(C¹,²)*** Diabetic ischemia-reperfusion (Diabetic IR) group, ***(D)*** Diabetic IR + Mg-P group and ***(E)*** Diabetic IR + Mg-T group. The iNOS immune positive cells were observed in different quantities for all groups in the proximal and distal tubule epithelial cells. The positive immunostained cells are marked with *black arrow* (→) in sections ***A, B, C, D*** and ***E*** (Original magnification ×100).

ing reperfusion and continue progressively correlated with high levels of BUN and serum creatinine[14,26,32,33]. In the present study, histomorphological, immunohistochemical (caspase-3, iNOS) and biochemical (BUN, Cr) methods were used to evaluate the effect of magnesium sulfate on renal I/R injury.

Renal I/R injury can be induced in experimental animal models using two different methods; either by unilateral nephrectomy and clamping the contralateral renal pedicle/artery or by clamping the bilateral renal pedicles/arteries. The duration of renal ischemia is important and generally varies between 30 and 60 min in several studies[14,23,26,34]. Al-

Table IV. Biochemical values of the groups.

Groups	BUN (mg/dL)	Cr (mg/dL)
Group I (Nondiabetic Sham)	33.39 ± 17.74	0.550.43
(n=7)	28.80	0.40
	18.70-71.30	0.32-1.52
Group II (Diabetic Sham)	47.57 ± 12.26	0.43 ± 0.67
(n=6)	42.10	0.43
	38.50-70.10	0.35-0.52
Group III (Diabetic IR)	72.05 ± 10.11	1.45 ± 0.20
(n=6)	72.55	1.37
	56.6-86.0	1.28-1.84
Group IV (Diabetic IR+Mg-P)	72.26 ± 8.70	1.30 ± 0.24
(n=7)	69.5	1.28
	61.0-88.5	0.94-1.70
Group V (Diabetic IR+Mg-T)	66.73 ± 20.49	1.21 ± 0.52
(n=7)	70.40	1.21
	24.1-87.3	0.35-2.01
***p** values**		
$p_{1,2}$	0.032	0.774
$p_{1,3}$	0.010	0.022
$p_{1,4}$	0.009	0.018
$p_{1,5}$	0.035	0.073
$p_{2,3}$	0.016	0.004
$p_{2,4}$	0.015	0.003
$p_{2,5}$	0.063	0.027
$p_{3,4}$	0.001	0.283
$p_{3,5}$	0.001	0.391
$p_{4,5}$	0.949	0.848

Group I; Nondiabetic Sham, Group II; Diabetic Sham, Group III (Diabetic IR); Renal ischemia/reperfusion in diabetic rats, Group IV (Diabet IR+Mg-P); Renal ischemia/reperfusion diabetics rats with prophylactic Mg, Group V (Diabetic IR+Mg-T) Renal ischemia/reperfusion in diabetic rats with therapeutic Mg. Data are presented as mean ± SD, median, minimum and maximum. *Mann-Whitney U test. $p_{1,2}$: Comparison of Nondiabetic Sham and Diabetic sham; $p_{1,3}$: Comparison of Nondiabetic Sham and Diabetic IR; $p_{1,4}$: Comparison of Nondiabetic Sham and Diabetic IR + Mg-P; $p_{1,5}$: Comparison of Nondiabetic Sham and Diabetic IR+ Mg-T; $p_{2,3}$: Comparison of Diabetic sham and Diabetic IR; $p_{2,4}$: Comparison of Diabetic Sham and Diabetic IR + Mg-P; $p_{2,5}$: Comparison of Diabetic Sham and Diabetic IR + Mg-T; $p_{3,4}$: Comparison of Diabetic IR and Diabetic IR + Mg-P; $p_{3,5}$: Comparison of Diabetic IR and Diabetic IR + Mg-T; $p_{4,5}$: Comparison of Diabetic IR + Mg-P and Diabetic IR + Mg-T.

so, different reperfusion durations were used in rat renal I/R injury models in previous studies[12,14,32,34]. Williams et al[32] reported that following 45 min of renal ischemia, IR injury occurred earliest at the 4th h and peaked at the 24th h of reperfusion in nondiabetic rats. However, Melin et al[14] demonstrated that early signs of ischemic injury could be observed in the renal medulla after 2 h of reperfusion in diabetic rats. In this study, bilateral renal pedicles were clamped to induce ischemia for 45 min followed by reperfusion for 4 h. Histomorphological injury scores (total cell injury and proximal tubulus scores) and biochemical values (for both BUN and Cr) were significantly higher in the Diabetic IR group than in the Diabetic sham group. Although there was a remarkable increase in the immunostaining intensities (for both caspase-3 and iNOS) in the Diabetic IR group, the difference was not significant (p=.056). In contrast with the results of some studies[21], significant increases determined in BUN and Cr values in an early postischemic period (4th hour of reperfusion) suggest that these biochemical markers can be reliable in acute renal injury[32]. These findings demonstrate that renal I/R injury was successfully induced in diabetic rat groups.

Pharmacological conditioning is one of the most commonly preferred methods used to reduce the effects of I/R injury and may be used as a prophylactic (preconditioning) and/or therapeutic (postconditioning) method[15]. Magnesium sulfate is a noncompetitive antagonist of *N*-methyl-D-aspartate (NMDA) receptor and renal I/R injury leads to NMDA receptor activation or up-

regulation in damaged kidneys[34,35]. It rearranges microcirculation during reperfusion, increases the total blood flow to the organ, and reduces edema[36]. Magnesium plays a fundamental role in many aspects of cellular functions besides the noncompetitive NMDA receptor and endogenous Ca^{2+} channel blockage. It may cause vasodilatation by stimulating endothelial prostacyclin release, and decrease I/R injuries by directly inhibiting lipid peroxidation[37]. The protective effect of $MgSO_4$ on I/R injury of different organs was investigated in several experimental models, but the results are inconsistent according to several factors including the dose or administration route. Kao et al[22] showed that $MgSO_4$ (50, 100 mg/kg, i.v.) administered just after reperfusion reduced lung I/R injury, and Kaplan et al[36] established that $MgSO_4$ (35 mg/kg/h, i.v.) administered before and following the ischemia as continuous infusion mitigated spinal I/R injury. In contrast, Hwang et al[37] reported that $MgSO_4$ (30, 100, 300 mg/kg, i.v.) administered before ischemic insult did not prevent spinal I/R injury, and Gormus et al[38] showed that $MgSO_4$ (5 mg/kg, i.v.) had no protective effect on muscle I/R injury. Similarly, the results of the clinical studies about the effect of $MgSO_4$ on I/R injury are inconsistent, as positive[35,39-41] and negative[42,43] outcomes were reported in the literature.

There are limited experimental studies on the protective effect of $MgSO_4$ on renal I/R injury. Xiao et al[24] administered $MgSO_4$ (25 mg/kg/h, i.v.) before and following the ischemia as a continuous infusion in a rat renal I/R injury model and reported that $MgSO_4$ reduced renal I/R injury. In another study, de Araujo et al[17] investigated the effects of magnesium on renal IR injury and established that although magnesium had a protective effect on renal functions (GFR), it could not prevent tubular damage. Besides, the authors determined higher histomorphological vasodilatation scores in rats treated with magnesium and attributed this to the vasodilator effect of magnesium. Pundir et al[20] administered $MgSO_4$ (600 mg/kg, i.p.) for five days before ischemic insult and conducted a rat renal I/R study with 40 min ischemia/24 h reperfusion periods. They showed that $MgSO_4$ reduced renal I/R injury according to biochemical (BUN, Cr) and histomorphological assessment. In addition, Pundir et al[20] established that the protective effect of $MgSO_4$ was related to its antagonistic action on NMDA receptors, consistent with the results of several studies[34,35].

To our best knowledge, the effect of $MgSO_4$ on renal I/R injury in diabetic kidney models has not been investigated previously. In the present study, $MgSO_4$ was administered before (prophylactic) or following ischemic insult (therapeutic) as it can potentially be used in clinical practice. Histomorphological injury scores (total cell injury, MNC infiltration and proximal tubulus scores) and immunostaining intensities (for both caspase-3 and iNOS) were significantly lower in the both $MgSO_4$ administered groups (Mg-P and Mg-T) than the Diabetic IR group. We could not detect a significant difference in capillary vasodilatation scores between the $MgSO_4$ administered groups and the Diabetic IR group that might be related to the vasodilator effect of $MgSO_4$. In contrast with the study of Pundir et al[20] in which they used a higher $MgSO_4$ dose and a longer reperfusion period, significant differences were not found in biochemical parameters (BUN, Cr) between the $MgSO_4$ administered groups and the Diabetic IR group in our study. In a comparison of the two administration methods (prophylactic or therapeutic), the iNOS immunostaining intensity and MNC infiltration scores were significantly lower in the therapeutic (Mg-T) group, but caspase-3 immunostaining intensity was significantly lower in the prophylactic (Mg-P) group. Thus, we can assume that therapeutic administration of $MgSO_4$ was slightly superior to prophylactic administration in this renal I/R injury model.

This study has some limitations. Reperfusion period might be longer as 24 h/48 h to determine the late histomorphological and biochemical changes. In addition, we did not perform Western blotting or quantitative polymerase chain reaction analyses to confirm the immunohistochemical methods.

Conclusions

Our histomorphological and immunohistochemical results established that preishemic (prophylactic) or postischemic (therapeutic) administration of $MgSO_4$ reduced renal I/R injury in this diabetic rat model. As $MgSO_4$ is a highly safe drug which has been commonly used in clinical practice for many years, we suggest conducting further clinical studies to determine the effective dose and administration methods of $MgSO_4$ in patients with diabetic nephropathy.

Declaration of Interest

The authors have no conflicts of interest to declare and there were no sources of funding that could have influenced the outcome of this study.

References

1) Legrand M, Mik EG, Johannes T, Payen D, Ince C. Renal Hypoxia and dysoxia after reperfusion of the ischemic kidney. Mol Med 2008; 14: 502-516.
2) Perico N, Cattaneo D, Sayegh MH, Remuzzi G. Delayed graft function in kidney transplantation. Lancet 2004; 364: 1814-1827.
3) Nigwekar SU, Kandula P, Hix JK, Thakar CV. Off-pump coronary artery bypass surgery and acute kidney injury: a meta-analysis of randomized and observational studies. Am J Kidney Dis 2009; 54: 413-423.
4) Mehta RL, Pascual MT, Soroko S, Savage BR, Himmelfarb J, Ikizler TA, Paganini EP, Chertow GM. Spectrum of acute renal failure in the intensive care unit: the PICARD experience. Kidney Int 2004; 66: 1613-1621.
5) Desai MM, Gill IS, Ramani AP, Spaliviero M, Rybicki L, Kaouk JH. The impact of warm ischemia on renal function after laparoscopic partial nephrectomy. BJU Int 2005; 95: 377-383.
6) Collard CD, Gelman S. Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. Anesthesiology 2001; 94: 1133-1138.
7) Tsutsui H, Sugiura T, Hayashi K, Ohkita M, Takaoka M, Yukimura T, Matsumura Y. Moxonidine prevents ischemia/reperfusion-induced renal injury in rats. Eur J Pharmacol 2009; 603: 73-78.
8) Faubel S, Edelstein CL. Caspases as drug targets in ischemic organ injury. Curr Drug Targets Immune Endocr Metabol Disord 2005; 5: 269-287.
9) Bagcik E, Ozkardesler S, Boztas N, Ergur BU, Akan M, Guneli M, Ozbilgin S. Effects of dexmedetomidine in conjunction with remote ischemic preconditioning on renal ischemia-reperfusion injury in rats. Rev Bras Anestesiol 2014; 64: 382-390.
10) Viñas JL, Sola A, Genescà M, Alfaro V, Pí F, Hotter G. NO and NOS isoforms in the development of apoptosis in renal ischemia/reperfusion. Free Radic Biol Med 2006; 40: 992-1003.
11) Villanueva S, Escobar P, Jacubovsky I, Irarrázabal C, Carreño JE, Erpel JM, Céspedes C, González AA, Vio CP, Velarde V. Cyclooxygenase-2 and hypoxia-regulated proteins are modulated by basic fibroblast growth factor in acute renal failure. Biol Res 2012; 45: 51-60.
12) Abu-Saleh N, Awad H, Khamaisi M, Armaly Z, Karram T, Heyman SN, Kaballa A, Ichimura T, Holman J, Abassi Z. Nephroprotective effects of TVP1022, a non-MAO inhibitor S-isomer of rasagiline, in an experimental model of diabetic renal ischemic injury. Am J Physiol Renal Physiol 2014; 306: F24-33.
13) Oldfield MD, Cooper ME. The biochemistry and pathophysiology of renal lesions in type 2 diabetes. In: C.E. Mogensen, Ed., Diabetic Nephropathy in Type 2 Diyabetes. Science Press, London, UK, 1st edition, 2002; pp. 41-56.
14) Melin J, Hellberg O, Funa K, Hällgren R, Larsson E, Fellström BC. Ischemia-induced renal expression of hyaluronan and CD44 in diabetic rats. Nephron Exp Nephrol 2006; 103: e86-94.
15) Uysal AI, Ocmen E, Akan M, Ozkardesler S, Ergur BU, Guneli E, Kume T, Koca U, Unal Togrul B. The effects of remote ischemic preconditioning and N-acetylcysteine with remote ischemic preconditioning in rat hepatic ischemia reperfusion injury model. Biomed Res Int 2014; 2014: 892704.
16) Melin J, Hellberg O, Larsson E, Zezina L, Fellström BC. Protective effect of insulin on ischemic renal injury in diabetes mellitus. Kidney Int 2002; 61: 1383-1392.
17) de Araujo M, Andrade L, Coimbra TM. Magnesium supplementation combined with N-acetylcysteine protects against postischemic acute renal failure. J Am Soc Nephro 2005; 16: 3339-3349.
18) Mizutani A, Okajima K, Uchiba M, Noguchi T. Activated protein C reduces ischemia/reperfusion-induced renal injury in rats by inhibiting leukocyte activation. Blood 2000; 95: 3781-3787.
19) Fouad AA, Al-Mulhim AS, Jresat I, Morsy MA. Protective effects of captopril in diabetic rats exposed to ischemia/reperfusion renal injury. J Pharm Pharmacol 2013; 65: 243-252.
20) Pundir M, Arora S, Kaur T, Singh R, Singh AP. Effect of modulating the allosteric sites of N-methyl-D-aspartate receptors in ischemia-reperfusion induced acute kidney injury. J Surg Res 2013; 183: 668-677.
21) Si YN, Bao HG, Xu L, Wang XL, Shen Y, Wang JS, Yang XB. Dexmedetomidine protects against ischemia/reperfusion injury in rat kidney. Eur Rev Med Pharmacol Sci 2014; 18: 1843-1851.
22) Kao MC, Jan WC, Tsai PS, Wang TY, Huang CJ. Magnesium sulfate mitigates lung injury induced by bilateral lower limb ischemia-reperfusion in rats. J Surg Res 2011; 171: e97-e106.
23) Lee CY, Jan WC, Tsai PS, Huang CJ. Magnesium sulfate mitigates acute lung injury in endotoxemia rats. J Trauma 2011; 70: 1177-1185.
24) Xiao B, Ma LL, Xiao CL, Lu M, Xu W, Huang Y, Zhang SD, Hou XF. Protective effect of heat shock protein 70 and magnesium sulfate supplementation on renal ischemia reperfusion injury. Beijing Da Xue Xue Bao 2011; 43: 525-530.
25) Guneli E, Tugyan K, Ozturk H, Gumustekin M, Cilaker S, Uysal N. Effect of melatonin on testicular damage in streptozotocin-induced diabetes rats. Eur Surg Res 2008; 40: 354-360.
26) Ozbilgin S, Ozkardesler S, Akan M, Boztas N, Ozbilgin M, Ergur BU, Derici S, Guneli ME, Meseri R. Renal ischemia/reperfusion injury in diabetic rats: the role of local ischemic preconditioning. Biomed Res Int 2016; 2016: 8580475.

27) Zhou SP, Liao WT, Yang LK, Sun L. Effects of sevoflurane pretreatment on renal Src and FAK expression in diabetic rats after renal ischemia/reperfusion injury. Mol Cell Biochem 2013; 384: 203-211.

28) Shi H, Patschan D, Epstein T, Goligorsky MS, Winaver J. Delayed recovery of renal regional blood flow in diabetic mice subjected to acute ischemic kidney injury. Am J Physiol Renal Physiol 2007; 293: F1512-F1517.

29) Micili SC, Ergur BU, Ozo ul C, Sario lu S, Ba riyanik HA, Tu yan K, Pekcetin C, Sonmez U, Tekmen I, Erbil G, Akdo an GG, Ozyurt D, Buldan Z. Effects of lipoic acid in an experimentally induced hypertensive and diabetic rat model. Clin Exp Hypertens 2013; 35: 373-381.

30) Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. J Pathol 2000; 190: 255-266.

31) Kocoglu H, Ozturk H, Ozturk H, Yilmaz F, Gulcu N. Effect of deksmedetomidine on ischemia-reperfusion injury in rat kidney a histopathologic study. Ren Fail 2009; 31: 70-74.

32) Williams P, Lopez H, Britt D, Chan C, Ezrin A, Hottendorf R. Characterization of renal ischemia-reperfusion injury in rats. J Pharmacol Toxicol Methods 1997; 37: 1-7.

33) Billings FT, Chen SW, Kim M, Park SW, Song JH, Wang S, Herman J, D'Agati V, Lee HT. Alpha-2 adrenergic agonists protect against radiocontrast-induced nephropathy in mice. Am J Physiol Renal Physiol 2008; 295: 741-748.

34) Yang CC, Chien CT, Wu MH, Ma MC, Chen CF. NMDA receptor blocker ameliorates ischemia-reperfusion-induced renal dysfunction in rat kidneys. Am J Physiol Renal Physiol 2008; 294: F1433-1440.

35) Arora S, Kaur T, Kaur A, Singh AP. Glycine aggravates ischemia reperfusion-induced acute kidney injury through N-Methyl-D-Aspartate receptor activation in rats. Mol Cell Biochem 2014; 393: 123-131.

36) Kaplan S, Ulus AT, Tütün U, Aksöyek A, Ozgencil E, Saritas Z, Apaydin N, Pamuk K, Can Z, Surucu S, Katircioglu SF. Effect of Mg2SO4 usage on spinal cord ischemia-reperfusion injury: electron microscopic and functional evaluation. Eur Surg Res 2004; 36: 20-25.

37) Hwang J, Kim J, Park S, Cho S, Park S, Han S. Magnesium sulfate does not protect spinal cord against ischemic injury. J Invest Surg 2011; 24: 250-256.

38) Gormus ZI, Ergene N, Toy H, Baltaci AK, Mogulkoc R. Preventive role of magnesium on skeletal muscle ischemia-reperfusion injury-an experimental study. Biol Trace Elem Res 2009; 127: 183-189.

39) Kim JE, Jeon JP, No HC, Choi JH, Lee SH, Ryu KH, Kim ES. The effects of magnesium pretreatment on reperfusion injury during living donor liver transplantation. Korean J Anesthesiol 2011; 60: 408-415.

40) Lampl Y, Gilad R, Geva D, Eshel Y, Sadeh M. Intravenous administration of magnesium sulfate in acute stroke: a randomized double-blind study. Clin Neuropharmacol 2001; 24: 11-15.

41) Westermaier T, Stetter C, Vince GH, Pham M, Tejon JP, Eriskat J, Kunze E, Matthies C, Ernestus RI, Solymosi L, Roosen K. Prophylactic intravenous magnesium sulfate for treatment of aneurysmal subarachnoid hemorrhage: a randomized, placebo-controlled, clinical study. Crit Care Med 2010; 38: 1284-1290.

42) Singh H, Jalodia S, Gupta MS, Talapatra P, Gupta V, Singh I. Role of magnesium sulfate in neuroprotection in acute ischemic stroke. Ann Indian Acad Neurol 2012; 15: 177-180.

43) Saver JL, Starkman S, Eckstein M, Stratton SJ, Pratt FD, Hamilton S, Conwit R, Liebeskind DS, Sung G, Kramer I, Moreau G, Goldweber R, Sanossian N. Prehospital use of magnesium sulfate as neuroprotection in acute stroke. N Engl J Med 2015; 372: 528-536.