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Effects of trapidil on renal ischemia-reperfusion injury

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Abstract There is increasing evidence to suggest that reactive oxygen and nitrogen species play a role in the pathogenesis of renal ischemia-reperfusion (I/R) injury. This study was designed to determine the possible protective effects of trapidil treatment against oxidative and nitrosative tissue injury of kidney induced by I/R.

A renal I/R injury was induced by a left renal pedicle occlusion by ischemia for 45 minutes, followed by 1 hour of reperfusion with contralateral nephrectomy in I/R and I/R + trapidil groups. Trapidil (8 mg/kg intravenously) was administrated immediately before reperfusion phase. At the end of the reperfusion period, rats were killed. Then, renal tissue samples were taken for biochemical analysis and histopathological evaluation, and blood samples were obtained to determinate serum urea, aspartate aminotransferase (AST), and tumor necrosis factor–α (TNF-α) levels. Ischemia-reperfusion injury caused significant increases in myeloperoxidase activity and malondialdehyde and 3-nitrotyrozine levels in renal tissue and elevated serum urea, AST, and TNF-α levels. In addition, severe deterioration of renal morphology was seen in the I/R group. Trapidil treatment significantly reduced in biochemical parameters, as well as serum urea, AST, and TNF-α levels. Furthermore, renal tissue injury was markedly attenuated with trapidil treatment. These data suggest that *reactive oxygen species* and *reactive nitrogen species* play a causal role in I/R-induced renal tissue, and trapidil has a renoprotective effect against oxidative and nitrosative kidney damage.

Ischemia-reperfusion (I/R)–induced acute renal failure (ARF), occurring in many settings including shock, vascular surgery, and especially renal transplantation, is associated with a high mortality and morbidity rate. Subsequent to renal transplantation, I/R injury is a major cause of early graft rejection and adversely affects the long-term survival of transplanted kidney. The mechanisms underlying renal I/R injury are complex. Renal ischemia initiates a complex and interrelated sequence of events, resulting in the injury and death of renal cells. Although reperfusion is essential for the survival of ischemic renal tissue, it causes additional damage contributing to the renal dysfunction and injury associated with I/R of the kidney [1,2]. Especially, the cells of the proximal tubular epithelial is susceptible to I/R injury, leading to acute tubuler necrosis, which plays a pivotal part in the pathogenesis of ARF [3].

The pathophysiological mechanisms that cause the reperfusion injury of the kidney include adhesion and activation of polymorphonuclear leukocytes, the release of

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proinflammatory cytokines such as tumor necrosis factor–α (TNF-α), interleukin (IL)–1, as well as the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydroxyl radical, superoxide anion, hydrogen peroxide, nitric oxide (NO) and peroxynitrite ($ONOO^-$) [1,4,5]. It has been shown that I/R injury associated with lipid peroxidation, mediated by ROS, is believed to be an important cause of oxidative damage to cellular membranes and, eventually, cell death [6].

Furthermore, NO has been implicated in the pathogenesis of inflammatory processes including I/R injury, and it participates in the regulation of microcirculation in several systems in both physiologic and pathologic settings [7,8]. Although, the role of NO in I/R injury–induced ARF is controversial, there is an increasing body of evidence indicating that formation of NO plays an important role in the pathophysiology of renal I/R injury [9,10]. It has been demonstrated that inhibition of inducible NO synthase ameliorates reperfusion injury in kidney [5,11]. Thus, the mechanisms underlying renal I/R injury are most likely multifactorial and interdependent. A better understanding of the cellular and molecular mechanisms of injury may enable us to improve therapy.

Trapidil [5-methyl-7-diethylamino-*s*-triazolo (1,5 a) pyrimidin] is an inhibitor of phosphodiesterase and platelet-derived growth factor, which is clinically used as an antianginal drug [12]. The pharmacologic properties of trapidil include nitroglycerine-like vasodilatation, inhibition of platelet aggregation via thromboxane A_2 (TxA_2) inhibition, facilitation of the biosynthesis of prostacyclin, and reduction of lipid peroxidation [13,14]. Moreover, trapidil inhibits secretion of IL-6 and IL-12 and suppresses production of TNF-α [15].

Recently, it has been demonstrated that trapidil has protective effects of different experimental I/R and inflammatory models [16-18]. However, it appears as there is no published data about the effect of trapidil on renal I/R injury. For this reason, in this study, we aimed to investigate whether trapidil had any beneficial effect on I/R–induced renal damage in rat. Furthermore, we have also investigated the relationship between trapidil and NO and $ONOO^-$ pathway in present study.

1. Materials and methods

1.1. Animals

The experimental procedures performed in this study were in concordance with the guidelines of Turkish National Institutes of Health. The experimental protocol was approved by the Ethical Committee of Mersin University. Wistar rats, weighing between 200 to 250 g, were used in this study. The rats were housed at constant temperature with 12-hour periods of light-dark exposure. Animals were allowed to access to standard rat chow and water ad libitum. A 1-week period of acclimatization was used in this study.

1.2. Experimental design

Animals were anesthetized with intramuscular injections of ketamine hydrochloride (50 mg/kg). The abdominal region was shaved with a safety razor and sterilized with povidone iodine solution. A midline incision was made, and the abdominal viscera were retracted to the right side. The left renal hilus was dissected, the renal vascular pedicle was occluded temporarily using a microvascular clamp, and the intestine was replaced into the abdominal cavity. At the end of the ischemic period, reperfusion was established by removal of the clamp, and a contralateral right nephrectomy was performed. Animals were randomly divided into 4 groups, each consisting of 6 animals. Group 1 (sham group, n = 6) rats were subjected to identical surgical procedures described above, except for renal I/R. Group 2 (sham + trapidil group, n = 6) animals were subjected to sham procedure, and trapidil (Rocornal USB, Germany) (8 mg/kg) was given intravenously immediately before sham procedure. Group 3 (I/R group, n = 6) rats received 45 minutes of left renal ischemia, followed by 60 minutes of reperfusion. Group 4 (I/R + trapidil group, n = 6) animals were administered trapidil (8 mg/kg intravenously) immediately before the reperfusion phase. At the end of the reperfusion period, the samples of blood and tissue were harvested for biochemical, serological, and histopahtological evaluation. Blood (6 mL) was taken from the animals via cardiac puncture. Of the blood, 3 mL was used for biochemical analysis, and 3 mL was used for detection of TNF-α levels. Quarter of tissues were used for tissue biochemical analysis.

1.3. Biochemical analysis

Serum samples were used for the measurement of serum urea levels, which were used to assess renal function, and aspartate aminotransferase (AST), which was used as an indicator of renal I/R injury [19]. The levels of serum urea and AST were analyzed with Cobas Integra 800 biochemical analyzer (Roche Diagnostics, GmbH, Manheim, Germany).

1.4. Detection of malondialdehyde levels

A tissue specimen of 50 mg was homogenized in 0.15 mol/L KCI for malondialdehyde (MDA) determination. After the homogenate had been centrifuged at 3000 rpm, the MDA levels in tissue homogenate supernatant and 50 mL of plasma were determined by thiobarbituric acid reaction according to Yagi. The principle of the method depends on the colorimetric measurement of the intensity of the pink color produced by the interaction of the barbituric acid with MDA. The colored reaction 1,1,3,3 tetraetoxypropane was used as the primary standard [20].

1.5. Measurement of tissue myeloperoxidase activity

Myeloperoxidase (MPO) is a hem-containing enzyme within the azurophil granules of neutrophils; therefore,

Table 1 The results of serum urea and AST levels

Groups	Urea	AST
Sham	39.3 ± 6.59	167 ± 15.6
I/R	92.8 ± 60.3*	342 ± 101*
Sham + trapidil	39 ± 4.42	159 ± 12.5
I/R + trapidil	51.8 ± 2.13	250 ± 34

* $P < .05$, as compared with other groups.

measurement of MPO activity was used as a simple quantitative method for detecting leukosequestration. A tissue specimen of 50 mg was homogenized in 0.02 mol/L EDTA (pH 4.7) in a Teflon Potter homogenizer. Homogenates were centrifuged at 20,000 × *g* for 15 minutes at +40°C. After pellet was rehomogenized in 1.5 mL of 0.5% hexadecyltirimethylammonium bromide prepared in 0.05 mol/L KPO_4 (pH 6) buffer, it was recentrifuged at 20,000*g* for 15 minutes at +40°C. One hundred milliliters of homogenate supernatant and serum were determined. The determination of lung tissue and serum MPO activity depends on the fact that it reduces *o*-dianozidine, hence reduced *o*-dianozidine was measured at 410 nm by spectrophotometer [21].

1.6. 3-Nitrotyrosine detection

3-Nitrotyrozine (3-NT) was obtained from Sigma Chemical Co (St Louis, Mo). H_2O_2, sodium acetate, citrate, NaOH, MnO_2, H_3PO_4, KH_2PO_4 and K_2HPO_4 were purchased from Merck Chemical Co (Deisenhofen, Germany) for measurement of 3-NT/total tyrosine. All organic solvents were high-performance liquid chromatography (HPLC) grade. The tissues were homogenized in ice-cold phosphate-buffered saline (pH 7.4). Equivalent amounts (50 mg) of each sample were hydrolyzed in 6N HCI at 100°C for 18 to 24 hours, then samples were analyzed on an HPLC apparatus (Aqi 1100 series). The analytical column was a 5-μm, pore-size Spherisorb ODS-2 C18 reverse-phase column (4.6-250 mm; Alltech, Deerfield, Ill). Guard column was a C18 cartidge (Alltech). The mobile phase was 50 mmol/L sodium acetate/50 mmol/L citrate/8% methanol (pH 3.1); HPLC analysis was performed under isocratic conditions at a flow rate of 1 mL min^{-1}, and ultraviolet detector was set 274 nm. 3-Nitrotyrozine peaks were determined according to their retention time, and the peaks were either confirmed by spiking with added exogenous 3-NT (10 μmol/L) or reducing to aminotyrosine [22].

1.7. Analysis of serum TNF-α levels

Serum samples were stored at −70°C until used for assay. Tumor necrosis factor–α levels in the serum samples were measured by enzyme-linked immunosorbent assay method. Specific TNF-α enzyme-linked immunosorbent assay kits (BioSource), lot no. 042904) were then used for the serum TNF-α assays. According to the kit's protocol, cutoff value was determined as 1.7 pg/mL. The results were accepted as positive if above the cutoff value and negative if below the cutoff value.

1.8. Processing of histologic samples

The kidney tissues were fixed in a 10% neutral formalin solution, routinely processed and embedded in paraffin. Sections 5 μm thick were cut, deparaffinized, hydrated, and stained with H&E for histologic assessment. Histologic examination and scoring of the sections were performed on a blinded basis. Severity of renal damage in terms of morphological changes was scored with a grading system of 0 to 3, developed by Chatterjee et al [19], as follows: 0 = normal histology; 1 = tubuler cell swelling, brush border loss, nuclear condensation, with up to tubuler profile showing nuclear loss; 2 = as with score 1, but greater than one third and less than two thirds of tubular profile shows nuclear loss; and 3 = greater than 2/3 of tubuler profile shows nuclear loss.

1.9. Statistical analysis

Statistical evaluation for proportional comparisons for biochemical values was analyzed using 1-way analysis of variance, followed by Bonferroni post hoc test. All data were presented as mean ± SD for biochemical analysis. Statistical evaluation for positive TNF-α values in groups used *t* test for difference between 2 proportions. Comparison for renal injury score was analyzed by Kruskall-Wallis variance analysis, followed by Dunn test. *P* values less than .05 were considered as statistically significant for all tests. In all computations, SPSS (version

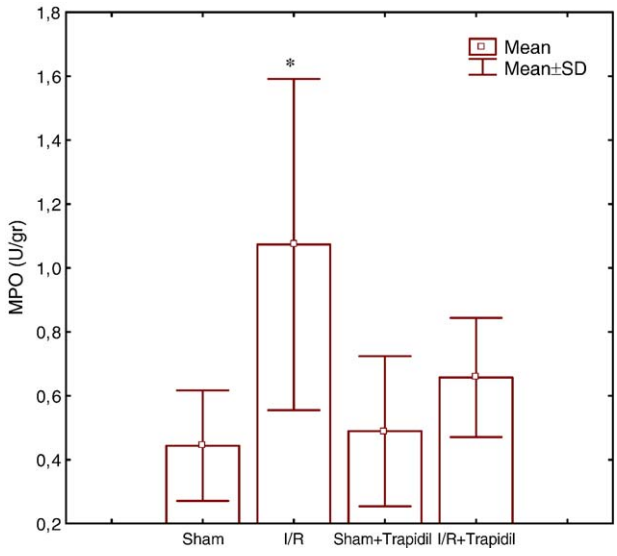

Fig. 1 Myeloperoxidase activities of kidney tissues in all groups. Data are expressed as mean ± SD. **P* < .05, compared with other groups.

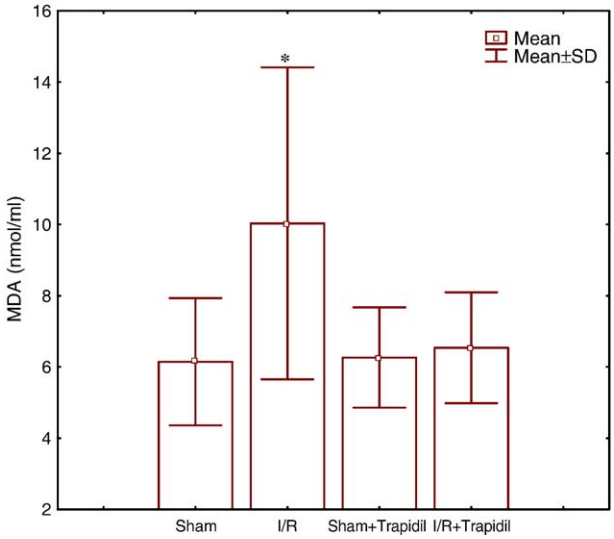

Fig. 2 Tissue MDA levels of kidney in all groups. All data are presented as mean ± SD. *$P < .05$, compared with other groups.

9.05) (SPSS, Chicago, Ill) statistical package program was used.

2. Results

2.1. Biochemical analysis

Animals that underwent renal I/R exhibited significant increase in the levels of serum urea and AST, as compared with sham and sham + trapidil groups ($P < .05$), suggesting a significant renal dysfunction mediated by I/R of kidney. Trapidil treatment produced significant reduction in the serum urea and AST levels in the I/R + trapidil group ($P < .05$) (Table 1).

Table 2 The results of serum TNF-α in study groups

N	Sham	I/R*	Sham + trapidil	I/R + trapidil
1	–	+	–	–
2	–	–	–	–
3	–	+	–	–
4	–	+	–	+
5	–	+	–	+
6	–	+	–	–

* $P < .05$ when compared with other groups.

2.2. Myeloperoxidase activity

Tissue MPO activity was significantly higher in the renal tissue of the I/R group when compared with sham and sham + trapidil groups ($P < .05$). Trapidil administration resulted in significantly reversed elevations in MPO activity, as compared with I/R group ($P < .05$) (Fig. 1).

2.3. Malondialdehyde levels

Malondialdehyde levels of tissue correlated with MPO activity. Tissue MDA levels in I/R group were elevated by I/R injury, as compared sham and sham + trapidil groups ($P < .05$). Treatment with trapidil produced a significant reduction in MDA levels in I/R + trapidil group ($P < .05$) (Fig. 2).

2.4. 3-Nitrotyrosine levels

3-Nitrotyrozine levels in tissues are demonstrated in Fig. 5. Renal I/R injury caused significantly an increase of 3-NT levels in the kidney, when compared with the sham group

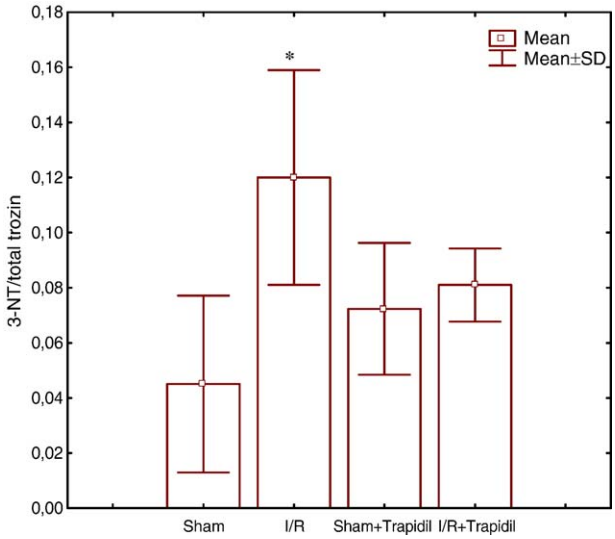

Fig. 3 3-Nitrotyrozine levels kidney tissues in all groups. Data are expressed as mean ± SD. *$P < .05$, compared with other groups.

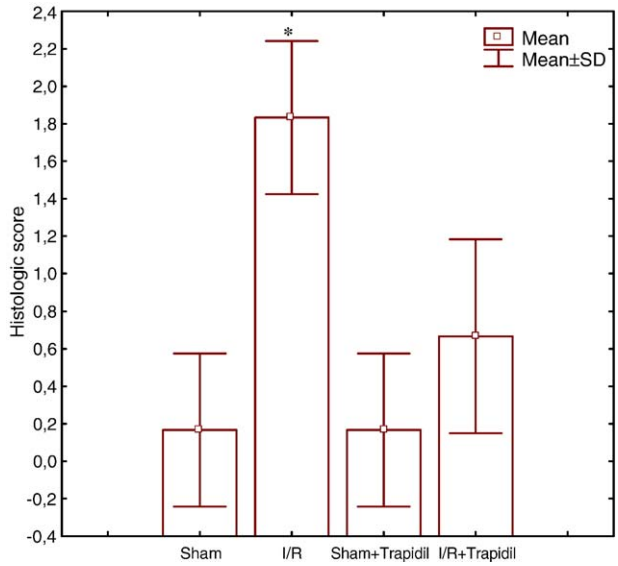

Fig. 4 Histopathological scores of renal tissue samples. Results are presented as mean ± SD. Ischemia-reperfusion group shows significantly higher injury scores when compared with scores of other groups. *$P < .05$.

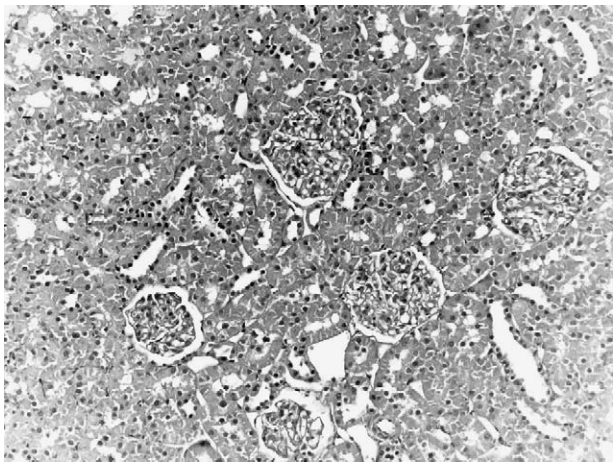

Fig. 5 Representative photomicrographs of rat kidneys in sham and sham + trapidil groups appeared as normal morphology (H&E stain; original magnification ×100).

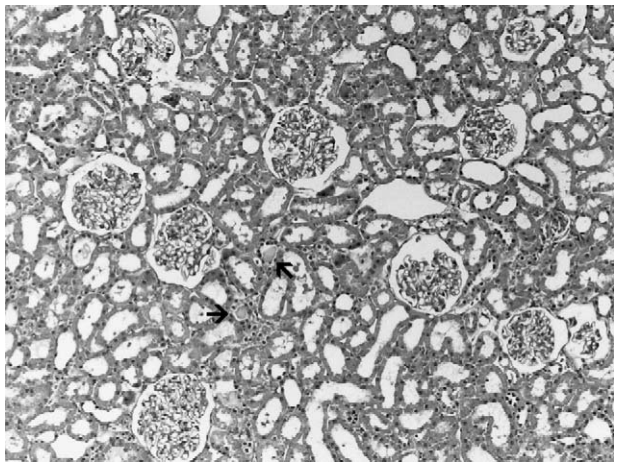

Fig. 7 Photomicrographs of I/R injury group treated with trapidil, in contrast, showed mild tubular injury such as tubular dilatation with hyalen casts in focal areas (arrowed), as shown here (H&E stain; original magnification ×100).

and sham + trapidil groups ($P < .05$). Trapidil treatment significantly reversed the elevations in renal 3-NT levels in I/R + trapidil group.($P < .05$) (Fig. 3).

2.5. Tumor necrosis factor–α values

Renal I/R injury significantly increased serum TNF-α levels. Tumor necrosis factor–α values were determined as positive in 5 rats (83.3%) in I/R group and in 2 rats (33.3%) in I/R + trapidil group. These results were significantly higher when compared sham and sham + trapidil groups ($P < .05$). Trapidil treatment inhibited TNF-α synthesis in treated group (Table 2).

2.6. Histopathological results

Histopathological scores in groups are summarized in Fig. 4. Sham and sham + trapidil group preserved almost normal morphology (Fig. 5). By contrast, in the kidney of I/R group, varying degrees of injury up to marked tubular necrosis was noted (Fig. 6). However, I/R-induced tissue injury was attenuated by trapidil of treatment group. In this

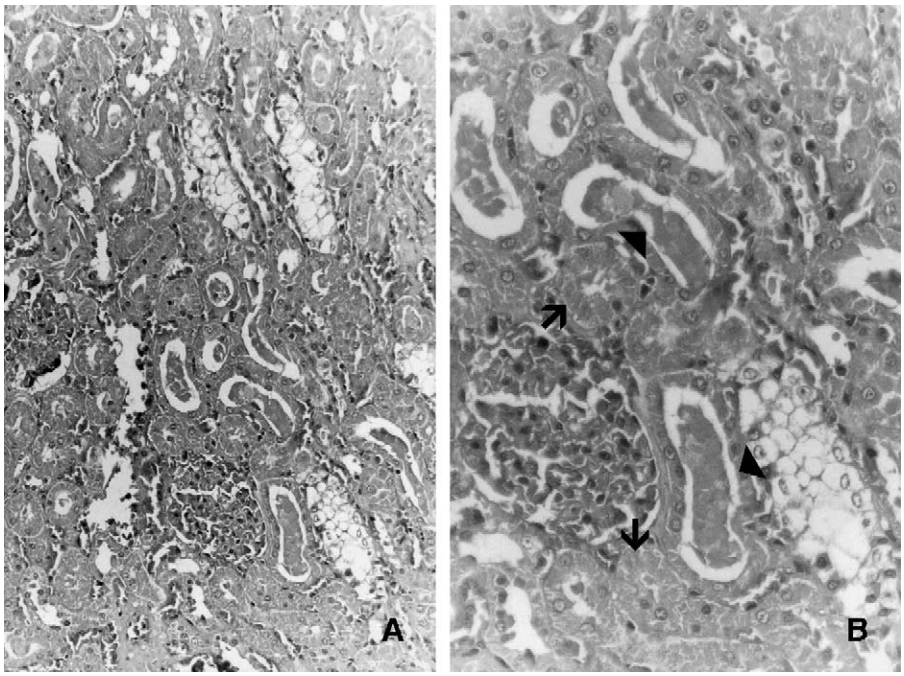

Fig. 6 Photomicrographs of I/R injury group showing the morphology of renal injury (A). In the higher magnification of the same area, marked tubular dilatation, cast formation (arrow head) and tubular necrosis (arrowed) are seen (H&E stain; original magnification ×100 [A] and × 400 [B]).

group, the areas of tubular necrosis and tubular dilatation and cast formations were more focal and mild (Fig 7).

3. Discussion

Renal I/R–induced acute tubular necrosis is observed most frequently in patients after cardiac and aortic operations, trauma, severe dehydratation, burns, and others. Because renal failure induced by these conditions is a devastating problem, evaluation of new theuropethic agents is essential. Thus, to assess its protective effect in renal I/R injury, we investigated trapidil in this study.

Renal I/R injury causes both glomerular and tubular dysfunction. In this study, the large increase in the serum levels of urea, indicating glomerular dysfunction mediated by renal I/R, suggested that I/R injury causes significant impairment of glomerular function. In addition, increased serum levels of AST confirmed renal reperfusion injury. The AST is not only specific to the liver but also found in other organs such as kidney and smooth muscle. This enzyme are especially elevated after renal tubular injury in rat [23]. Because AST is present within the proximal tubulus and is regarded as a nonspecific marker of extensive cellular damage [24], we have used serum AST in this study as a marker of reperfusion injury. Furthermore, evidence of tubular injury was supported by the histopathological scoring of renal injury as there was marked tubular injury.

The pathophysiology of renal I/R injury is still incompletely understood. However, an excessive inflammatory response is clearly recognized as a key mechanism of injury during reperfusion. Several proinflammatory cytokines such as IL-1 and TNF-α are generated mainly by macrophages [6]. Tumor necrosis factor–α is a critical early mediator of reperfusion-induced organ damage. This cytokine stimulates both neutrophil accumulation in tissue, playing an important role of reperfusion injury, and enhances the expression of intercellular adhesion molecule–1 (ICAM-1), playing a pivotal role the development of inflammatory reaction [25,26]. Donnahoo et al [26] have shown that I/R-induced renal TNF-α expression. In this study, we also found that renal I/R injury significantly elevated the serum TNF-α levels. Zhou et al has clearly revealed that trapidil inhibits the monocytic CD40 pathway, thereby reducing the production of TNF-α, IL-6, and IL-12 [15]. Tumor necrosis factor–α production was inhibited by trapidil treatment in the present study. These data indicate that trapidil has a potent inhibitor effect on TNF-α production in I/R injury. We believe that this effect of trapidil may contribute at reducing tissue damage.

Polymorphonuclear leukocytes accumulated during reperfusion have long been implicated as critical mediators of I/R injury. These cells are a potential source of ROS and RNS and has a major role in development of oxidative and nitrosative tissue injury [6]. Several studies showed that neutrophil accumulation in kidney may be involved in the pathogenesis of the early renal parenchymal injury in ARF [1,4,27]. In our study, MPO activity, primarily found in neutrophils and levels of MPO in tissues reflect neutrophil sequestration, significantly increased in kidney tissue, followed by 60 minutes reperfusion period. Trapidil treatment inhibited neutrophils infiltration, as evidenced by reduced MPO activity in treated group. Although there are no evident findings for explanation of inhibiting effect of trapidil, the relationship between trapidil and platelet-derived growth factor, which has chemotactic capacity for macrophages and neutrophils and strongly inhibited by trapidil, may play a pivotal role in this effect [12,28,29].

It has been shown that I/R injury is associated with lipid peroxidation, which is believed to be an important cause of oxidative damage to cellular membranes and, eventually, cell death. Many studies have clearly demonstrated that ROS and RNS play an important role in lipid peroxidation on renal I/R injury [4,5,9,10]. In our study, we observed that the levels of tissue MDA, which is the product of lipid peroxidation, were significantly increased in kidney tissues. In contrast, trapidil treatment inhibited MDA elevations significantly and reversed back to control levels. This set of data showed that lipid peroxidation was prevented with trapidil treatment. Thus, based on these findings, the protective effect of trapidil I/R-induced renal damage, in part, may be attributed to the membrane-stabilizing effect of trapidil by the inhibition of TxA_2 synthesis [13,14], and in part, trapidil may also be antogonized by the cytotoxicity of reactive oxygen and nitrogen species.

The contribution of NO in the pathogenesis of ARF is still controversial. However, it has been demonstrated that NO plays a biphasic role in pathophysiology of renal injury mediated by I/R [30]. In early phases, the increased oxidative and nitrosative damage was exacerbated by NO donors but ameliorated by NOS inhibition [5,30]. The toxicity of NO has been attributed to the $ONOO^-$, which was formed by reaction of NO with superoxide anion and called the “ugly face” of NO [31]. Nitric oxide and $ONOO^-$ react with cellular lipid and protein components, consequently disturbing their function. Another pathway of cell death, triggered by $ONOO^-$, leads to the loss of cellular energetics via the induction of DNA single-strand breaks and subsequent activation of the nuclear enzyme poly (ADP-ribose) synthetase. Poly (ADP-ribose) synthetase activation depletes the intracellular concentration of energetic substrate, nicotinamide dinucleotide, thus leading to cellular dysfunction and cell death [32,33].

The nitration of protein tyrosine residues by $ONOO^-$ results in the formation of 3-NT, the footprint of $ONOO^-$ and, indirectly, NO, and the appearance of this product in tissue samples is taken as a diagnostic tool for exposure to $ONOO^-$ and NO [33]. Recently, several studies have reported that $ONOO^-$ plays a devastating role in renal I/R injury. In these studies, increased level of 3-NT has been detected in the kidney of reperfusion injury, and inducible NO synthase inhibition reduced renal dysfunction and injury

associated with I/R of the kidney [5,10]. In the present study, we observed that the levels of 3-NT were increased in I/R-injured kidney tissues, and trapidil treatment significantly reversed these increases. These data support the hypothesis that $ONOO^-$ formation occurs during I/R and that trapidil treatment can reduce $ONOO^-$ formation and preserve renal function.

In addition, adhesion molecules, such as P-selectin, a member of selectin family and mediates rolling of leukocytes along the endothelium, and ICAM-1, which is one of the most important ligands for leukocytes and mediates binding of leukocytes on the endothelium during the reperfusion, are normally expressed at basal level, but their expression can be enhanced by several cytokines in renal I/R injury [34]. Furthermore, it has been shown that $ONOO^-$ enhances expression of P-selectin and ICAM-1 in I/R injury [35]. On the other hand, the use of TxA_2 inhibitors, such as trapidil, was demonstrated to attenuate diapedesis and ICAM-1 expression in I/R model [36]. In our study, although we did not study these adhesion molecules, trapidil treatment might also have reduced the expression of P-selectin and up-regulation of ICAM-1 in tissues in this experimental study.

In conclusion, the results of this study indicate that in the renal I/R injury, ROS and RNS play a causal role. Treatment with trapidil reduced I/R induced kidney damage by multiple effects on the lipid peroxidation, $ONOO^-$ pathway, and inflammatory cytokines such as TNF-α.Therefore, trapidil merits consideration as a potential therapeutic agent in renal I/R injury.

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