The Effects of Ascorbic Acid and U-74389G on Renal Ischemia-Reperfusion Injury in a Rat Model

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Abstract. Background/Aim: U-74389G and ascorbic acid protect the cells from oxidation. This study aimed to depict their role in ischemia–reperfusion injury in a renal rat model. Materials and Methods: Sixty Wistar rats were randomized into six groups of 10 animals each. Group A Ischemia 30 min, reperfusion 60 min; Group B Ischemia 30 min, reperfusion 120 min; Group C Ischemia 30 min, ascorbic acid administration, reperfusion 60 min; Group D Ischemia 30 min, ascorbic acid administration, reperfusion 120 min; Group E Ischemia 30 min, U-74389G administration, reperfusion 60 min; Group F Ischemia 30 min, U-74389G administration, reperfusion 120 min. We then collected tissue and blood samples. Results: Histology and the significantly decreased malondialdehyde and tumor necrosis factor-α levels indicated that ascorbic acid was superior to U-74389G, at pre-defined time intervals. Conclusion: Ascorbic acid and U-74389G ameliorated renal damage induced by ischemia–reperfusion injury, suggesting a therapeutic effect.

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Renal ischemia–reperfusion injury (RIRI) is an essential clinical concern. Clinically, it commonly presents as either an embolic or a thrombotic episode related to acutely or chronically established hypotension or as a postoperative complication following cardiac bypass surgery. The most prominent renal damage is related to kidney transplantation that affects short-term and long-term graft function and patient morbidity and mortality (1). RIRI can cause significant acute kidney damage and tubular cell death either by necrosis or apoptosis (2). The kidneys are strongly predisposed to ischemia. The discrepancy between blood oxygen supply and functional need, results in a decrease in oxidative metabolism, and a progressive damage and death of tubular epithelial cells (3). Consequently, the kidneys lose their homeostatic regulation of metabolic waste products, resulting in water and electrolyte imbalance.

Free oxygen radicals are mainly responsible for RIRI. Studies (4, 5) have reported that ischemia and reperfusion generate a systemic inflammatory cascade in various organs (e.g., pancreas, liver, and intestines) by free oxygen radical and cytokine (6-8) production.

To describe and delineate the complexity of the pathogenetic mechanisms in the post-ischemic kidney, many experimental studies have examined the efficacy of different potentially therapeutic agents that could prevent renal damage. Various studies have suggested efficient or inefficient RIRI treatments; most such studies used experimental animal models, and the substances tested remain to be examined by human controlled trials.
Many of the agents tested were oxygen-free radical scavengers that have a short half-life and are unable to circulate and enter the target cell, e.g. the renal tubular cell (9). Failure may also be due to antioxidants having a relatively high molecular weight and thus, cannot readily enter the renal tubular cells in adequate concentrations.

U-74389G resides in lazaroids that are low molecular weight antioxidants. Lazaroids are synthesized from aminosteroids. They are deprived of glucocorticoids and mineralocorticoids in nature and act by inhibiting iron-dependent lipid peroxidation. Other potential mechanisms of action include oxygen-free radical scavenging, iron-chelating, and membrane-stabilizing (10-12). Moreover, lazaroids inhibit cytokine release, adhesion molecule expression, and infiltration or activation of neutrophils. Various experimental studies have shown that antioxidants and specific lazaroids attenuate IRI in vital organs (13-23).

Vitamin C (or ascorbic acid) is a water-soluble antioxidant involved in the formation of collagen, the metabolism of carnitine and catecholamine and in dietary iron uptake. Humans cannot synthesize ascorbic acid, and thus it is crucial to include it in their diet. Finally, ascorbic acid is responsible for the hydroxylation of proline residues in procollagen to form mature collagen with a stable triple-helix (24).

Tumor necrosis factor-alpha (TNF-α) is an inflammatory cytokine. Therapeutic attempts aim to neutralize TNF-α by targeting antibodies or inhibiting the TNF-α receptor. Malondialdehyde (MDA) is an aldehydic lipid hydroperoxide decomposition product synthesized upon oxygen-free radical-induced damage of cellular lipids. Considering that the lazaroids’ mechanism of action is free radical scavenging, we hypothesized that MDA levels could accurately estimate lazaroid’s effect in experimental studies, such as those using RIRI models. Moreover, in experimental studies of IRI, U-74389G decreased TNF-α levels (6-8).

In light of these findings, our study aimed to assess the potential effect of lazaroid U-74389G and ascorbic acid as therapeutic agents following RIRI in a Wistar rat model; several clinicopathological and biochemical parameters, TNF-α, and MDA were examined.

To our knowledge, information is lacking regarding the simultaneous administration of U-74389G and ascorbic acid in a common experimental animal model of RIRI.

The deep understanding of the molecular mechanisms involved in RIRI and their impact on long-term kidney function are fundamentally important in for the development of novel therapeutic approaches.

Materials and Methods

Experimental animals. The Pasteur Institute supplied the laboratory male (3-to-4-month old) Wistar rats. Experiments were performed at the accredited laboratory of the Experimental, Educational and Research Center (ELPEN) in Athens, Greece. All animals were acclimatized in conventional environmental conditions [temperature (22-25°C), humidity (55%-58%) and lighting (12 h light/dark cycles)], with free access to food and water. The experimental procedures strictly conformed to the National Research Council Guide for the Care and Use of Laboratory Animals and the Directive 86/609 of the European Union, protocol number K/2284.

All experiments were approved by ELPEN Laboratories and veterinary authorities of East Attica Region in accordance with the principles of the Helsinki Declaration and Greek Law No. 160, A-64, May 1991, European Union regulations.

Study design. Sixty male Wistar rats, weighting 280-350 g, were used in this study. The animals were distributed randomly into the following six experimental groups: Group A (control group, n=10): 30-min kidney ischemia and 60-min reperfusion, Group B (n=10): 30-min kidney ischemia and 120-min reperfusion, Group C (n=10) 30-min kidney ischemia and inferior vena-cava IV ascorbic acid injection (10 mg/kg), followed by 60-min reperfusion, Group D (n=10) 30-min kidney ischemia, inferior vena-cava IV ascorbic acid injection (10 mg/kg), followed by 120-min reperfusion, Group E (n=10) 30-min kidney ischemia, inferior vena-cava IV U-74389G injection (10 mg/kg), and 60-min reperfusion, Group F (n=10) 30-min kidney ischemia, inferior vena-cava IV U-74389G injection (10 mg/kg), and 120-min reperfusion. We sampled tissues and blood at 30-min intervals, 0-120 min following reperfusion.

The U-74389G dose was 10 mg/kg body weight (CALBIOCHEM® Company, Cat. No. 153190-29-5, for experimental use). This lazaroid dose has also been used in previous experimental studies from the same laboratory using U-74389G (6-8); this dose has also been used in various other experimental models (6-8, 23) and appears to be beneficial. The ascorbic acid dose (VITAMIN-C-LOGES Optipharm Co, 1 ampule 100 mg/ml) was also 10 mg/kg body weight. Before the experiment, the rats were naturalized to the laboratory for 7 days.

Anesthesia protocol and operative technique. Animals were placed in a glass cage where the anesthesia protocol was followed. Anesthesia included isoflurane administration, following a 0.25 ml (10 mg/ml) subcutaneous injection of the opioid butorphanol as an analgesic (Dolorex; Intervet, Schering-Plough Animal Health, Boxmeer, the Netherlands). After 3 min, the state of anesthesia of the animals was confirmed by lack of swallowing movements, immobility, and loss of the tail flick reflex. Afterward, we placed the animals on the operating table and performed endotracheal intubation (EI) under direct laryngoscopy using a 16 Fr venous catheter. Indeed, rat experiments can also be performed without EI using rat facemasks or ketamine alone; however, EI was the preferred method of controlled mechanical ventilation. EI is proposed by most researchers in their experimental protocols. On the operating table, animals were placed in the supine position; they were immobilized, and their mandible was stabilized. The endotracheal tube and the anesthetic gas tubing were stabilized with adhesive bands.

Abdominal hair was shaved, and the skin was disinfected with 10% iodine povidone. A midline incision was made from the xiphoid process, 3 cm caudally of the linea alba. The abdominal cavity was then entered, and the kidney was mobilized and freed from its attachments. The renal artery and vein were prepared for complete occlusion with surgical bulldog clips to apply the ischemia–reperfusion protocol. Following U-74389G and ascorbic acid administration via the inferior vena cava, the clips were removed. The abdominal cavity was closed with 2-0 Vicryl sutures and the animals remained anesthetized for 60 or 120 min (in the
Histochemical examination of the tissue specimens was performed using a scale ranging from 0-3; 0=absent, 1=1-2 degenerated renal tubules per HPF, 2=2-4 degenerated renal tubules per HPF, 3=more than four degenerated renal tubules per HPF.

Two experienced pathologists (TP and IS) examined the specimens independently and in a blinded manner, under a Nikon eclipse 50i microscope.

Biochemical parameters, MDA, and TNF-α Measurements. To evaluate MDA, N-methyl-2-phenyldihydridine (MPI), which is a chromogenic agent, reacted with MDA at 45°C. The reagents were: MPI reagent, 10.3 mmol/l MPI in acetonitrile, MDA standard, 10 mmol/l 1,1,3,3-tetramethoxypropane in 20 mmol/l Tris-HCl, 500 mmol/l butylated-hydroxytoluene in acetonitrile, Tris buffer pH 7.4, 0.9% NaCl, 37% (12 mol/l) high-pressure liquid chromatography (HPLC) grade methanol, and acetonitrile. Based on standard practice, one volume of 100% methanol was diluted with three volumes of the MPI reagent. We rinsed the tissue samples with ice-cold isotonic saline and homogenized them in Tris-buffer 20 mmol/l pH 7.4 with an Ultra-Turrax (Ika–Labortechnik) blender (1 ml Tris/0.1 g of tissue). For sample oxidation prevention, we added 10 ml butylated hydroxylutene (500 mmol/l) to 1 ml tissue homogenate. The homogenate was centrifuged at 3000 rpm with a 10 cm radius centrifuge that delivered a centrifugal force of 1000×g for 10 min. Then, 0.2 ml of the supernatant of the tissue homogenate was maintained in a polypyrrole microcentrifuge tube, and 0.65 ml of the diluted MPI reagent was vortexed. We extracted the mixture and added 0.15 ml of HCl (12 mol/l). We incubated the tubes at 45°C for 60 min and then centrifuged them at 6000 rpm with a 10 cm radius centrifuge that delivered a centrifugal force of 4025×g for 15 min; 0.8 ml of the supernatant was used to measure MDA at 586 nm. The MDA standard curve was prepared by successive dilutions of the stock tetramethoxypropane solution (10 mmol/l). The final standard concentrations were 2.08, 4.16, 8.33, 12.5, and 16.66 μmol/l, and the absorbance values were 0.059, 0.124, 0.264, 0.4 and 0.545, respectively (6-8, 13).

We measured TNF-α using an enzyme-linked immunosorbent assay in tissue homogenate. A swine, not a human, anti-TNF-α antibody was used (anti-swine; BIO SOURCE Co., Carlsbad, CA, USA) (13) to avoid cross reactions that may interfere with the results.

Further, we measured the complete blood cell count and serum biochemical parameters (i.e., sodium, potassium, glucose, creatinine, urea, SGOT/AST, SGPT/ALT, gamma-GT, total bilirubin, direct bilirubin levels) at 30 min intervals (0-120 min) after reperfusion (13).

Statistical analysis. Data are expressed as mean±standard deviation (SD) for continuous variables and as percentages for categorical variables. We used the Shapiro-Wilk test to measure the normal distribution of the variables.

Comparison of variables between the three intervention groups (control vs. U-74389G vs. ascorbic acid) was performed using one-way analysis of variance followed by post-hoc comparisons using the Bonferroni test. Kruskal-Wallis and Mann-Whitney tests were used for non-parametric analyses as needed.

Comparison of the variables between the two reperfusion times (60 min vs. 120 min) per intervention group was performed using the t-test or Mann-Whitney test (in case of violation of normality).

All tests were two-sided. A p<0.05 was considered significant. All analyses were performed using SPSS version 21 (IBM Corp., Armonk, NY, USA).

Results

Pathology results. The pathology score analysis revealed a statistically significant difference (p=0.007) regarding necrosis and degeneration of renal tubules. Comparative analysis revealed a statistically significant difference between group A (necrosis score 2.6/degeneration of renal tubules score 2.7) and group B (necrosis score 2.7/degeneration of renal tubules score 3) (p=0.013) and C (necrosis score 1.4/degeneration of renal tubules score 1) (p=0.022) (Table I, and Figures 1 and 2).

A statistically significant difference was observed in the pathology score (p=0.007) among groups D (necrosis score 1.7/degeneration of renal tubules score 1), E (necrosis score 1.1/degeneration of renal tubules score 1) (Figures 3 and 4) and F (necrosis score 1.5/degeneration of renal tubules score 1) (Figure 5). The multiple comparisons showed a difference between group D and groups E (p=0.013) (Figures 6 and 7) and F (p=0.022) (Table I, Figure 8).

MDA and TNF-α per group results. There was no significant difference among groups A, B, and C regarding MDA-μM and TNF-α (p=0.678). Conversely, we noted a statistically significant difference among groups D, E, and F for MDA-μM (p=0.010) and TNF-α (p=0.0005) (Figure 3).
Figure 1. Main variables among the groups expressed as MEAN+SD after 60 min and 120 min of reperfusion.

Figure 2. Main variables among the groups expressed as MEAN+SD after 60 min and 120 min of reperfusion.
Results at 60 min reperfusion. No statistically significant difference was observed among the groups for MDA (μM) \((p=0.085)\), TNF-α \((p=0.546)\), and SGPT \((p=0.820)\). The ALP values were significantly higher in the U 74389 G group than in the control \((p=0.002)\) and ascorbic acid \((p<0.001)\) groups. The γ-GT values were significantly lower in the control group than in the ascorbic acid \((p=0.051)\) and U74389G \((p=0.231)\) groups (Figures 1 and 2). The ascorbic-acid group had statistically significant higher values of SGOT than the control \((p=0.002)\) and U74389G \((p<0.001)\) groups (Table II).

Results at 120 min reperfusion. The TNF-α values were significantly higher in the U74389G group than in the control \((p<0.001)\) and ascorbic acid \((p<0.001)\) groups. The SGPT values were significantly higher in the U74389G group than in the control \((p=0.046)\) and ascorbic acid \((p=0.001)\) groups. The ALP values were significantly higher in the U74389G group than in the control \((p<0.001)\) and ascorbic acid \((p<0.001)\) groups. The creatinine values were significantly higher in the U74389G group than in the control \((p=0.004)\) and ascorbic acid \((p=0.001)\) groups. The
TNF-α, SGPT, ALP, values were significantly higher in the U74389G group than in the control \((p<0.01)\) and ascorbic acid \((p<0.01)\) groups. The TNF-α value was significantly higher in the U74389G group than in the ascorbic acid group \((p=0.049)\) (Figures 1 and 2).

The SGOT values were significantly higher in the ascorbic acid group than in the control \((p<0.001)\) and U74389G \((p<0.001)\) groups (Table III).

Finally, there was no statistically significant difference between the two reperfusion times \((60 \text{ min vs. } 120 \text{ min})\) for all variables in the control and ascorbic acid groups \([\text{only the } \gamma\text{-GT value } (p=0.005) \text{ was significantly different}].\) There was a statistically significant difference between the two reperfusion times \((60 \text{ min vs. } 120 \text{ min})\) for TNF-α \((p=0.0005),\) SGOT \((p=0.022),\) SGPT \((p=0.022),\) \(\gamma\text{-GT} \((p=0.006),\) and creatinine \((p=0.035)\) (Table IV).

In the control group, renal damage was exacerbated in all the estimated variables. A statistically significant difference was found regarding renal damage among groups E and F compared with the control and ascorbic acid groups.

**Discussion**

The complex cascade of ischemia, reperfusion, and inflammatory processes in kidney cells could result in entirely opposite outcomes, namely injury or recovery. IRI is an inflammatory process involving various factors and molecules that may contribute to cellular dysfunction. Renal endothelial and tubular epithelial cells are predisposed to IRI \((25-28).\)

Following ischemia, oxidant attack stimulates cytokine or chemokine upregulation, activating inflammatory cells to mediate injury \(\text{via TNF-} \alpha \text{ and IFN-} \gamma \text{ expression} \,(29, 30).\)
Currently, no specific treatments exist to reduce IRI, as most of the pathogenetic mechanisms involved have not been thoroughly elucidated. The most prominent mechanisms are alterations in hemodynamics and in the microvasculature of the renal tubules. Other mechanisms include alterations in cellula metabolism and gene expression. In addition, IRI in clinical practice is usually multi-factorial, with concurrent components such as ischemia, nephrotoxicity, and sepsis (31). It has been indicated that nuclear factor-κB (NF-κB) is instrumental in IRI pathogenesis (32-36). NF-κB is principally responsible for producing reactive oxygen species (ROS), chemokines, and cytokines and control pro- or anti-apoptotic signaling, eventually crucial for IRI generation (32, 34, 35). NF-κB acts as a factor of transcription in tubular epithelial and inflammatory cells, bridging the equilibrium between cell death signaling pathways and inflammatory processes. In addition, it was shown in a mouse model of renal IRI that NF-κB activation of renal tubular epithelial cells impaired tubular injury and accentuated the inflammatory process (36). More research may lead to IRI treatment centered on NF-κB (37).

Efforts have been made to prevent IRI that impairs renal graft function after kidney transplantation with the use of calcium-channel blockers and L-arginine. Other substances that have been administered and proven to be beneficial in diminishing acute kidney damage in experimental models are dopamine, fenoldopam, and atrial natriuretic peptide (1). Many other antioxidants have been successfully used in experimental models but only few have been tested on humans undergoing kidney transplantation. It has been shown that pretreatment with specific antioxidants could ameliorate the damage of IRI in kidney transplantation. Lazaroids have been suggested to have a therapeutic effect on IRI in various organs (7, 8). Using our current experimental model, we examined the antioxidant protective function of lazaroïds and ascorbic acid against IRI. N-acetylcysteine and L-arginine are also potential therapeutic factors. Other auspicious results have been obtained in experimental models with vitamin E peroxinitrite, nitrosithiols, ligustrazine, allopurinol, and folate. However, scarce clinical transplantation-related studies have been conducted using few of these agents. We are far from determining whether pretreatment with these antioxidants can ameliorate renal function in kidney transplantation. Other researchers have reported failure in administrating antioxidants to pathologies where the pivotal role of ROS was considered the principal pathogenetic mechanism, such as in pancreatic IRI (6).

A study by Koul et al. that employed a rat model concluded that ascorbic acid protects against IR-induced acute kidney injury (38). Similar studies to ours have shown the protective role of vitamins C and E in attenuating oxidative stress following renal ischemia in a rat model (39), probably by radical scavenging and expressing antioxidant properties (40). Different parameters were evaluated but similar results to our study were shown by Ergin et al. concerning renal microcirculation after IRI (41).
One of the strengths of our study is that we attempted a more holistic approach to examine the potentially beneficial effect of both molecules (ascorbic acid and U-74389G) instead of using a fragmental evaluation of some parameters. In contrast, a limitation of our study is that we did not evaluate IRI for 24 h. Moreover, experimental studies in larger mammals than rats are anticipated.

Numerous issues still remain unanswered, such as the different functions of inflammatory cells. According to experimental studies, targeting inflammatory pathways has a protective action in rodents; conversely, clinical studies have shown that targeting the same pathways in humans has no therapeutic effect. Moreover, bridging the underlying mechanisms of acute rejection and ischemic graft injury with intervention with novel therapeutic agents would be of paramount importance (42, 43).

The results of our study emphasize the effect of the inherent immune response on the pathogenesis of IRI, leading to potential therapeutic options. However, based on our search, these potentially therapeutic strategies have not been incorporated into clinical practice for IRI evaluation.

Equally, U-74389G and ascorbic acid that were found to be protective in our experimental rat model, should be applied to clinical scenarios.

Conclusion

In conclusion, this study, which consolidated statistically significant histopathological results (p<0.01) in renal tubular degeneration or necrosis and found a substantial decrease in MDA and TNF-α levels, indicates the superiority of ascorbic acid at 120 min of reperfusion to lazaroid U-74389G in repairing renal damage from IRI. In contrast, there was no significant difference in MDA and TNF-α among the groups at 60 min of reperfusion.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors’ Contributions

AEP, KZ, AC, DC: conception and design of the study; KZ, VK, DC, AC, KP, MS: acquisition of data, drafting the article; AG: statistical analysis; TP, IS: histological study, analysis of histological scores; DC, MS, AEP: drafting the article, revising it critically for important intellectual content; AEP, EF, GZ, DC: final approval of the version to be submitted.

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