

TITLE PAGE

Urolithin A mitigates cisplatin-induced nephrotoxicity by inhibiting renal inflammation and apoptosis in an experimental rat model

Melissa Guada, Raghu Ganugula, Manicka Vadhanam and Majeti N. V. Ravi Kumar

Department of Pharmaceutical Sciences, College of Pharmacy, Texas A&M University,
College Station, TX 77843, USA (MG, RG, MRK).

Department of Medicine, University of Louisville, Louisville, KY 40202, USA (MV).

RUNNING TITLE PAGE

Urolithin A prevents cisplatin-induced nephrotoxicity

Corresponding author: Majeti N. V. Ravi Kumar

Address: Department of Pharmaceutical Sciences, College of Pharmacy, Texas A&M University, Reynolds Medical Building, Mail Stop 1114, College Station 77843, Texas, USA

Phone number: +1-979-436-0721; e-mail: mnvrkumar@tamhsc.edu

Number of text pages: 23

Number figures: 6

Number references: 45

Number of words in the Abstract: 250

Number of words in the Introduction: 521

Number of words in the Discussion: 981

Abbreviations: CIS, cisplatin; EA, ellagic acid; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; H&E, hematoxylin-eosin; Iba1, ionized calcium-binding adapter molecule 1; IL, interleukin; INF- γ , interferon gamma; iNOS, inducible nitric oxide synthase; KIM-1, kidney injury molecule-1; MCP-1, monocyte chemoattractant protein-1; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; NOS3, nitric oxide synthase 3; PAS, periodic acid-Schiff; TBST, tris-buffered saline and Tween 20; TIM-1, T cell Ig and mucin domain-containing protein-1; TNF- α , tumor necrosis factor alpha; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; UA, urolithin A

Section assignment: Toxicology

Abstract

Cumulative kidney toxicity associated with cisplatin is severe and there is no clear consensus on the therapeutic management of the same. The pathogenesis involves activation of inflammatory and apoptotic pathways, therefore regulating these pathways offers protection. Given the anti-inflammatory and antioxidant effects of urolithin A, a gut microbial metabolite of ellagic acid, our aim was to explore the potential of urolithin A for prevention of cisplatin-induced nephrotoxicity in an experimental rat model. For this purpose, animals received a single intraperitoneal dose of cisplatin (5 mg/kg body weight). Six hours prior to cisplatin administration, rats were orally treated with either ellagic acid or urolithin A (50 mg/kg body weight), followed by a daily dose of these compounds during the next 5 days. At the end, plasma and kidneys were collected for analysis. Cisplatin-induced kidney damage was revealed by a significant rise in the plasma creatinine levels accompanied by significant morphological changes in tubules, T cell Ig and mucin domain-containing protein-1, ionized calcium-binding adapter molecule 1, as well as a marked increase in the number of apoptotic cells localized in tubules. Cisplatin also reduced nitric oxide synthase 3 and nuclear factor kappa-light-chain-enhancer of activated B cells resulting in regulation of various inflammatory cytokines. Urolithin A effectively attenuated cisplatin-induced kidney damage and showed significantly greater effect than its precursor ellagic acid on preserving the normal kidney architecture by downregulating the pro-inflammatory cytokines. In summary, urolithin A mitigates cisplatin-induced nephrotoxicity in rats by modulation of the inflammatory cascade and inhibition of the pro-apoptotic pathway.

Introduction

Increasing the efficacy of cytotoxic drugs without compromising non-target tissue safety is a critical issue in the field of oncology, and is still a major therapeutic approach to treat both localized and metastasized cancers (Mathijssen et al., 2014). Platinum-based drugs such as cisplatin are widely used in the treatment of human and veterinary neoplasms' alike (Barabas et al., 2008; Dilruba and Kalayda, 2016). However, their severe side effects and resistance to the treatment (Barabas et al., 2008; Apps et al., 2015; Dilruba and Kalayda, 2016) limit clinical success. Among the undesirable effects, nephrotoxicity negatively impact patient morbidity and mortality (Yao et al., 2007; Bhat et al., 2015). Over the years, a range of strategies such as, re-formulating cisplatin, pharmacogenomics based personalized dose regimens, use of adjuvants, etc, have been applied in clinical practice to mitigate cisplatin-induced nephrotoxicity (Pabla and Dong, 2008; Boulikas, 2009; Ciccolini et al., 2011). However, kidney related toxicity remains an issue for the patient health care and thus their quality of life.

The plant polyphenols are extensively investigated as adjuvants for their ability to sensitize drug-resistant cancer cells to the chemotherapy as well as protect non-target tissues from damage by their antioxidant and anti-inflammatory properties (Koyner et al., 2008; Lecumberri et al., 2013). Earlier, we have reported that ellagic acid, a natural compound found in certain nuts, fruits and vegetables, can protect kidney against cyclosporine-induced nephrotoxicity in experimental rat model (Sharma et al., 2007; Sonaje et al., 2007). Similarly, the protective effect of ellagic acid against cisplatin-induced kidney injury has also been experimentally investigated (Ateşşahin et al., 2007; Al-Kharusi et al., 2013; El-Garhy et al., 2014). The beneficial effects reported are due to

the reduction of oxidative stress and inhibition of pro-inflammatory and pro-apoptotic pathways e.g., monocyte chemoattractant protein (MCP)-1, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), tumor necrosis factor (TNF)- α , inducible nitric oxide synthase (iNOS) and nitric oxide (NO) (Ateşşahin et al., 2007). Recent reports suggest that most of the pharmacological properties associated to ellagic acid may be attributed to its gut microbial metabolites (urolithins) that are found in higher levels in circulation compared to their precursor, after consumption of food rich in ellagic acid content (Kang et al., 2016; Ryu et al., 2016; Saha et al., 2016; Yuan et al., 2016). There is a significant interest in therapeutic applications of urolithin A, such as prostate, bladder, colon cancers as well as cardiovascular conditions (Zhou et al., 2016; Liberal et al., 2017; Tang et al., 2017). Further, the availability of chemical synthesis for urolithin A makes it an attractive candidate for investigating therapeutic applications.

To our knowledge, this is the first study to examine the potential of urolithin A treatment for the prevention of cisplatin-induced nephrotoxicity in a rat model using the precursor compound ellagic acid as a reference. In order to provide an insight of the mechanism of the renal protection associated to urolithin A, kidney injury was examined by histology, renal function, apoptosis, as well as different molecular mediators, including T cell Ig and mucin domain-containing protein (TIM)-1, NF- κ B, nitric oxide synthase 3 (NOS3), ionized calcium-binding adapter molecule 1 (Iba1) and various inflammatory cytokines.

Materials and methods

REAGENTS

Cisplatin (Shaanxi Sciphar Hi-Tech Industry Co., Ltd., China); urolithin A (AGN-PC-0LQZ0Q, Angene International Limited, UK); ellagic acid (Sigma-Aldrich, USA); anti-TIM 1 antibody [EPR6383(2)] - N-terminal ab190696 (ABCAM, MA, USA); anti- β -actin loading control monoclonal antibody (BA3R), goat anti-rabbit and goat anti-mouse IgG (H+L) secondary antibodies, HRP (Thermo Fisher Scientific, IL, USA); Coomassie Protein assay reagent (Bio-Rad, CA, USA); NOS3 (C-20): sc-654 and NF- κ B p65 (F-6): sc-8008 (Santa Cruz Biotechnology, Inc., TX, USA); anti-Iba1 antibody (Wako Chemicals USA, Inc., VA, USA).

ANIMALS AND EXPERIMENTAL DESIGN

Male Sprague Dawley rats (200 – 300 g) were purchased from Harlan, USA. All experimental procedures were approved by Texas A&M University Institutional Animal Care and Use Committee (protocol number IACUC 2014-0106). Animals were maintained in standard housing environment and had free access to food and water during the study. After acclimatization, rats were randomly divided into four groups (n=6) as follows: group I, negative control; group II, positive control (cisplatin, CIS); groups III and IV, ellagic acid/cisplatin (EA/CIS) and urolithin A/cisplatin (UA/CIS), respectively. The first day of the experiment, the groups II, III and IV received a single intraperitoneal dose of cisplatin (5 mg/kg body weight) dissolved in saline. Six hours prior to cisplatin administration, groups III and IV were orally treated with either ellagic acid or urolithin A (50 mg/kg body weight) suspended in 0.5% (w/v) carboxymethyl cellulose aqueous

solution, followed by a daily dose of these compounds during the next 5 days. Animal weights were recorded throughout the experiment. On day 7, the animals were euthanized by CO₂ asphyxiation followed by cervical dislocation. Blood samples were taken by heart puncture, collected into K₂EDTA coated tubes and centrifuged at 956 × g/4 °C for 30 min for plasma separation (stored at -80 °C until analysis). After perfusion with saline via circulatory system, both kidneys were collected, weighed and longitudinally cut into two sections, one section was used for histology (stored in 10% formalin) and the other section was used for molecular analysis (frozen immediately in liquid nitrogen and stored at -80 °C).

BIOCHEMICAL ANALYSIS

Creatinine levels in plasma were measured using a quantitative ELISA kit specific to rat following manufacturer's protocol (MyBioSource, Inc., CA, USA). Plasma samples were diluted at a ratio of 1:4 using the sample dilution buffer provided by the manufacturer.

HISTOLOGICAL EXAMINATION

Formalin-fixed kidneys were embedded in paraffin and sectioned into 4 μm thickness. These sections were stained with hematoxylin-eosin (H&E) and periodic acid–Schiff (PAS) reagents for histological examination. Tissue sections were evaluated using bright field microscopy at 40 X original magnification (ACCU-SCOPE® 3000-LED Microscope). Five representative microscopy images taken from different rats within the same group were analyzed using ImageJ software in order to measure PAS positive area and results were expressed as average percentage of total area.

WESTERN BLOT

Kidneys were homogenized in PBS at final tissue concentration of 10% (w/v) and centrifuged at $20817 \times g/4$ °C for 30 min. Supernatant was collected and protein concentration was determined using Coomassie protein assay. Approximately 20 µg of protein were loaded into a 4-15% 1-D SDS polyacrylamide gel and subjected to electrophoresis. Next, the proteins were transferred to a nitrocellulose membrane and unreacted sites were blocked with 5% non-fat milk for 1 h at room temperature. The membranes were incubated with the primary antibody (TIM-1 at 1:10000 and β-actin at 1:2000) overnight at 4 °C, and then washed with TBST (mixture of tris-buffered saline (TBS) and Tween 20) and incubated with the appropriate secondary antibody for another 2 h at room temperature. Chemiluminescent detection method was used to visualize protein signals. The band images were obtained by ChemiDoc imaging system (Bio-Rad) and were analyzed by densitometry using ImageLab software. β-actin was used to normalize the protein loading for each sample.

IMMUNOHISTOCHEMISTRY

Paraffin-kidney sections were heated at 60 °C for 1 h, and then were washed twice with xylene for 10 min to ensure paraffin removal. The sections were rehydrated in serial ethanol dilutions (100%, 95%, 70%). After washing the slides in water, antigen retrieval was achieved by boiling the tissue sections in sodium-citrate buffer (0.01 M, pH 6.0) 3 times for 5 min in a microwave. The slides were cooled down to room temperature, washed with PBS and then, blocked and stained using a commercial kit for detecting primary antibodies (ImmunoCruz™ rabbit ABC Staining System: sc-2018, Santa Cruz Biotechnology, Inc., TX, USA). Kidney sections were incubated with primary antibodies

specific to TIM-1 (1:200), NF- κ B p65, NOS3 and Iba1 (1:100) overnight at 4 °C and all other staining steps were performed according to the manufacturer's instructions.

Tissue sections were counterstained with hematoxylin, dehydrated and mounted for microscopy analysis. Images were taken with a bright field microscope at original magnification of 40 X (ACCU-SCOPE® 3000-LED Microscope).

MULTIPLEX IMMUNOASSAY

The quantification of different cytokines in plasma and kidney homogenates were performed using ProcartaPlex™ Immunoassay Kit specific to rat, based on magnetic beads technology (eBioscience, USA), following the manufacturer's instructions. The cytokine concentrations on each sample were measured using a microplate reader (Bio-Plex® 200 System, Bio-Rad) and the values obtained were normalized by the protein content in case of kidney homogenates.

IMMUNOFLUORESCENCE

Kidney sections were deparaffinized, rehydrated and washed as previously described for immunohistochemistry staining. After antigen retrieval, tissue section were stained with Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture using the in situ cell death detection kit (Roche, Mannheim, Germany). Slides were mounted using Vectashield Antifade mounting media containing DAPI. Tissue sections were imaged using a confocal laser scanning microscope (Zeiss LSM 780) at 40 X original magnification. TUNEL positive cells were quantified using ImageJ software and results were expressed as average of at least 22 images for each group.

STATISTICAL ANALYSIS

The data are expressed as mean values \pm standard deviation. Statistical analysis was performed using GraphPad Prism version 5.00 (GraphPad Software, CA, USA). For plasma creatinine levels and quantification of PAS positive area, Mann–Whitney *U* test was used to compare different groups. For all other experiments, one-way analysis of variance followed by Tukey's multiple comparison test was performed. Significant differences were considered when $p < 0.05$.

Results

EFFECT OF CISPLATIN ON BODY AND KIDNEY WEIGHTS

Body weight of control rats significantly increased (~17%) during the experiment. However, this increase in body weight was marginal in animals that received cisplatin and cisplatin with intervention (~5%). The relative kidney weights for all cisplatin groups, including those received intervention were significantly higher than control animals indicative of cisplatin-induced renal damage (Supplemental Tables 1 and 2).

RENAL FUNCTION AND HISTOLOGICAL FINDINGS

Cisplatin controls showed a significant rise in plasma creatinine levels compared to negative control group. A significant reduction was observed in creatinine levels in rats treated with ellagic acid or urolithin A (Figure 1A). Specifically, kidney histological examination revealed degeneration of the tubules leading to cell detachment towards to the tubular lumen in cisplatin controls, and such changes were not observed in the intervention groups (Figure 1B). The morphological tubular damage was further confirmed by PAS-staining showing epithelial necrosis reflected by loss of brush border

membrane, prominent PAS staining in tubular cells located toward to the lumen of the tubules and significant accumulation of PAS positive material in the medullary region of kidneys in cisplatin control (Figure 2A). Animals treated with ellagic acid showed moderate protection, while urolithin A offered significant protection (Figure 2B).

IMMUNOLOGICAL AND ANTI-INFLAMMATORY FINDINGS

Renal expression of TIM-1 was found to be significantly decreased in rats treated with ellagic acid and urolithin A (Figure 3A-C). Additionally, the immunohistochemical staining for NF- κ B, a transcription factor, was prominent in cells detached from renal tubules and localized into the lumen, as can be observed in cisplatin control kidney sections (Figure 4A). In contrast, intervention groups displayed decreased staining in kidney sections suggesting the prevention of NF- κ B expression (Figure 4A). The expression of NOS3 was preserved in renal tubules in the intervention groups, while a decrease in the expression of the enzyme was observed in kidneys of cisplatin controls (Figure 4B). Furthermore, the immunohistochemical analysis for Iba1, a protein specifically expressed in macrophages, showed a marked staining in renal tubular cells of cisplatin control animals, indicating the enhanced macrophage infiltration in the damaged tissue (Figure 5A). Interestingly, rats treated with urolithin A dramatically reduced the Iba1 expression at the tubular region of the kidney, which was almost comparable to negative control animals, while ellagic acid failed to attenuate (Figure 5A). These findings further correlated with the expression of various inflammatory cytokines in kidney homogenates (Figures 5B-J and Supplemental Figure 1). Specifically, urolithin A treatment decreased the expression of pro-inflammatory cytokines, such as TNF- α , IL-6, INF- γ , IL-1 α , IL-1 β , IL-13, IL-17A and IL-2. In contrast,

ellagic acid treatment failed to reduce these levels (Figures 5B-I). Further, both urolithin A and ellagic acid treatments showed a marked increase in the levels of the anti-inflammatory cytokine, IL-10 (Figure 5J). These changes in the cytokine levels were localized in kidney tissue and no systemic inflammatory response was observed in plasma samples reflected by the absence of these cytokines.

APOPTOSIS EVALUATION

As shown in Figure 6A, a significant increase in the number of apoptotic cells localized in the tubular area was found in cisplatin control kidneys. In contrast, treatment with urolithin A and ellagic acid considerably decreased the tubular apoptotic cells in the kidney sections Figure 6B, suggesting the anti-apoptotic activity of these compounds in tubular injury. Overall, urolithin A offered better protection compared to ellagic acid in preventing cisplatin-induced nephrotoxicity by inhibiting inflammation and apoptosis.

Discussion

Cisplatin, since its first licensed use in 1978, has been widely established in the treatment of various cancers. However, to date there has been a limited advance in addressing the concerns related to cisplatin-induced nephrotoxicity (Loehrer and Einhorn, 1984). Alternative safer platinum-based analogs were developed but they were not as effective as cisplatin (Ho et al., 2016). The pathogenesis of cisplatin nephrotoxicity is characterized by a renal dysfunction based on a rise of creatinine levels in plasma due to the decrease in the glomerular filtration rate, and is attributed to different mechanisms involving pro-apoptotic signaling, oxidative stress and inflammation (Ramesh and Reeves, 2002; Hanigan and Devarajan, 2003; Yao et al.,

2007). Overcoming inflammatory response through effective therapeutic agent is considered a significant approach to prevent nephrotoxicity (Ramesh and Reeves, 2002; Pabla and Dong, 2008). However, to date, there is no clear consensus on the therapeutic management of cisplatin-induced nephrotoxicity (Weijl et al., 2004; Baek et al., 2015; Yamamoto et al., 2016).

The dose of cisplatin used to induce renal damage in this study (5 mg/kg) was selected based on previous literature (El-Garhy et al., 2014; Kursunluoglu et al., 2014). The doses of urolithin A and ellagic acid were selected based on prior studies (Espín et al., 2013; García-Nino and Zazueta, 2015) as well as keeping in mind the translational feasibility for conventional dosage forms for oral administration (50 mg/kg, Human Equivalent Dose:~520 mg for 65 kg person (FDA, 2005).

The histological and biochemical markers studied are reliable indicators in the experimental model (Nematbakhsh et al., 2013), which is also evident in our cisplatin-treated animals. Urolithin A and, to a lesser extent, ellagic acid improved renal function along with kidney morphology by attenuating the cisplatin-induced nephrotoxicity, which is localized in tubules and reflected by severe loss of brush borders membrane, detachment of cells towards the lumen of the tubules and also prominent accumulation of PAS positive material in the site of the renal injury.

TIM-1, also known as kidney injury molecule-1 (KIM-1), is a protein markedly expressed by proximal tubular cells in the injured kidney. It has been proposed as potential biomarker to detect and predict acute tubular injury in different stages of drug development and patient care since this protein is also found in urine and is stable over a prolong period of time (Vaidya et al., 2010). TIM-1 signaling has been involved in

promoting cisplatin nephrotoxicity by T-cell activation and cytokine secretion and its inhibition is demonstrated to be highly protective (Nozaki et al., 2011). In our findings we showed that the administration of urolithin A or its precursor, ellagic acid, was able to suppress the renal protein expression of TIM-1, suggesting it as a potential target. The transcription factor NF- κ B has been implicated in the pathogenesis of cisplatin as a regulator of inflammation. The oxidative stress generated by this drug in the renal cells activates NF- κ B pathway leading to stimulation of synthesis of many pro-inflammatory mediators (Schrier, 2002; Ozkok and Edelstein, 2014). Interestingly, both interventions, urolithin A and ellagic acid, showed marked reduction of NF- κ B signaling in the renal tissue, however, only urolithin A was also able to downregulate various cytokines involved in the pro-inflammatory cascade and immune response. This observation demonstrated that urolithin A specifically targeted NF- κ B-induced pro-inflammatory response pathway. Since macrophages are involved in the release of cytokines, such inhibitory activity of these mediators obtained with urolithin A may be due to its capacity to reduce macrophage infiltration in the tubular area of the kidney in cisplatin-induced nephrotoxicity. Moreover, it has been suggested that the upregulation of endogenous anti-inflammatory cytokine IL-10 produced by dendritic cells protect the kidney against cisplatin damage and its contribution to suppress pro-inflammatory cytokines (Tadagavadi and Reeves, 2010). Our observations showed that both urolithin A and ellagic acid treatments led to significant increase in the renal IL-10 levels suggesting that these compounds may have direct anti-inflammatory effect in the pro-inflammatory response produced by cisplatin in the kidney tissue. Along with these findings, the interventions were also able to reduce the number of apoptotic cells in the renal tubules

probably mediated by the inhibition of NF- κ B, which is involved in the cell survival pathway. Interestingly, this anti-apoptotic effect was more evident in case of urolithin A, suggesting that NF- κ B is direct target and through modulating this pathway, it is able to attenuate the damage in cisplatin-induced nephrotoxicity.

NOS3, known as endothelial nitric oxide synthase, is highly expressed in renal vascular endothelium but also is expressed in the cortex and medulla of proximal tubules (Mount and Power, 2006). Its activity has been associated to the renal protective effect of ischemic preconditioning against the ischemia/reperfusion-induced acute renal failure in a mouse model (Yamasowa et al., 2005). Here we demonstrate that cisplatin reduced the NOS3 expression in the kidney explained by a decrease in the renal blood flow likely to occur in early cisplatin-induced acute renal failure (Winston and Safirstein, 1985). Since nitric oxide synthase system is responsible for NO production, which regulates sodium and water homeostasis in the kidney (Mount and Power, 2006), any alteration in this pathway contributes to worsening of the tubular damage. This finding is in agreement with previous studies that showed decreased expression of NOS3 in cisplatin-induced acute kidney injury in rats, and such expression was enhanced by α -lipoic acid (Bae et al., 2009) and vitamin E (Darwish et al., 2017), suggesting the beneficial role of this enzyme in cisplatin-induced nephrotoxicity. Urolithin A and ellagic acid were able to reverse the effect of cisplatin-induced NOS3 modulation.

In summary, our data support the hypothesis that urolithin A is a better therapeutic agent than its precursor ellagic acid, in mitigating cisplatin-induced nephrotoxicity. However, further studies are needed to understand the intricacies and inter-play of the molecular mechanism proposed in this research work. Overall, urolithin A demonstrates

a significant potential as a good adjuvant for renal protection in patients receiving nephrotoxic drugs, such as cisplatin.

Acknowledgments

We are grateful to Dr. Gus A. Wright, Department of Veterinary Pathobiology (VTPB), Texas A&M University, for providing access to the Digital Imaging Core Facility and Dr. Andrew Hillhouse, Texas A&M Institute for Genome Sciences and Society, for providing access to Bio-plex 200 system.

Authorship Contribution

Participated in research design: Guada, Ganugula, Vadhanam and Majeti

Conducted experiments: Guada, Ganugula

Performed data analysis: Guada, Ganugula and Majeti

Wrote or contributed to the writing of the manuscript: Guada, Ganugula, Vadhanam and Majeti

References

- Al-Kharusi N, Babiker H, Al-Salam S, Waly M, Nemmar A, Al-Lawati I, Yasin J, Beegam S and Ali B (2013) Ellagic acid protects against cisplatin-induced nephrotoxicity in rats: a dose-dependent study. *Eur Rev Med Pharmacol Sci* 17:299-310.
- Apps MG, Choi EH and Wheate NJ (2015) The state-of-play and future of platinum drugs. *Endocr Relat Cancer* 22:R219-233.
- Ateşşahin A, Çeribaşı AO, Yuce A, Bulmus Ö and Çikim G (2007) Role of ellagic acid against cisplatin-induced nephrotoxicity and oxidative stress in rats. *Basic Clin Pharmacol Toxicol* 100:121-126.
- Bae EH, Lee J, Ma SK, Kim IJ, Frokiaer J, Nielsen S, Kim SY and Kim SW (2009) alpha-Lipoic acid prevents cisplatin-induced acute kidney injury in rats. *Nephrol Dial Transplant* 24:2692-2700.
- Baek SH, Kim SH, Kim JW, Kim YJ, Lee KW and Na KY (2015) Effects of a DPP4 inhibitor on cisplatin-induced acute kidney injury: study protocol for a randomized controlled trial. *Trials* 16:239.
- Barabas K, Milner R, Lurie D and Adin C (2008) Cisplatin: a review of toxicities and therapeutic applications. *Vet Comp Oncol* 6:1-18.
- Bhat ZY, Cadnapaphornchai P, Ginsburg K, Sivagnanam M, Chopra S, Treadway CK, Lin HS, Yoo G, Sukari A and Doshi MD (2015) Understanding the Risk Factors and Long-Term Consequences of Cisplatin-Associated Acute Kidney Injury: An Observational Cohort Study. *PLoS One* 10:e0142225.

Boulikas T (2009) Clinical overview on Lipoplatin: a successful liposomal formulation of cisplatin.

Expert Opin Investig Drugs 18:1197-1218.

Ciccolini J, Mercier C, Dahan L and Andre N (2011) Integrating pharmacogenetics into

gemcitabine dosing--time for a change? Nat Rev Clin Oncol 8:439-444.

Darwish MA, Abo-Youssef AM, Khalaf MM, Abo-Saif AA, Saleh IG and Abdelghany TM (2017)

Vitamin E mitigates cisplatin-induced nephrotoxicity due to reversal of oxidative/nitrosative stress, suppression of inflammation and reduction of total renal platinum accumulation. J Biochem Mol Toxicol 31:1-9.

Dilruba S and Kalayda GV (2016) Platinum-based drugs: past, present and future. Cancer

Chemother Pharmacol 77:1103-1124.

El-Garhy AM, Abd El-Raouf OM, El-Sayeh BM, Fawzy HM and Abdallah DM (2014) Ellagic acid

antiinflammatory and antiapoptotic potential mediate renoprotection in cisplatin nephrotoxic rats. J Biochem Mol Toxicol 28:472-479.

Espín JC, Larrosa M, García-Conesa MT and Tomás-Barberán F (2013) Biological significance of

urolithins, the gut microbial ellagic acid-derived metabolites: the evidence so far. Evid Based Complement Alternat Med 2013.

FDA (2005) Guidance for industry: estimating the maximum safe starting dose in initial clinical

trials for therapeutics in adult healthy volunteers. Rockville, MD, USA.

García-Nino WR and Zazueta C (2015) Ellagic acid: pharmacological activities and molecular

mechanisms involved in liver protection. Pharmacol Res 97:84-103.

Hanigan MH and Devarajan P (2003) Cisplatin nephrotoxicity: molecular mechanisms. Cancer

Ther 1:47-61.

- Ho GY, Woodward N and Coward JI (2016) Cisplatin versus carboplatin: comparative review of therapeutic management in solid malignancies. *Crit Rev Oncol Hematol* 102:37-46.
- Kang I, Buckner T, Shay NF, Gu L and Chung S (2016) Improvements in Metabolic Health with Consumption of Ellagic Acid and Subsequent Conversion into Urolithins: Evidence and Mechanisms. *Adv Nutr* 7:961-972.
- Koyner JL, Sher Ali R and Murray PT (2008) Antioxidants. Do they have a place in the prevention or therapy of acute kidney injury? *Nephron Exp Nephrol* 109:e109-117.
- Kursunluoglu G, Kayali HA and Taskiran D (2014) The effect of cisplatin toxicity and capsaicin on electron transport chain in liver and kidney of sprague dawley rats. *Cell Biochem Biophys* 69:707-716.
- Lecumberri E, Dupertuis YM, Miralbell R and Pichard C (2013) Green tea polyphenol epigallocatechin-3-gallate (EGCG) as adjuvant in cancer therapy. *Clin Nutr* 32:894-903.
- Liberal J, Carmo A, Gomes C, Cruz MT and Batista MT (2017) Urolithins impair cell proliferation, arrest the cell cycle and induce apoptosis in UMUC3 bladder cancer cells. *Invest New Drugs*.
- Loehrer PJ and Einhorn LH (1984) Drugs five years later. Cisplatin. *Ann Intern Med* 100:704-713.
- Mathijssen RH, Sparreboom A and Verweij J (2014) Determining the optimal dose in the development of anticancer agents. *Nat Rev Clin Oncol* 11:272-281.
- Mount PF and Power DA (2006) Nitric oxide in the kidney: functions and regulation of synthesis. *Acta Physiol (Oxf)* 187:433-446.

- Nematbakhsh M, Ashrafi F, Nasri H, Talebi A, Pezeshki Z, Eshraghi F and Haghghi M (2013) A model for prediction of cisplatin induced nephrotoxicity by kidney weight in experimental rats. *J Res Med Sci* 18:370-373.
- Nozaki Y, Nikolic-Paterson DJ, Yagita H, Akiba H, Holdsworth SR and Kitching AR (2011) Tim-1 promotes cisplatin nephrotoxicity. *Am J Physiol Renal Physiol* 301:F1098-1104.
- Ozkok A and Edelstein CL (2014) Pathophysiology of cisplatin-induced acute kidney injury. *Biomed Res Int* 2014:967826.
- Pabla N and Dong Z (2008) Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. *Kidney Int* 73:994-1007.
- Ramesh G and Reeves WB (2002) TNF-alpha mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. *J Clin Invest* 110:835-842.
- Ryu D, Mouchiroud L, Andreux PA, Katsyuba E, Moullan N, Nicolet-Dit-Felix AA, Williams EG, Jha P, Lo Sasso G, Huzard D, Aebischer P, Sandi C, Rinsch C and Auwerx J (2016) Urolithin A induces mitophagy and prolongs lifespan in *C. elegans* and increases muscle function in rodents. *Nat Med* 22:879-888.
- Saha P, Yeoh BS, Singh R, Chandrasekar B, Vemula PK, Haribabu B, Vijay-Kumar M and Jala VR (2016) Gut Microbiota Conversion of Dietary Ellagic Acid into Bioactive Phytochemical Urolithin A Inhibits Heme Peroxidases. *PLoS One* 11:e0156811.
- Schrier RW (2002) Cancer therapy and renal injury. *J Clin Invest* 110:743-745.
- Sharma G, Italia JL, Sonaje K, Tikoo K and Ravi Kumar MN (2007) Biodegradable in situ gelling system for subcutaneous administration of ellagic acid and ellagic acid loaded

nanoparticles: evaluation of their antioxidant potential against cyclosporine induced nephrotoxicity in rats. *J Control Release* 118:27-37.

Sonaje K, Italia JL, Sharma G, Bhardwaj V, Tikoo K and Kumar MN (2007) Development of biodegradable nanoparticles for oral delivery of ellagic acid and evaluation of their antioxidant efficacy against cyclosporine A-induced nephrotoxicity in rats. *Pharm Res* 24:899-908.

Tadagavadi RK and Reeves WB (2010) Endogenous IL-10 attenuates cisplatin nephrotoxicity: role of dendritic cells. *J Immunol* 185:4904-4911.

Tang L, Mo Y, Li Y, Zhong Y, He S, Zhang Y, Tang Y, Fu S, Wang X and Chen A (2017) Urolithin A alleviates myocardial ischemia/reperfusion injury via PI3K/Akt pathway. *Biochem Biophys Res Commun* 486:774-780.

Vaidya VS, Ozer JS, Dieterle F, Collings FB, Ramirez V, Troth S, Muniappa N, Thudium D, Gerhold D, Holder DJ, Bobadilla NA, Marrer E, Perentes E, Cordier A, Vonderscher J, Maurer G, Goering PL, Sistare FD and Bonventre JV (2010) Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. *Nat Biotechnol* 28:478-485.

Weijl NI, Elsendoorn TJ, Lentjes EG, Hopman GD, Wipkink-Bakker A, Zwinderman AH, Cleton FJ and Osanto S (2004) Supplementation with antioxidant micronutrients and chemotherapy-induced toxicity in cancer patients treated with cisplatin-based chemotherapy: a randomised, double-blind, placebo-controlled study. *Eur J Cancer* 40:1713-1723.

Winston JA and Safirstein R (1985) Reduced renal blood flow in early cisplatin-induced acute renal failure in the rat. *Am J Physiol* 249:F490-496.

Yamamoto Y, Watanabe K, Tsukiyama I, Yabushita H, Matsuura K and Wakatsuki A (2016) Hydration with 15 mEq Magnesium Is Effective at Reducing the Risk for Cisplatin-induced Nephrotoxicity in Patients Receiving Cisplatin (≥ 50 mg/m²) Combination Chemotherapy. *Anticancer Res* 36:1873-1877.

Yamasowa H, Shimizu S, Inoue T, Takaoka M and Matsumura Y (2005) Endothelial nitric oxide contributes to the renal protective effects of ischemic preconditioning. *J Pharmacol Exp Ther* 312:153-159.

Yao X, Panichpisal K, Kurtzman N and Nugent K (2007) Cisplatin nephrotoxicity: a review. *Am J Med Sci* 334:115-124.

Yuan T, Ma H, Liu W, Niesen DB, Shah N, Crews R, Rose KN, Vatter DA and Seeram NP (2016) Pomegranate's Neuroprotective Effects against Alzheimer's Disease Are Mediated by Urolithins, Its Ellagitannin-Gut Microbial Derived Metabolites. *ACS Chem Neurosci* 7:26-33.

Zhou B, Wang J, Zheng G and Qiu Z (2016) Methylated urolithin A, the modified ellagitannin-derived metabolite, suppresses cell viability of DU145 human prostate cancer cells via targeting miR-21. *Food Chem Toxicol* 97:375-384.

Footnotes

Melissa Guada and Raghu Ganugula contributed equally.

Conflict of interest

The authors declare no conflicts of interest.

FIGURES LEGENDS

Figure 1. Preventive effect of urolithin A and ellagic acid on cisplatin-induced renal dysfunction and histopathological damage. **(A)** Plasma creatinine levels and **(B)** histological changes in kidney tissue were observed by hematoxylin and eosin staining from each group (n=6) on day 6 after cisplatin administration to rats (arrows indicate tubular injury). Images were captured using bright field microscope at 40 × magnification. * = $p < 0.05$ compared to cisplatin group; Mann–Whitney *U* test.

Figure 2. Protective effect of urolithin A and, in lesser extent, ellagic acid on tubular morphology of kidneys injured by cisplatin exposure. **(A)** Kidney sections stained with Periodic Acid–Schiff (PAS) obtained from each group (n=6) on day 6 after cisplatin administration to rats (arrows indicate tubular damage and magenta colored-spots represent PAS positive area). Representative images are shown at 40 × original magnification. **(B)** Quantitative analysis of PAS positive area reflecting the degree of tubular injury. ** = $p < 0.01$ compared to cisplatin group; Mann–Whitney *U* test.

Figure 3. Urolithin A and ellagic acid regulate TIM-1 protein expression in cisplatin-induced nephrotoxicity. **(A)** Representative image of western blot of TIM-1 expression in kidney homogenates for each group (n=6) on day 6 after cisplatin administration to rats. **(B)** Relative band intensity analysis of TIM-1 expression of western blots normalized by β -actin. **(C)** Immunohistochemical staining of TIM-1 in kidney sections (golden brown colored-areas indicate positive staining; 40 × magnification). ** = $p < 0.01$ and *** = $p <$

0.001 compared to cisplatin group; one-way analysis of variance followed by Tukey's multiple comparison test.

Figure 4. Anti-inflammatory and pro-survival effects of urolithin A and ellagic acid in cisplatin-induced kidney damage. **(A,B)** Representative images of immunohistochemical staining of NF-kB p65 and NOS3 in kidney sections, respectively, for each group (n=6) on day 6 after cisplatin administration to rats (golden brown colored-areas indicate positive staining). Images were captured using bright field microscope at 40 × magnification.

Figure 5. Effect of urolithin A and ellagic acid on macrophage infiltration and modulation of inflammatory cytokines in cisplatin induced-nephrotoxicity. **(A)** Representative image of immunohistochemical staining of Iba1 in kidney sections (golden brown colored-areas indicate positive staining; 40 × original magnification). **(B-J)** Cytokine levels in kidney homogenates for each group (n=6) on day 6 after cisplatin administration to rats. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ compared to cisplatin group; one-way analysis of variance followed by Tukey's multiple comparison test.

Figure 6. Protective effect of urolithin A and ellagic acid on tubular cell apoptosis in cisplatin-induced kidney injury. **(A)** Apoptotic cells were determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) (green); nuclei were visualized by DAPI staining and shown in blue from each group (n=6) on day 6

after cisplatin administration to rats. Representative images are shown at 40 × original magnification. **(B)** Quantification of TUNEL positive cells. *** = $p < 0.001$ compared to cisplatin group; one-way analysis of variance followed by Tukey's multiple comparison test.

Figure 1.

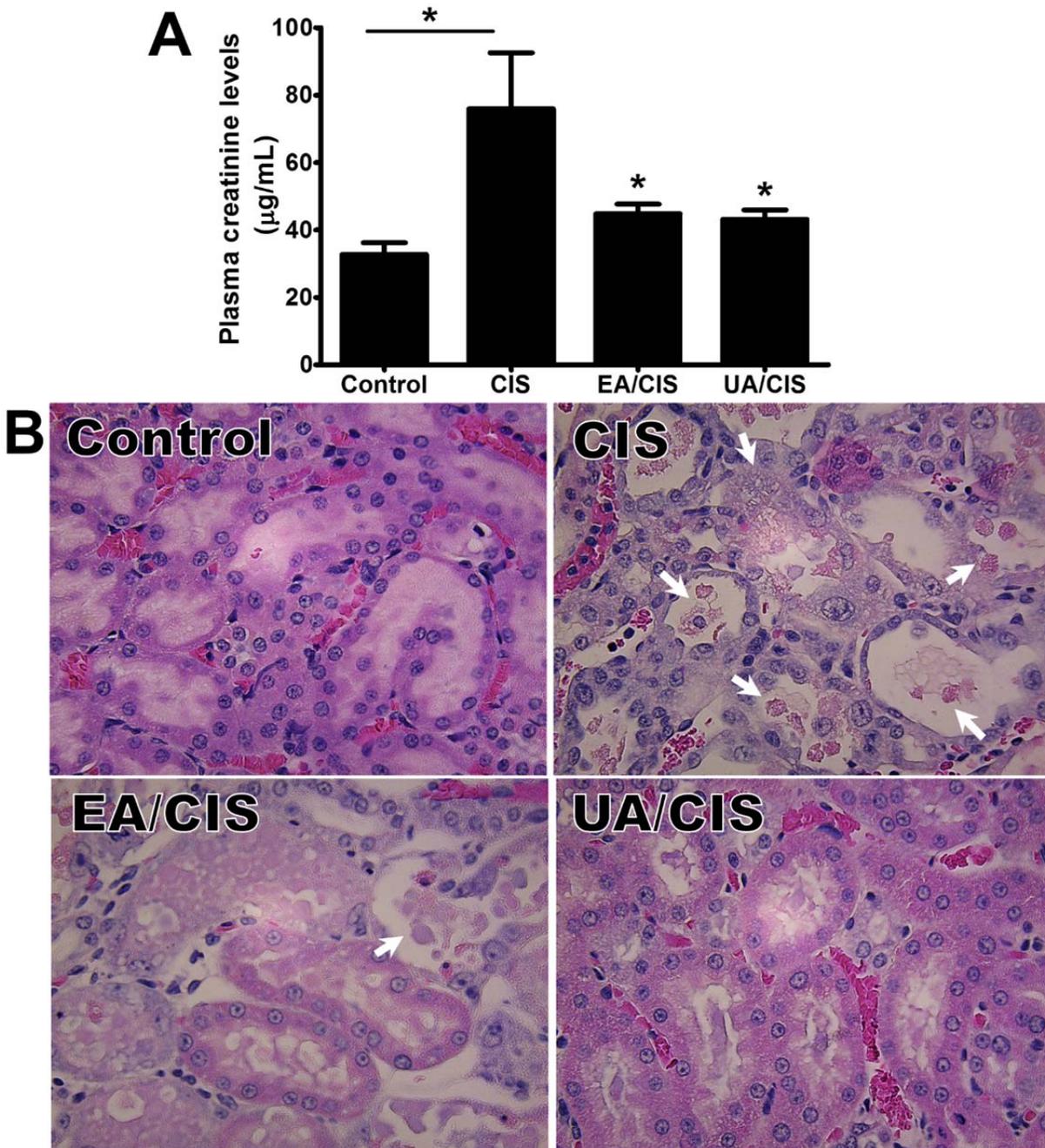


Figure 2.

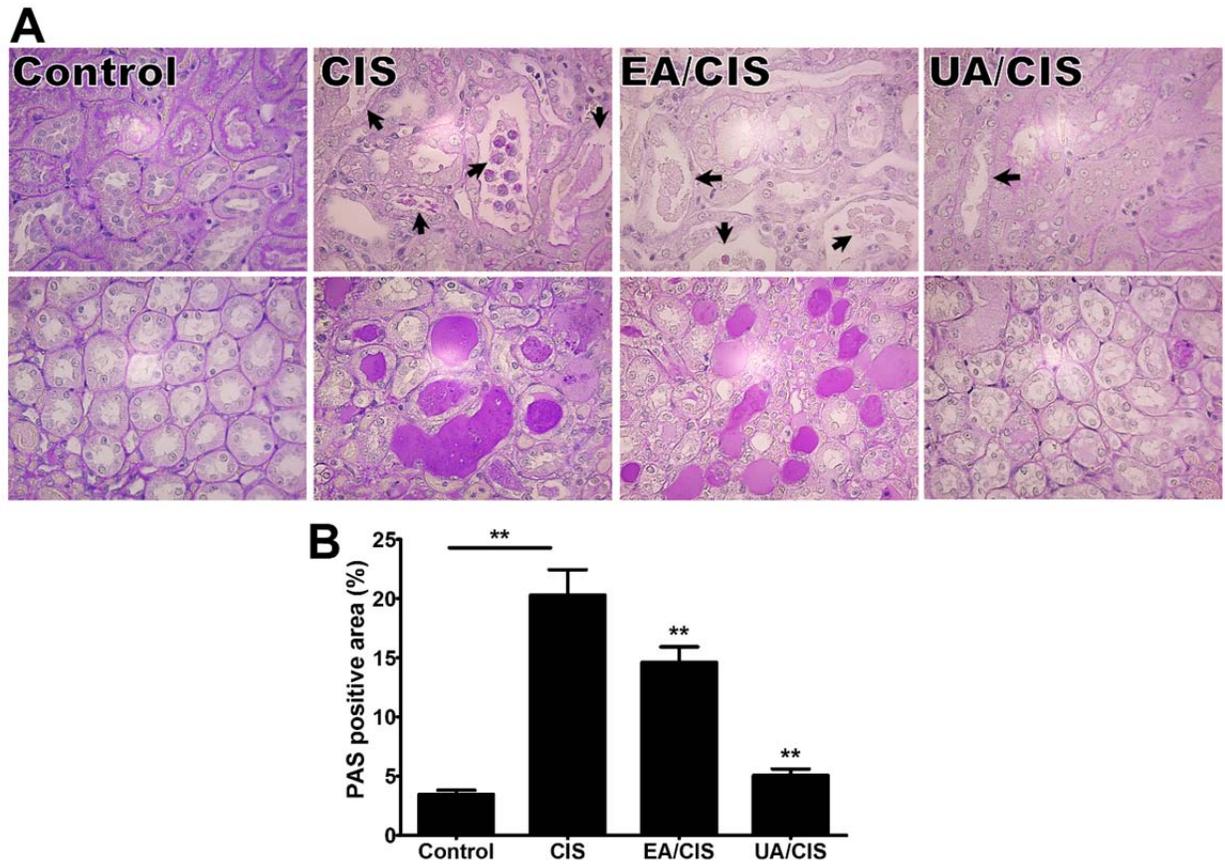


Figure 3.

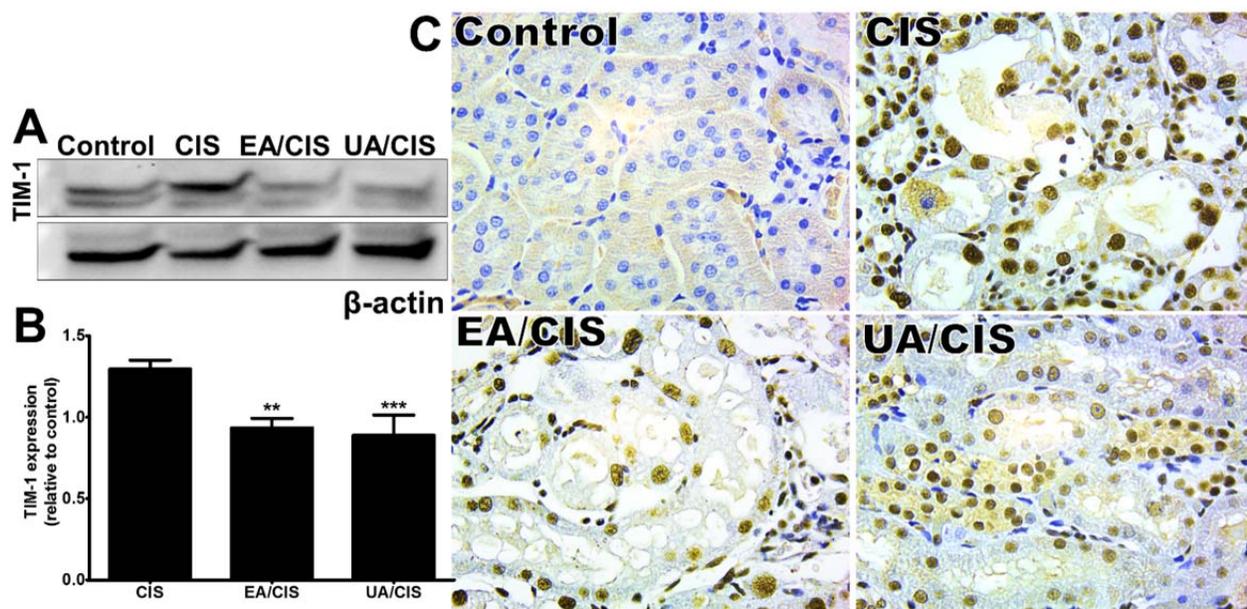


Figure 4.

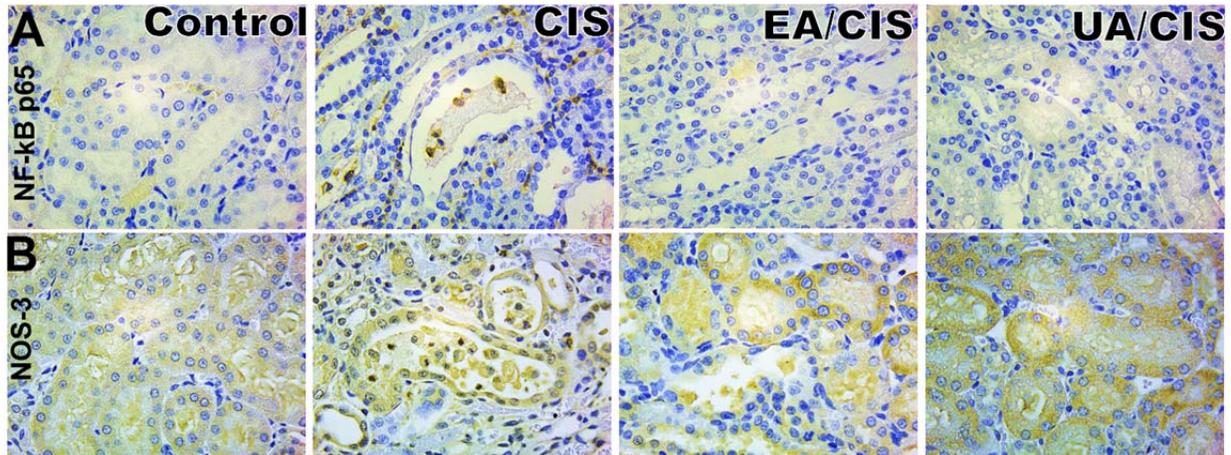


Figure 5.

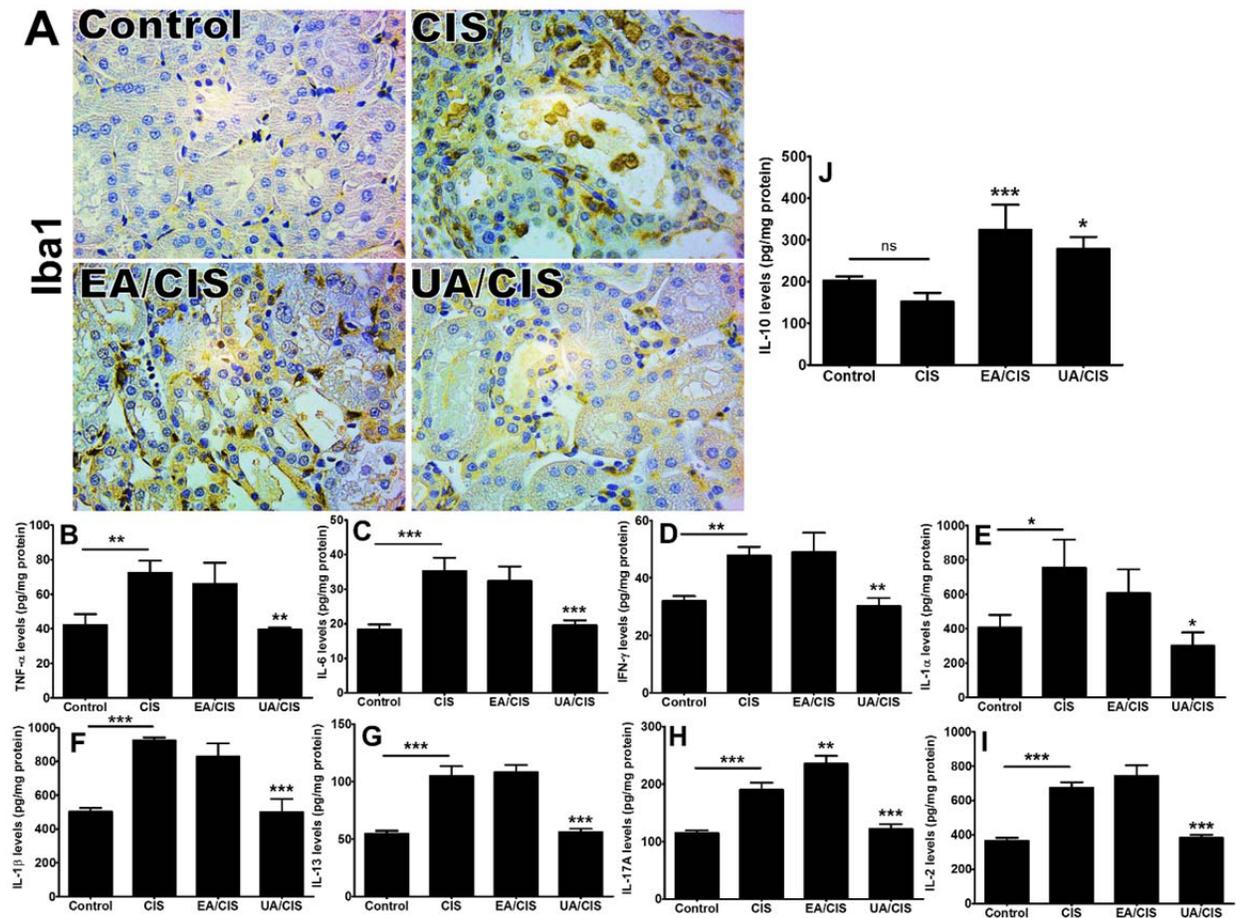


Figure 6.

