Screening for Early Biomarkers of Cisplatin-Induced Acute Kidney Injury in Rats Through Serum Metabolomics Technology

Jinru Yang[1], Mingkang Zhang[2], Fenglin Ran[2], Xueyan Gou[1], Yanrong Ma[3] and Xinan Wu[1,3]

[1]*The First Clinical Medical College of Pharmacy, Lanzhou University, Gansu, China*
[2]*The Pharmacy of Lanzhou University, Gansu, China*
[3]*Department of Pharmacy, The First Hospital of Lanzhou University, Gansu, China*

ABSTRACT

Objective: To systematically identify early biomarkers of cisplatin-induced acute kidney injury (AKI) in rats.
Study Design: An experimental study.
Place and Duration of the Study: Experimental Animal Laboratory of Lanzhou University, Gansu, China, and the Department of Pharmacy, The First Hospital of Lanzhou University, Gansu, China, from July 2022 to October 2023.
Methodology: In this study, an AKI model was established by continuously injecting cisplatin into rats at a dose of 1 mg/kg once a day for control group and for 2, 3, 4, and 5 days to other four groups, respectively. Subsequently, rat plasma samples were collected for metabolomics analysis to identify early differentiated metabolites in the plasma prior to creatinine elevation. Furthermore, accurate HPLC-MS/MS methods were developed to validate the biomarker variation in other AKI models.
Results: The occurrence of time-dependent renal cortical injury and significant alterations of creatinine (Cr) concentration were observed on day-4 and 5, which demonstrated successful model construction. Sixty-six compounds changed on Day-2 while 61 compounds changed on Day-3. Eleven compounds with variable importance in projection (VIP) >1.5 and false discover rate (FDR) <0.2 were selected and identified by HPLC-MS/MS. Among these, *N*-acetylglutamine and citramalic acid changed earlier than serum creatinine (sCr) in the AKI model.
Conclusion: *N*-acetylglutamine and citramalic acid may serve as early biomarker of cisplatin-induced AKI.

Key Words: *Acute kidney injury, Biomarker, Cisplatin, Metabolomics, LC-MS/MS, Rats.*

How to cite this article: Yang J, Zhang M, Ran F, Gou X, Ma Y, Wu X. Screening for Early Biomarkers of Cisplatin-Induced Acute Kidney Injury in Rats Through Serum Metabolomics Technology. *J Coll Physicians Surg Pak* 2024; **34(08)**:936-941.

INTRODUCTION

Acute kidney injury (AKI) is a common clinical emergency characterised by increased serum creatinine (sCr) levels and decreased urine output within 7 days, leading to a rapid decline in kidney function.[1] Despite advances in medical science, the mortality rate associated with AKI remains high and has been increasing over the past 50 years, resulting in significant socio-economic and public health impacts.[2] The clinical assessment of AKI largely depends on the sCr level,[3] a breakdown product of creatine and phosphocreatine, most of which is freely filtered by the glomeruli and has been the gold standard for nearly a century.[4] However, sCr has several limitations as an indirect indicator of renal injury, particularly a time lag in identifying damage.

Correspondence to: Dr. Xinan Wu, The First Hospital of Lanzhou University, Gansu, China
E-mail: wuxa@lzu.edu.cn
Received: April 20, 2024; Revised: July 02, 2024; Accepted: July 15, 2024
DOI: https://doi.org/10.29271/jcpsp.2024.08.936

Additionally, sCr levels may remain unaltered even in cases of severe renal impairment if the patient has sound underlying renal function.[5] Therefore, identifying additional markers to assess the pathological condition of the kidney and supplementing and confirming creatinine (Cr) evaluation is positively significant for the prevention and treatment of AKI.

Cisplatin, a highly effective metal-containing chemotherapeutic agent, is extensively used to treat various types of solid tumours. Reports indicate that the annual global sales of platinum chemotherapeutic agents exceed two billion dollars, with nearly 50% of cancer patients being treated with cisplatin.[6,7]

Metabolomics, which analyses small molecular metabolites, uses advanced techniques in analytical chemistry to detect alterations in metabolic profiles. The rapid development of meta-bolomics has led to the discovery of numerous disease bio-markers and markers for various organ injuries. In the case of cisplatin-induced AKI, specific proteins and metabolites are produced and accumulate in urine. These metabolites may serve as early markers reflecting cisplatin-induced AKI.[8,9] However, no studies identified to identify early or predictive metabolomic markers for cisplatin-induced AKI. The objective of this study was to systematically identify early biomarkers of cisplatin-induced AKI in rats.

METHODOLOGY

This experimental study was conducted from 1st July 2022 to 30th October 2023 in the Experimental Animal Laboratory of Lanzhou University, and the Department of Pharmacy, The First Hospital of Lanzhou University. Review Committee's approval was obtained (Approval no. LDYYLL2021-168, Dated: 3rd March 2021).

Citramalic acid, acetonitrile, and methanol were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Cisplatin was purchased from the First Hospital of Lanzhou University. Proline, leucine, *N*-acetylglutamine, threonic acid, 1-methylhistidine, glyceric acid, 3-Hydroxybutyric acid, glutaconic acid, and L-isovelarylcarnitine were purchased from Energy Chemical Co., Ltd. Shanghai, China. Chromatography-grade acetonitrile and methanol were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), along with all other analytical-grade chemicals.

Specific pathogen-free (SPF) male SD rats, weighing 222 - 235 g, were obtained from the Veterinary Research Institute, Academy of Agricultural Sciences (license number: SCXK-2022-0002). The rats were housed in suitable enclosures under conditions of 24°C temperature and 40% humidity, with a light / dark cycle of 12 hours. They had unrestricted access to water and food.

After 7 days of adaptation, the rats were randomly divided into five groups; a control group and four model groups. The control group was injected with saline 0.9% once, the Day 2 group was injected with cisplatin (1 mg/kg) once a day for 2 days, while the Day 3, Day 4, and Day 5 groups were injected with cisplatin (1 mg/kg) once a day for 3 days, 4 days, and 5 days, respectively. Each rat was anaesthetised and weighed, and then blood samples were collected *via* the abdominal aortic method 12 hours after the last injection of cisplatin. The kidneys were dissected at the same time.

Additionally, other rats were divided into five groups: The control group received intraperitoneal injections of 0.9% normal saline; the model group received intraperitoneal injections of lipopolysaccharide (LPS, 10 mg/kg) to induce septic AKI. The control group underwent anaesthesia, and blood collection occurred 4 hours after injection, while the model groups received LPS injections at 1 hour, 2 hours, and 3 hours, respectively. Blood collection took place after a total of 4 hours under anaesthesia.

Similarly, rats were divided into five groups. The control group received a single intraperitoneal injection of 0.9% normal saline, while the model groups received intraperitoneal injections of gentamicin (80 mg/kg) once daily for 2, 3, 5, and 7 consecutive days, respectively. Anaesthesia and blood collection were performed 12 hours after the final administration.

The kidney tissues were carefully collected and subsequently fixed in a 4% paraformaldehyde solution for morphological analysis using haematoxylin and eosin (H&E) staining. Following centrifugation of the blood samples at 3,000 rpm for 10 minutes at 4°C, plasma was separated and stored at -80°C for metabolic analysis.

The kidney tissues were fixed in a 4% formaldehyde solution for a duration of 7 days. Following fixation, the tissues were embedded in paraffin and subsequently sliced into sections measuring 5 µm in thickness. These sections were then subjected to staining with H&E and examined under a BX51 light microscope. Subsequently, an impartial researcher, who was unaware of the experimental groupings evaluated the pathological alterations observed in the kidney tissue.

All endogenous compounds were quantified using UPLC. For liquid chromatography, a Xevo TQS series UPLC system with an ACQUITY UPLC™ BEH C18 1.7 µm analytical column (2.1*150 mm, 3.5µm) was used. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile (B) with a flow rate of 0.4 ml/min. The elution procedure was as follows: 0-1 min (95% A), 1.1-11min (95-22% A), 11.1-13.5 min (22-5% A), 13.6-14 min (5-0% A), and finally from 14.l-16 min to reach back to the initial condition of the mobile phase at a concentration of 95% A until the end of the run at the 18th minute. Column temperature was maintained at a constant value of 30 ℃.

The quantification of the eleven potential differential metabolites was performed by HPLC using an HPLC system from Waters, MA, USA, equipped with a Waters HPLC Agilent C18 analytical column measuring dimensions of 2.l *150 mm and particle size being equal to 3.5 µm. The mobile phase consisted of water containing 0.l % formic acid (A) and methanol (B). Column temperature was maintained at a constant value of 30℃.

The levels of kidney injury molecule-1 (KIM-1) were quantified using the enzyme-linked immunosorbent assay (ELISA). Biochemical indicators, such as creatinine, cystatin C (Cys-C), and blood urea nitrogen (BUN), were measured using reagent test kits and a biochemical automatic analyzer. The experimental procedures followed the manufacturer's instructions. Briefly, sCr levels were determined by employing the sarcosine oxidase substrate method, while BUN levels were assessed using urease-glutamate dehydrogenase substrates.

Statistical analysis of potential biomarkers was conducted using SPSS 19.0 (IBM Corp., USA). The data were analysed using the independent samples t-tests, non-parametric tests, and one-way ANOVA analysis. Results with p-values less than 0.05 or 0.01 were considered statistically significant.

RESULTS

As depicted in Figure 1A, the examination of body weight indicated that cisplatin exhibited a decelerating effect on the decline in body weight when compared to the control group rats. By day 5, the body weight of rats in the model group was comparatively lower.

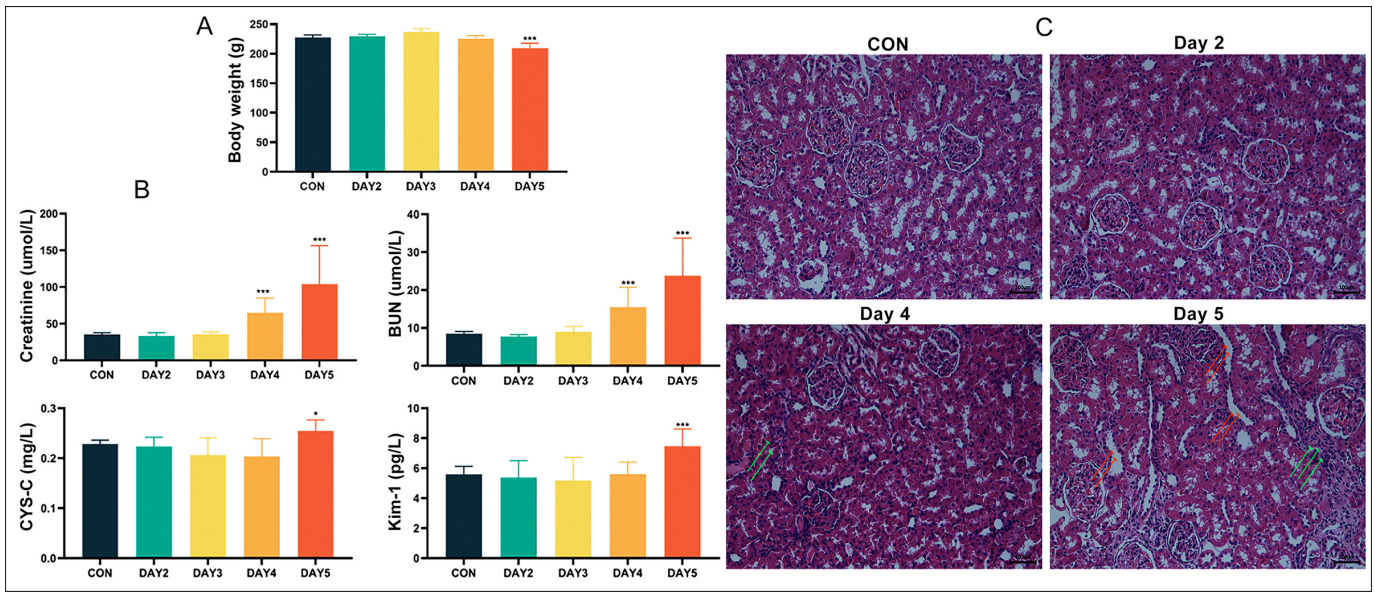

Figure 1: Physiological and pathological evaluation of cisplatin-induced AKI (A) Analysis of body weight between control and treatment groups; (B) Determination of serum biochemical indices sCr, BUN, Cys-C, and KIM-1 in rats treated with cisplatin; (C) Histological observation by H&E staining of the kidney. (n = 6, *p <0.05, **p <0.01, ***p <0.001, *vs.* control).

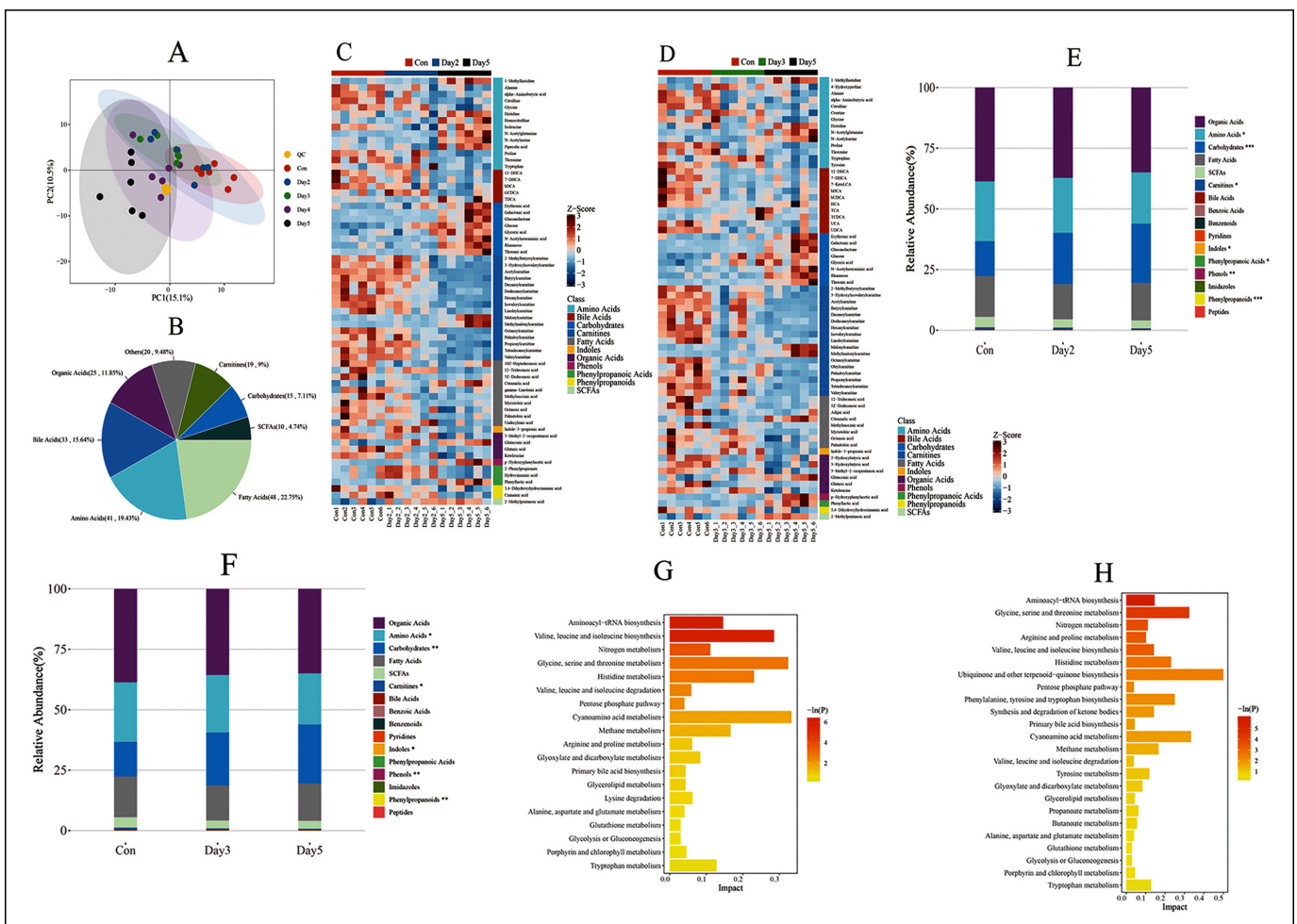

Figure 2: Metabonomics of rat serum (A) PCA score plots of experimental rats after cisplatin treatment; (B) The types of compounds in the differential metabolome; (C) Heatmaps of metabolic profiles in the serum of day 2; (D) Heatmaps of metabolic profiles in the serum of day 3. Red colours indicate an increase while blue indicates a decrease; (E) Relative abundance changes in serum of day 2; (F) Relative abundance changes in serum of day 3; (G) Metabolic pathway analysis in the serum of day 2; (H) Metabolic pathway analysis in the serum of day 3 (n = 5).

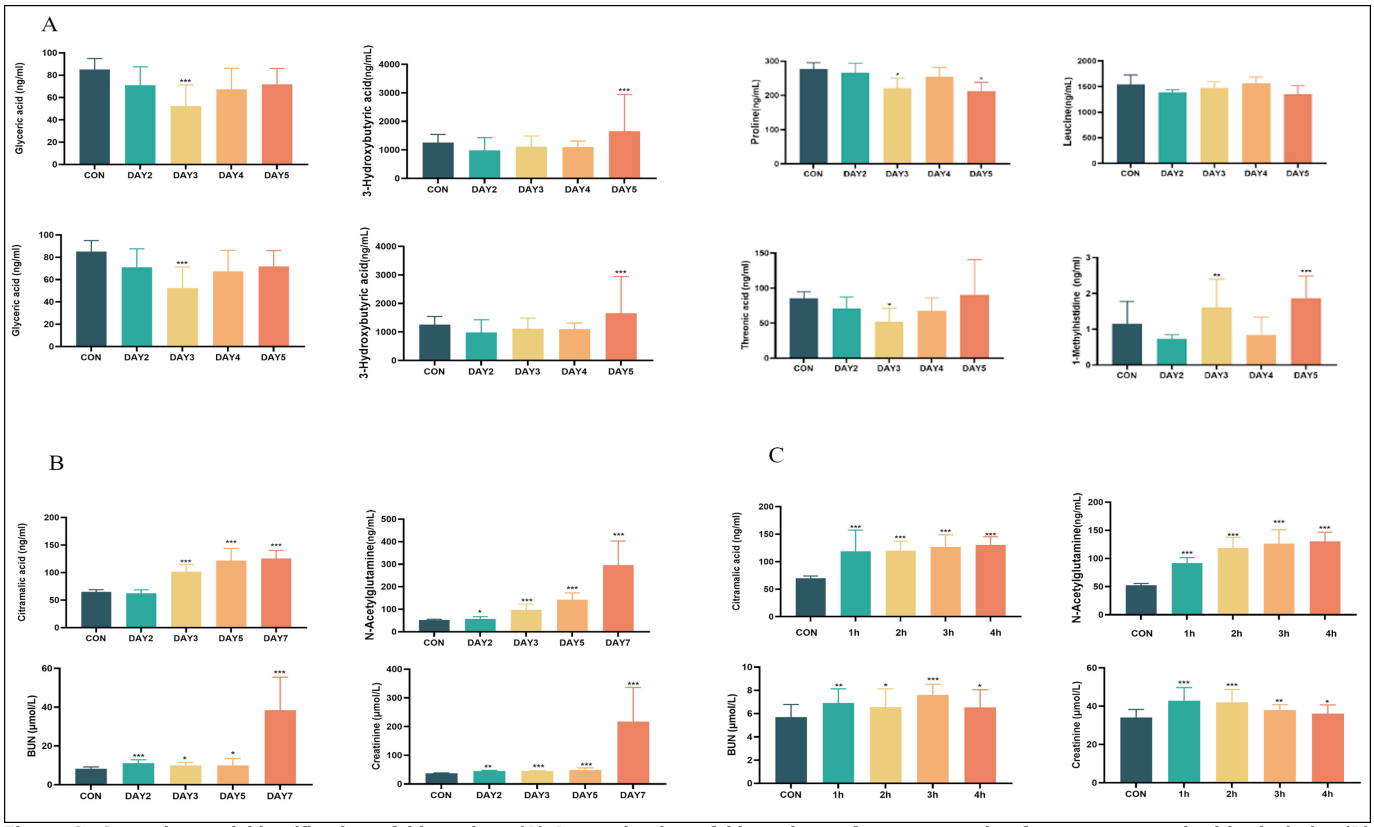

Figure 3: Screening and identification of biomarker; (A) Determination of biomarkers of serum samples from rats treated with cisplatin; (B) Determination of available biochemical indices: *N*-acetylglutamine and citramalic acid of serum samples from rats treated with gentamicin; (C) *N*-acetylglutamine and citramalic acid of serum samples from rats treated with LPS (n=6, *p <0.05, **p <0.01, ***p <0.001, *vs.* control).

The concentration of Cr and BUN was increased significantly on day 4 and day 5, while the concentration of Cys-C and KIM-1 significantly increased on day-5 with rats in the control group (Figure 1B). Reversible changes in kidney morphology were observed over time, indicating the presence of injury. By day 5, there was obvious inflammation (green arrow) and tubular dilatation (red arrow). This was consistent with the trend in sCr (Figure 1C).

To assess changes in biochemical parameters due to cisplatin, serum was collected for metabolomic analysis. Cisplatin treatment on day 5 resulted in a distinct separation of principal component analysis (PCA) score plots for the control group (Figure 2A). The proportion of differential metabolite components extracted from the metabolome is illustrated in Figure 2B. Moreover, an examination uncovered alterations in the metabolic profile over time when exposed to cisplatin-induced toxicity, with some metabolites significantly altered earlier than creatinine on day 2 (Figure 2C).

On day 2 after cisplatin administration, a total of 66 serum metabolites exhibited significant differences based on the p <0.05, FDR <0.2, and VIP >1. To investigate their evolutionary patterns, correlated heat maps were generated. Figure 2D depicts changes in the relative abundance of various small molecules. The time dependence of carbohydrate, carnitine, and phenylpropionic acid was significantly reduced, while amino acid and carbohydrate showed a significant increase. Similarly, on day 3, a total of 61 different metabolites were identified from serum (Figure 2 E and F), meeting the inclusion criteria: p <0.05, FDR <0.2, and VIP >1.

Furthermore, Metaboanalyst was utilised to perform metabolic pathway analysis, which revealed changes in metabolites present in the serum and identified modified pathways within the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Figure 2G and H). The analysis revealed alterations in the biosynthesis of aminoacyl-tRNA, as well as the biosynthesis and breakdown of valine, leucine, and isoleucine. Additionally, changes were observed in the metabolism of glycine, serine, and threonine along with cyanoamino acid metabolism during day 2 and 3.

To identify a biomarker with better sensitivity than creatinine, eleven biomarkers were screened that changed significantly and simultaneously after day 2 and day 3 of cisplatin administration. An HPLC-MS/MS method was established to identify and quantify these 11 biomarkers. The authors demonstrated that threonic acid and citramalic acid exhibited a time-dependent increase, starting on day 3

compared to the controls; *N*-acetylglutamine also showed an increase from day 3 onwards; erythronic acid, proline, leucine, and serine underwent slight changes on day 2, 3, and 4 (Figure 3A). To verify the universality of threonic acid, citramalic acid and *N*-acetylglutamine, sepsis- and gentamicin-induced AKI models were established. The results show that the concentrations of *N*-acetylglutamine and citramalic acid remained significantly increased after AKI, while threonic acid did not change (Figure 3 B and C).

DISCUSSION

In this study, a systematic biological approach was employed to analyse key metabolites and their metabolic pathways by assessing the plasma of rats treated with cisplatin. There were significant changes in metabolites in the plasma of rats after cisplatin treatment, but pathological damage and creatinine levels did not show significant differences. Compared with the control group, 66 and 61 compounds on day 2 and day 3 were changed in AKI rats, respectively. After validation, the authors found that changes in *N*-acetylglutamine and citrulline levels preceded changes in Cr levels and could potentially be an early biomarker.

The VIP of tryptophan and rhamnose were greater than those of *N*-acetylglutamine and citramalic acid, which suggested a greater contribution in AKI. However, the authors did not identify these as a potential biomarker because of an inconsistent trend, in which they increased on day 2 and decreased on day 5. Similarly, the VIP of carnitine was greater than that of *N*-acetylglutamine, but its trace amount in the measured plasma and its time-dependent decline suggested that it is not suitable as a biomarker. Threonic acid showed a time-dependent increase in rat plasma (Figure 3A), but there were large individual differences in human plasma, limiting its value as a biomarker. As the concentration of *N*-acetylglutamine is stable in healthy people, they have the best potential to serve as an early biomarker of AKI.

Citramalic acid is a fatty acid and has long been found to increase significantly in concentration in patients with bacterial meningitis.[10] Zhang *et al.* reported that the concentration of citramalic acid in the serum of diabetic nephropathy patients is increased, and it may serve as a potential marker of diabetic nephropathy.[11] Sirtuin 3 (sirt3), nuclear farnesoid X, and Sirtuin 5 (sirt5) modulate fatty acid oxidation and attenuate cisplatin-induced AKI by protecting the proximal tubule,[12-14] while fatty acid-binding protein 4 modulates fatty acid levels and attenuates sepsis-induced AKI.[15] This study found that citramalic acid was increased in cisplatin-induced AKI and sepsis AKI, indicating that the above proteins may contribute to the metabolism of citramalic acid.

Arendowski *et al.* reported that *N*-acetylglutamine can be used as a metabolic biomarker for renal cancer.[16] It was also reported that its levels were significantly increased in the urine of mice with sepsis-induced AKI, a finding consistent with this study's results; its plasma concentration was also significantly increased in rats with sepsis-induced AKI (Figure 3C). The potential of glucose as a marker of AKI has been reported,[17] but *N*-acetylglutamine appears to have better specificity. Unfortunately, the upstream and downstream metabolic pathways for this compound are still unknown.

The molecular mechanism of cisplatin-AKI has not been determined. There are possible mechanisms by which organic transporters increase drug uptake by the kidney;[18] this was followed by cisplatin-mediated decreases in the expression and function of the sodium-dependent glucose and amino acid transporter.[19] In addition, cisplatin is metabolised to cisplatin-glutathione and the cisplatin-cysteinyl-glycine conjugate is also a factor.[20] Production of reactive oxygen species may also contribute to AKI.[21] Portilla *et al.* reported that inhibition of fatty acid oxidation in the proximal tubule induces hyperlipidaemia and accumulation of triglycerides and non-esterified fatty acids in the kidney.[22] Luo *et al.* demonstrated that the mRNA level and enzyme activity of acyl-CoA dehydrogenase in mitochondria were significantly decreased.[23] Hu *et al.* showed that the activation of MYH9 by upregulation of APE2 expression is an important cause of cisplatin-induced AKI.[24] The role and mechanism of citramalic acid and *N*-acetylglutamine in AKI remain to be determined.

CONCLUSION

Analysis of the metabolic pathways of differentiation metabolites in the plasma found that a change in *N*-acetylglutamine and citramalic acid levels preceded the change in creatinine in cisplatin-AKI. An LC MS/MS method developed and replicated this finding in sepsis- and gentamicin-induced AKI models which suggest that these metabolites may serve as an early biomarker of AKI.

ETHICAL APPROVAL:
The present study received an ethical approval from the Ethics Committee of the First Hospital of Lanzhou University, Gansu, China with an assigned (Approval letter number: LDYYLL2021-168).

PATIENTS' CONSENT:
Informed and written consent was obtained from the patients.

COMPETING INTEREST:
The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:
XW: Conceptualisation, formal analysis, project administration, and resources.
JY, MZ, XG: Data curation.

FR: Investigation.
JY: Methodology and writing the original draft.
XW, JY, MZ, YM: Writing, reviewing, and editing.
All authors approved the final version of the manuscript to be published.

REFERENCES

1. Khwaja A. KDIGO clinical practice guidelines for acute kidney injury. *Nephron Clin Pract* 2012; **120(4)**:c179-84. doi: 10.1159/000339789.
2. Verma S, Kellum JA. Defining acute kidney injury. *Crit Care Clin* 2021; **37(2)**:251-66. doi: 10.1016/j.ccc.2020.11.001.
3. Wu I, Parikh CR. Screening for kidney diseases: Older measures *versus* novel biomarkers. *Clin J Am Soc Nephrol* 2008; **3(6)**:1895-901. doi: 10.2215/CJN.02030408.
4. Ronco C, Bellomo R, Kellum JA. Acute kidney injury. *Lancet* 2019; **394(10212)**:1949-64. doi: 10.1016/S0140- 6736 (19)32563-2.
5. Kashani K, Rosner MH, Ostermann M. Creatinine: From physiology to clinical application. *Eur J Intern Med* 2020; **72**:9-14. doi: 10.1016/j.ejim.2019.10.025.
6. Care W, Grenet G, Schmitt C, Michel S, Langrand J, Le Roux G, *et al.* Adverse effects of licorice consumed as food: An update. *Rev Med Interne* 2023; **44(9)**:487-94. doi: 10.1016/j.revmed.2023.03.004.
7. Ghosh S. Cisplatin: The first metal based anticancer drug. *Bioorg Chem* 2019; **88**:102925. doi: 10.1016/ j.bioorg. 2019.102925.
8. Sanz AB, Sanchez-Nino MD, Ramos AM, Ortiz A. Regulated cell death pathways in kidney disease. *Nat Rev Nephrol* 2023; **19(5)**:281-99. doi: 10.1038/s41581-023-00694-0.
9. Lin W, Mousavi F, Blum BC, Heckendorf CF, Moore J, Lampl N, *et al*. Integrated metabolomics and proteomics reveal biomarkers associated with hemodialysis in end-stage kidney disease. *Front Pharmacol* 2023; **14**:1243505. doi: 10.3389/fphar.2023.1243505.
10. Perlman S, Carr SA. Citramalic acid in cerebrospinal fluid of patients with bacterial meningitis. *Clin Chem* 1984; **30(7)**:1209-12. doi: 10.1093/clinchem/30.7.1209.
11. Zhang B, Wan Y, Zhou X, Zhang H, Zhao H, Ma L, *et al*. Characteristics of serum metabolites and gut microbiota in diabetic kidney disease. *Front Pharmacol* 2022; **13**: 872988. doi: 10.3389/fphar.2022.872988.
12. Li M, Li CM, Ye ZC, Huang J, Li Y, Lai W, *et al*. Sirt3 modulates fatty acid oxidation and attenuates cisplatin-induced AKI in mice. *J Cell Mol Med* 2020; **24(9)**:5109-21. doi: 10.1111/jcmm.15148.
13. Chiba T, Peasley KD, Cargill KR, Maringer KV, Bharathi SS, Mukherjee E, *et al*. Sirtuin 5 regulates proximal tubule fatty acid oxidation to protect against AKI. *J Am Soc Nephrol* 2019; **30(12)**:2384-98. doi: 10.1681/ASN. 2019020163.
14. Xu S, Jia P, Fang Y, Jin J, Sun Z, Zhou W, *et al*. Nuclear farnesoid X receptor attenuates acute kidney injury through fatty acid oxidation. *Kidney Int* 2022; **101(5)**: 987-1002. doi: 10.1016/j.kint.2022.01.029.
15. Wang B, Xu J, Ren Q, Cheng L, Guo F, Liang Y, *et al*. Fatty acid-binding protein 4 is a therapeutic target for septic acute kidney injury by regulating inflammatory response and cell apoptosis. *Cell Death Dis* 2022; **13(4)**:333. doi: 10.1038/s41419-022-04794-w.
16. Arendowski A, Ossolinski K, Niziol J, Ruman T. Screening of urinary renal cancer metabolic biomarkers with gold nanoparticles-assisted laser desorption/ionization mass spectrometry. *Anal Sci* 2020; **36(12)**:1521-5. doi: 10. 2116/analsci.20P226.
17. Izquierdo-Garcia JL, Nin N, Cardinal-Fernandez P, Rojas Y, de Paula M, Granados R, *et al*. Identification of novel metabolomic biomarkers in an experimental model of septic acute kidney injury. *Am J Physiol Renal Physiol* 2019; **316(1)**:F54-62. doi: 10.1152/ajprenal.00315.2018.
18. McSweeney KR, Gadanec LK, Qaradakhi T, Ali BA, Zulli A, Apostolopoulos V. Mechanisms of cisplatin-induced acute kidney injury: Pathological mechanisms, pharmacological interventions, and genetic mitigations. *Cancers (Basel)* 2021; **13(7)**:1572. doi: 10.3390/cancers13071572.19.
19. Xu EY, Perlina A, Vu H, Troth SP, Brennan RJ, Aslamkhan AG, *et al*. Integrated pathway analysis of rat urine metabolic profiles and kidney transcriptomic profiles to elucidate the systems toxicology of model nephrotoxicants. *Chem Res Toxicol* 2008; **21(8)**:1548-61. doi: 10.1021/tx 800061w.
20. Townsend DM, Deng M, Zhang L, Lapus MG, Hanigan MH. Metabolism of cisplatin to a nephrotoxin in proximal tubule cells. *J Am Soc Nephrol* 2003; **14(1)**:1-10. doi: 10.1097/ 01. asn.0000042803.28024.92.
21. Mirzaei S, Hushmandi K, Zabolian A, Saleki H, Torabi SMR, Ranjbar A, *et al*. Elucidating role of reactive oxygen species (ROS) in cisplatin chemotherapy: A focus on molecular pathways and possible therapeutic strategies. *Molecules* 2021; **26(8)**:2382. doi: 10.3390/molecules26082382.
22. Portilla, D, Li, S. Y, Nagothu, K, Ranganathan, G, Ali, S. M, Shank, B, *et al.* Reduced kidney lipoprotein lipase and renal tubule triglyceride accumulation in cisplatin-mediated acute kidney injury. *Am J Physiol Renal* 2012; **303(1)**:3-10. doi: 10.1152/ajprenal.00111.2012 .1931-12.
23. Luo R, Onyshchenko K, Wang L, Gaedicke S, Grosu AL, Firat E, *et al*. Necroptosis-dependent immunogenicity of cisplatin: Implications for enhancing the radiation-induced abscopal effect. *Clin Cancer Res* 2023; **29(3)**:667-83. doi: 10.1158/1078-0432.CCR-22-1591.
24. Hu Y, Yang C, Amorim T, Maqbool M, Lin J, Li C, *et al*. Cisplatin-mediated upregulation of APE2 binding to MYH9 provokes mitochondrial fragmentation and acute kidney injury. *Cancer Res* 2021; **81(3)**:713-23. doi: 10.1158/0008-5472.CAN-20-1010.