Urinary metabolomic markers of aminoglycoside nephrotoxicity
in newborn rats

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Abstract

Background—Aminoglycoside exposure is a common cause of acute kidney injury (AKI). Delay in the diagnosis of AKI using conventional biomarkers has been one of the important obstacles in applying early effective interventions. We tested the hypothesis that urinary metabolomics could identify novel early biomarkers for toxic renal injury.

Methods—3 days old rats were divided into 3 groups; they received a single daily injection of vehicle (0.9% NaCl solution) or gentamicin at a dose of 10 or 20 mg/kg/day for 7 days. Urine and blood were collected after 3 and 7 days of injections. Urinary metabolites were evaluated using High-performance liquid chromatography and gas chromatography/mass spectrometry.

Results—A distinct urinary metabolic profile characterized by glucosuria, phosphaturia and aminoaciduria was identified preceding changes in serum creatinine. At both gentamicin doses, urinary tryptophan was significantly (p<0.05) increased; fold change (1.91 and 2.31 after 3d, 1.81 and 1.93 after 7d). Similarly, kynurenic acid, a tryptophan metabolite, showed a significant (p<0.05) decrease, fold change (0.26 and 0.24 after 3d, 0.21 and 0.52 after 7d), suggesting interruption of the normal tryptophan metabolism pathway.

Conclusion—We conclude that urinary metabolomic profiling provides a robust approach for identifying early and novel markers of gentamicin induced AKI.

Introduction

Acute kidney injury (AKI) in the intensive care setting is multifactorial and is an important and common contributor to morbidity and mortality (1). The current approach to determine renal injury is based upon the two classical biochemical markers, serum creatinine and blood
urea nitrogen (BUN). Although these indices are valid indicators of renal function, they have several limitations including: the long time lag between initial renal injury and creatinine rise, inability to differentiate specific site of renal injury and the unreliability of creatinine measurement with increasing degree of renal injury. In the neonatal population these issues are compounded by initial postnatal creatinine reflecting maternal values and the changes in creatinine clearance as growth and renal development occur. Thus, kidney injury goes undetected until there is a highly significant reduction in renal function. Early, sensitive and specific markers of kidney injury are therefore needed (2,3). Furthermore, understanding pathologic pathways underlying the development of AKI in neonates and other high-risk populations is critical for uncovering new, targeted treatment modalities that may alter the disease progression by allowing for earlier intervention.

Metabolomics refers to the systematic study of small-molecule metabolites and their changes in biological samples due to physiological stimuli or genetic modification (4). As an emerging field, metabolomics has a potential essential role in the search for useful biomarkers of kidney injury (5). Mass spectrometry (MS) is used to identify metabolites after separation by either gas chromatography (GC), or high-performance liquid chromatography (HPLC) (6). The combination of these techniques is well suited for probing a very large part of the urine metabolome as they are capable of detecting both lipophilic and hydrophilic metabolites (7).

Nephrotoxic-medication exposure is a common cause of AKI. Gentamicin is one of the most effective and widely used antibiotic agents against gram-negative bacterial infections. However, it induces toxic damage to the proximal convoluted tubules in the kidney. The aim of this discovery study was 1) to demonstrate a proof of concept that global metabolomic profiling of the urine of rats is a reliable and consistent tool to identify metabolites, 2) to identify and characterize changes in urinary metabolites following gentamicin-induced kidney injury in newborn rats. Rats were selected to study because the rat kidney continues to develop until about 3 weeks after birth, in a similar pattern to the postnatal renal development that occurs in preterm neonates (8).

RESULTS

Histopathology and clinical chemistry

Low dose gentamicin (10 mg/kg/day) had very little effect on the histology of the kidneys, only minimal focal tubular necrosis was observed after 7 days of injections. However, the rats treated with the higher dose of gentamicin (20 mg/kg/day) exhibited more distinguishable changes after 7 days of exposure (Figure 1). These changes included degeneration of some proximal convoluted tubules with loss of the brush borders on the surface, damaged mitochondria and tubular casts.

In both gentamicin injected groups, a significant increase in BUN and serum creatinine compared to controls was not evident after 3 days of injections, but was noted after 7 days. These data are summarized in Table 1.
Metabolomic analysis of urine by HPLC/MS

The urinary metabolomic data obtained via LC/MS revealed marked metabolic alterations related to gentamicin injections at both day 3 and day 7 time points, in both positive and negative modes. The identity of four metabolites was confirmed by HPLC/MS/MS product ion analysis of the putative biomarkers and comparison with authentic standards. Table 2 lists the metabolites that showed a statistically significant fold-change difference between control and treated rats with their corresponding retention time and postulated identity.

The LC/MS peak at retention time 9.1 min with an (M+H)+ ion of mass-to-charge ratio (m/z) 204 showed a significant (p<0.05) increase after gentamicin injection; therefore it was investigated further by tandem mass spectrometry (MS/MS) and was identified as tryptophan. The two principal ions showing a reduction in positive-ion ESI after gentamicin injections were those for m/z 190 and m/z 206, eluting at 9.75 min and 10.1 min, The (MS/MS) analysis confirmed the identity of these metabolites as kynurenic acid and xanthurenic acid, respectively. The ion of m/z 178 in negative ion mode that decreased significantly (p<0.05) was identified as hippuric acid. These changes in metabolic profile are consistent with those observed by Lenz et al. (9) in adult rats.

Metabolomic analysis of urine by GC/MS

Data analysis from GC/MS was also able to separate treated animals from controls, clear time-dependent effect was evident. Metabolites that showed changes to gentamicin administration are listed in Table 3. Identification was performed by comparing mass spectral fragmentation patterns with those found in a NIST (National Institute of Standards and Technology) mass spectral database and confirmed by coeluting authentic reference standards, when available. The results showed a marked differentiation in the abundance of several metabolites after the administration of the drug, suggesting that several biochemical metabolic pathways are significantly altered after 3 days of the administration of gentamicin. Care must be applied with this interpretation as glucosuria and phosphaturia may simply reflect tubular injury due to aminoglycoside toxicity. In the case of our data, the presence of a reciprocal change in tryptophan metabolites between control and the 3 day gentamicin injected group suggests an interruption of the metabolic pathway itself. The identified metabolites belonged to several distinct chemical families: carbohydrates, lipids, amino acids, organic acids and nucleotides. At day 3 after gentamicin injection there was a 3.15-fold increase in urine glucose levels in the low dose group, and a 3.12-fold increase in the high dose group when compared to control rats. The significant increase in oligosaccharides induced by gentamicin after 3 days is an indicator of tubular necrosis. Several amino acids including glutamine, glycine and alanine showed a significant increase in both gentamicin injected rats starting from day 3, the pattern of metabolites changes was similar at the two time points.

Multivariate data analysis

The integrated peak areas of the chromatographic profiles (all masses in the corresponding mass spectral profile contributes to the area) from all samples in all time windows were combined to form a data matrix, which was subjected to multivariate statistical analysis in order to visualize clusters and to detect the metabolites that are able to differentiate...
gentamicin injected rats from controls. Metabolite fingerprinting of samples using LC-MS followed by unsupervised principal component analysis (PCA) showed strong discrimination between gentamicin treated groups and control group (Figure 2A and B). The evident separation of days 3 and 7 in the control group indicates that time factors also changed metabolic profiles. PCA of GC-MS data also showed a clear time-dependent separation of controls and treated animals (Figure 2 C and D). The supervised method, PLS-DA, was then used to isolate the variables responsible for differences among the various group. The mass spectrometry signals responsible for this differentiation are characterized by a variable importance plot (VIP). Variables that had significant contributions to discriminate among the groups were considered as potential biomarkers. A total of 10 metabolites are noted to show the most marked perturbations over the entire time-course of the sampling. These 10 metabolites by characterizing variable importance plots are found to contribute to significant perturbations of biochemical metabolism induced by gentamicin (Figure 3), they are identified as: phosphate, glucose, myo-inositol, alanine, isocitric acid, N-acetylglucosamine, glycine, glutamine, uridine and succinate. The PLS-DA score plot revealed good fitness and high predictability of the PLS-DA model with high statistical values of R² and Q², respectively.

**Metabolic pathway analysis**

In order to better understand complex relationships among various metabolites, a more detailed analysis of pathways and networks influenced by gentamicin was performed by Ingenuity Pathway Analysis (IPA). MetPA (http://metpa.metabolomics.ca) is a free, web-based tool that combines results from powerful pathway enrichment analysis with pathway topology analysis to identify the most relevant pathways. The metabolic pathways are obtained from the KEGG database and presented as a network of chemical compounds. The p value is calculated from the enrichment analysis, the Impact is the pathway impact value calculated from pathway topology analysis. Pathway analysis revealed that metabolites that were identified are responsible for tryptophan metabolism, aminoacylRNA biosynthesis, cyanoamino acid metabolism, glycine, serine and threonine metabolism and sphingolipid metabolism with p < 0.05 (Figure 4). Thus, different functional pathways contribute to the perturbations of metabolic signature induced by gentamicin by revealing the time-dependent changing pattern of the individual targeted metabolites.

**DISCUSSION**

Rats provide a good predictive human surrogate for the nephrotoxicity of aminoglycosides because of the close pharmacokinetic as well as toxicologic similarities of aminoglycosides in rats and humans (10). Most studies in the literature assessing nephrotoxicity of gentamicin have been performed on adult rats that were given large multiples of the human dose. The rat kidney development is not complete until 20 days postpartum. The present study was designed to assess the value of metabolomics approach for detection of gentamicin induced toxic injury in the phase of a developing kidney in neonatal rats. The two drug dosage levels used correspond to 2 and 4 times the dose recommended for newborn infants on a weight basis.
The aim of this study was to use metabolomic profiling as a tool for detection of kidney injury in newborn rats using the aminoglycoside antibiotic gentamicin as a model. Urine has evolved as one of the most appealing body fluids in metabolomics as it can be easily obtained and is stable compared to other biofluids. Furthermore, it is clear that a number of metabolites that can be easily detected in urine could provide a reliable indication of organ dysfunction that is not necessarily confined to the kidney injury. Validation of these metabolic biomarkers with respect to preclinical and clinical use, sensitivity, and specificity will allow delineation of early AKI identification and therefore prompt earlier intervention.

It is important to note that the present study was designed as a discovery study and that the data did not allow for estimating sensitivity and/or specificity of the gentamicin-induced urine metabolite changes identified here as diagnostic tools. However, metabolic changes identified in the urine were consistent among all animals in each group as evident by the multivariate data analysis. Another limitation of this study is the lack of correlation of gentamicin concentration in the blood (not measured) with metabolic changes in the urine. Our emphasis is on the potential use of changes in urine metabolite patterns as a diagnostic tool to monitor kidney function and gentamicin-induced nephrotoxicity. Importantly, our study confirmed that gentamicin administration cause multiple metabolic derangements in different pathways.

Metabolomic data sets are intrinsically multi-dimensional, with the number of metabolites ranging from a few dozen to hundreds. They represent snapshots of global biochemical profiles. Most of these features are expected to be within normal physiological variations, and only a few may be significantly associated with the conditions under study. In this study, mass spectrometry based metabolomics combined with multivariate recognition analyses were applied to investigate changes of the metabolic profile in neonatal rat urine. A comprehensive analysis of the urine obtained from gentamicin injected rats compared to control rats led to the identification of a large and diverse set of perturbed metabolites. A clear time-dependent separation of gentamicin-dosed animals from controls was observed by multivariate statistical analysis of MS data with significant changes occurring as early as day 3. Importantly, this was well before significant alterations in serum creatinine or BUN were detectable. The most marked changes induced by gentamicin, as revealed by MS were evident by 3 days post-treatment and were characterized by increased urinary concentrations of glucose, phosphate and amino acids such as glycine, glutamine and tryptophan.

Putative nephrotoxicity biomarkers identified in this study showed a persistent increase or decrease over time, from day 3 to day 7, reflecting the ongoing toxic effects of the medication on the kidneys with prolonged dosing. Some of the metabolites that are shown in table 3 are known to change when proximal tubular kidney injury occurs. This typically includes an increase in urine glucose and phosphate secondary to decreased tubular reabsorption. These results are consistent with earlier literature on glucosuria and phosphaturia associated with nephrotoxicity (10). Gentamicin has been shown to decrease DNA and protein synthesis in the proximal tubules (10) and to increase mitochondrial energy metabolism by enhancing the oxidative phosphorylation in the cortical mitochondria. Our results confirmed these effects as evident by the decrease in succinate and the increase in uridine in the urine of gentamicin injected rats compared to controls.
Tryptophan is an essential amino acid that cannot be synthesized by the body; it is either incorporated into proteins or broken down for energy and metabolic intermediates. It is involved in two metabolic pathways: serotonin and kynurenine pathways. In our study tryptophan is significantly increased at both low and high dose groups. Kynurenic acid and xanthurenic acid are both tryptophan metabolites. Kynurenic acid is one of the few known endogenous excitatory amino acid receptor blockers with a broad spectrum of antagonistic properties in supraphysiological concentrations (11,12). The lower levels of kynurenic acid that were noted in the urine of gentamicin injected rats, coinciding with higher levels of tryptophan, suggest a degrading effect of gentamicin toxicity on tryptophan metabolism pathway. Zhao et al. (13) demonstrated similar findings in a chronic renal failure animal model. It has also been reported that tryptophan metabolism is impaired by puromycin aminonucleoside (PAN)-induced nephrotoxicity (14). While the significant glucosuria and phosphaturia noted in the urine of gentamicin injected rats, can be attributed to the tubular injury that is expected to occur with aminoglycoside nephrotoxicity, the reciprocal change in tryptophan metabolites suggests an interruption of the metabolic pathway. Further cytological studies are required to confirm these findings using the harvested kidney tissues.

An advantage of the metabolomics approach is that it can determine marker metabolites that contribute to the differentiation between drug injected rats and controls through the supervised multivariate analysis. Subsequently, the variable importance plot arranges the importance of these identified metabolites in the contribution to metabolic perturbation induced by gentamicin.

In summary, our analysis by mass spectrometry demonstrates that exposure of newborn rat pups to high-dose gentamicin results in a marked change in the urinary metabolic profile preceding changes in the classically used biomarkers of nephrotoxicity, such as BUN and serum creatinine. Early diagnosis of nephrotoxicity is essential because dosage adjustment or discontinuation of renally excreted drugs may prevent further iatrogenic damage. Furthermore, ingenuity network analysis showed the different metabolic pathways that are affected by gentamicin administration. Using IPA the phenotypic data of the metabolites were correlated with the potential metabolic pathways, and related to biochemical functions. Validation of these metabolic biomarkers with respect to preclinical and clinical use, sensitivity, and specificity will provide additional tools in the early detection of kidney injury. We are currently conducting a prospective study to develop a normative renal metabolomics data set for preterm infants. It will be interesting to see if these pattern changes will translate into preterm infants and if they can provide the basis for a toxicodynamic drug monitoring strategy.

**METHODS**

**Animal Housing and Treatment**

Housing and treatment of the animals was performed in accordance with the USDA Animal Welfare Act. Institutional Animal Care and Use Committee (IACUC) approval at the University of Iowa was obtained for all experiments. Pregnant female Sprague Dawley rats obtained at gestation day 16 from Harlan Laboratories (Madison, WI) were allowed to spontaneously deliver. After birth pups were housed with their dams and were treated
beginning at 3 days of age. Newborn rats were equally divided into three groups of 6 pups each; the control group received a single daily intraperitoneal injection of vehicle (0.9% NaCl solution), the two drug treated groups received daily gentamicin (Sigma Chemical Co, St. Louis, MO) at a dose of 10 mg/kg/day (low dose) or 20 mg/kg/day (high dose) for 7 days. After 3 and 7 days of injections 200 μl of urine were collected by straight voiding while holding abdominal pressure and 100 μl of blood was sampled from the facial vein from all pups. Twenty-four hours after the last injection, all groups were euthanized by exsanguination. At necropsy the right kidney was removed, weighed, flash frozen in liquid nitrogen and stored at−80°C for future cytological studies. The left kidney was fixed in paraformaldehyde for 72 hours and then embedded in paraffin blocks. Thin cuts (2-3 microns of thickness) were sectioned then examined under Transmission Electron Microscope with sectioning and protocol guidance from the microscopy core at the University of Iowa.

**Clinical Chemistry**

Blood urea nitrogen (BUN) and serum creatinine were measured using the VITROS 350 chemistry system (Ortho Clinical Diagnostics, Rochester, NY) with VITROS calibrators, standards, and supplies (Fisher Scientific, Pittsburgh, PA).

**Sample Preparation of Urine for Metabolomics**

Following collection, urine samples were stored frozen at −80 C until analysis. All urine samples were kept on ice until frozen. 15 units of urease (Calzyme Laboratories, San Luis Obispo, CA) were added to 100 μl of urine and immediately incubated at 37°C for 15 min to decompose and remove excess urea present. Then 100 μl of methanol, containing two internal standards was added, after which the solution was centrifuged at 15,000 rpm for 10 min to remove particulate debris. The supernatant was split into 4 aliquots and transferred to autosampler vials for mass spectrometry analysis. The internal standards to monitor LC/MS performance were dl-chlorophenylalanine and amitriptyline (Sigma Chemical Co, MO).

**High-performance liquid chromatography/mass spectrometry (HPLCIMS) analysis of urine**

HPLC/MS was carried out using Thermo LCQ Deca mass spectrometer which consists of a Dionex Ultimate liquid chromatograph (LC) interfaced with a quadrupole ion trap (Q) mass analyzer (Thermo Fisher Scientific, Waltham, MA). An electrospray ionization (ESI) interface was used. The mobile phase consisted of 0.1% formic acid in H2O (solvent A) and 0.1% formic acid in methanol (solvent B). The sample was loaded onto a Supelco Discovery C18 column (100 × 2.1 mm, 5 μm particle size) via an autosampler and gradient eluted (0% B, four minutes; 0%-50% B, two minutes; 50%-80% B, five minutes; 80%-100% B, one minute; maintain 100% B, two minutes) directly into the mass spectrometer at a flow rate of 200 μl/min. Data was collected over the mass range 99 to 800. Polarity switching was used to monitor both positive and negative ions. The spray voltage used for positive ion mode was 4.25 kV, and 3.75 kV spray voltage for negative ion mode. Each scan was composed of two microscans with a max ion trap fill time of 200 msec. The inlet capillary and sheath gas were maintained at 275°C and 200°C respectively, throughout the analysis.
Gas chromatography/mass spectrometry (GC/MS) metabolic profiling of urine

For GC/MS, the internal standard was myristic acid (1mg/ml). The supernatant was evaporated to dryness under nitrogen at room temperature. 15 μl of methoxyamine in pyridine was added to each GC vial. Then the solution was vigorously vortexed for 10 min. After methoximation reaction for 16 hours at room temperature, the samples were trimethylsilylated for another 1 h by adding 15 μl of N-methyltrimethylsilyltrifluoroacetamide (MSTFA). At last, 20 μl of heptane was added to the GC vial, and the solution was vigorously vortexed again for 10 min before GC/MS analysis.

Data analysis

The MS spectra processing was carried out using the XCMS software (15). Peak detection, alignment and retention time correction was performed sequentially. The ion intensity of each mass signal detected was normalized to construct data matrices, after which a data integrity check was performed to ensure that the data are valid and suitable for subsequent analysis. This data integrity check included checking for data values, sample size and group labels. Absolute value changes between the gentamicin and control groups means were compared by fold-change analysis. Unsupervised principal component analysis (PCA) was performed using the prcomp software; the data was summarized into much fewer variables called scores which are weighted averages of the original variables. Supervised partial least-squares discriminates analysis (PLS-DA) was performed to identify and characterize metabolic perturbations signatures induced by gentamicin. To assess the significance of class discrimination, a permutation test was performed. Variable Importance in Projection (VIP) is a variable importance that was measured in PLS-DA, it represents a weighted sum of squares of the PLS loadings (16).

Acknowledgments

STATEMENT OF FINANCIAL SUPPORT: This research was supported by a pilot grant from the University of Iowa Institute for Clinical and Translational Science UL1RR024979 (ICTS-CTSA) and a grant from the Peregrine Research Foundation.

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Figure 1.
Transmission electron micrographs (low magnification x4000) of the kidney from a control rat (A), a rat treated with low-dose gentamicin showing few necrotic cells (B), and a rat treated with high-dose gentamicin showing severe tubular necrosis and vacuolization (C). The scale bar reflects 2 μm.
Figure 2.
Multivariate analysis by PCA. Score plots generated from HPLC/MS and GC/MS metabolites separating gentamicin injected and control rats using the low dose (A, C) and the high dose (B, D) respectively. PCA refers to principle component analysis, HPLC refers to high-performance liquid chromatography, MS refers to mass spectometry and GC refers to gas chromatography. Squares represent samples from the control group after 3 days of injections, circles represent samples from the gentamicin group after 3 days of injections, triangles represent samples from the control group after 7 days of injections and times represent samples from the gentamicin group after 7 days of injections.
Figure 3.
PLS-DA score plots generated from GC/MS spectra for the low dose (A) and the high dose (C) gentamicin with the corresponding VIP scores (B and D), respectively. PLS-DA refers to supervised partial least-squares discriminates analysis, MS refers to mass spectometry, GC refers to gas chromatography and VIP refers to Variable Importance in Projection. Squares represent samples from the control group after 3 days of injections, circles represent samples from the gentamicin group after 3 days of injections, triangles represent samples from the control group after 7 days of injections and times represent samples from the gentamicin group after 7 days of injections.
Figure 4.
Summary of Pathway Analysis with MetPA (internet software analysis program), tryptophan metabolism (A), aminoacyl tRNA biosynthesis (B), glycine, serine and threonine metabolism (C), cyanoamino acid metabolism (D), and sphingolipid metabolism (E).
Table 1

Summary of biochemical changes after 3 and 7 days of gentamicin injection

|         | Low dose  | High dose |
|---------|-----------|-----------|
|         | 3d        | 7d        | 3d        | 7d        |
|         | Cont      | Gent      | Cont      | Gent      | Cont      | Gent      | Cont      | Gent      |
| BUN     | 28.2±1.3  | 27.1±1.3  | 28.3±1.7  | 46.4±5.1 *| 25.3±1.8  | 28.7±2.6  | 24.1±1.9  | 47.7±3.1 *|
| Creatinine | 0.37±0.1  | 0.38±0.1  | 0.35±0.1  | 0.6±0.1 * | 0.4±0.1   | 0.4±0.1   | 0.42±0.1  | 1.5±0.3 * |

* Data are presented as mean ± SD (n=6). Statistically significant changes compared with controls are indicated as P < 0.05

BUN: blood urea nitrogen, Cont: control, Gent: gentamicin.
Table 2

Metabolic changes in urine detected using LC/MS

| Metabolite mass (m/z) | Retention time (min) | Postulated identity | Low dose  | High dose |
|-----------------------|----------------------|---------------------|-----------|-----------|
|                       |                      |                     | 3d        | 7d        | 3d        | 7d        |
| 204                   | 9.1                  | Tryptophan          | 1.91      | 1.81      | 2.31      | 1.93      |
| 190                   | 9.75                 | Kynurenic acid      | 0.26      | 0.21      | 0.4       | 0.52      |
| 206                   | 10.1                 | Xanthurenic acid    | 0.45      | 0.39      | 0.34      | 0.32      |
| 178                   | 9.55                 | Hippuric acid       | 0.49      | 0.24      | 0.3       | 0.38      |

Fold changes between drug-treated and control group are shown (P < 0.05)
## Table 3

Metabolic changes in urine detected using GC/MS

| Metabolite     | Pathway     | Low dose 3d | Low dose 7d | High dose 3d | High dose 7d |
|----------------|-------------|-------------|-------------|--------------|--------------|
| Glucose        | Carbohydrate| 3.15        | 2.74        | 3.12         | 3.88         |
| N-acetylglucosamine | Carbohydrate     | 3.5         | 2.13        | 2.92         | 3.45         |
| D-glucopyranose | Carbohydrate   | 2.16        | 2.64        | 2.18         | 1.67         |
| Glutamine      | Amino acid    | 2.38        | 2.22        | 3.1          | 3.35         |
| Glycine        | Amino acid    | 2.24        | 2.54        | 2.42         | 2.71         |
| Alanine        | Amino acid    | 2.73        | 2.2         | 2.92         | 3.35         |
| Myo-inositol   | Lipid         | 3.24        | 4.03        | 2.17         | 3.25         |
| Butanoic acid  | Lipid         | 1.49        | 2.81        | 2.43         | 2.98         |
| 3-hydroxybutyrate | Lipid       | 2.59        | 2.41        | 2.56         | 2.89         |
| Succinate      | Organic acid  | 0.18        | 0.4         | 0.36         | 2.89         |
| Isocitric acid | Organic acid  | 2.02        | 2.49        | 3.1          | 1.9          |
| Gluconic acid  | Organic acid  | 3.16        | 2.85        | 2.51         | 2.86         |
| Uridine        | Nucleotide    | 2.35        | 3.31        | 2.1          | 1.7          |
| Phosphate      | Energy        | 2.93        | 4.67        | 3.23         | 4.95         |

Fold changes between drug-treated and control group are shown (P < 0.05)