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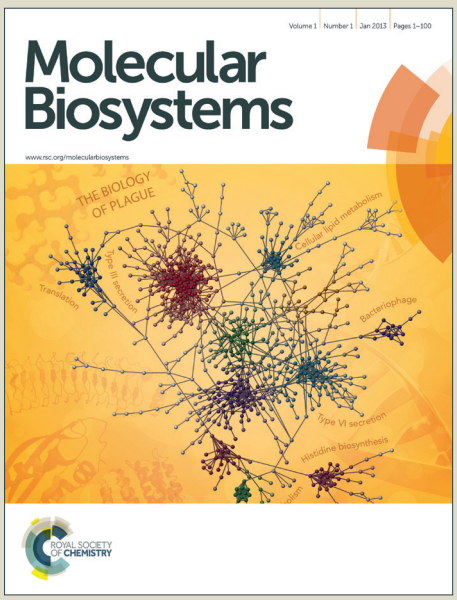
Accepted Manuscript



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Assessment of early triage for acute radiation injury in rat model based on urinary amino acid target analysis

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Running Title: amino acid target analysis to the acute radiation injury early triage

Abstract

Rapid radiation injury early triage after a radiological or nuclear exposure is vital for treatment of a large number of wounded. Owing to the high-throughput analysis and minimally invasive of collection sample, radiation metabolomics has been recently applied to radiation damage researches. In the present study, exploring the feasibility of estimating the acute radiation injury early triage by means of urinary amino acid target analysis was attempted using high performance liquid chromatography electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) technique combined with multivariate statistics analysis. The nonlinear kernel partial least squares (KPLS) model was used to separate the control and different radiation dose groups. The classification in different groups was performed after feature selection instead of before feature selection because of its better separation. The classification accuracy in various radiation injury levels at different time points (5, 24, 48 and 72 h) post-irradiation exposure was investigated. For most of radiation damage levels, the classification accuracy at 72 h after exposure was superior to earlier time points. Additionally, the potential radiation injury biomarkers selected suggested urea cycle, glycine, serine and threonine metabolism, Alanine, aspartate and glutamine metabolism and related metabolic pathways were involved. The finding suggests noninvasive urinary biomarkers have great potential for serving as an effective tool for rapid triage mass casualties in nuclear accident and understanding the pathogenesis of radiation injury.

Keywords radiation injury, triage, radiation metabolomics, amino acids, HPLC-MS

Introduction

With the development of nuclear energy and the global rise in terrorism, radiological events are more likely to occur. In a large-scale radiological event, mass radiological triage will thus be critical for optimizing the allocation of scarce medical countermeasures and preventing development of multi-organ failure after a large-scale event. [1,2] Currently, effective dosimetry measurement in large populations mainly includes physical dose estimates, biodosimetry and clinical parameters. Clinical parameters assess exposure dose base on time-developed symptoms includes time-to-emesis, lymphocyte depletion, and the manifestation of individual acute radiation syndrome (ARS). [3,4] However, these symptoms cannot be applied immediately to large numbers of the affected population because of individual variations or delayed response. [4,5] Biodosimetry is the preferred method to assess the exposure doses because it can reflect the actual amount of biological damage to the individual. As the gold standard for radiation biodosimetry, the dicentric assay is currently impractical for mass triage because it is too time consuming and requires highly trained personnel for scoring. [6] Recently, Gene expression signatures,[7,8] proteomics, [9,10] metabolomic[11,12] and microRNA[13] that are based on the omics have been developed. The omics methods are suitable for rapid assessing the radiation doses received by a large number of individuals because of the high throughput technique. In these omics methods, the smallest number of variables is determined to classify different groups of individuals using multivariate statistical analysis tools,

which overcome the shortcomings of insufficient sensitivity and specificity of single radiation injury biomarkers.

Radiation metabolomics is defined the changes in the relative levels of small molecule metabolites that are associated with exposure to radiation[14]. Radiation exposure triggers a complex network of molecular and cellular responses that impacts metabolic processes and alters the levels of metabolites. Such metabolites have potential capacity as biomarkers of radiation damage[15]. Owing to the high-throughput analysis and minimally invasive of collection sample (such as plasma,urine), radiation metabolomics has been recently applied to select radiation injury biomarkers. [14, 16-20] In order to summarize and interpret the complex data in metabolomics, efficient and robust modeling, analysis and interpretation methods are needed. The popular ones included principal components analysis (PCA), partial least squares regression (PLS). [17,18,20] PLS consists of ordinary linear and nonlinear models. Linear PLS, has been widely applied in metabolomics research due to the simplicity and convenience.[21,22] However, in complex biological research, the relationship between the response variable and explanatory variables may exhibit significant nonlinear characteristics. To tackle the issue of data nonlinearity, nonlinear kernel partial least squares (KPLS) combined with a preprocessing technique of orthogonal signal correction (OSC) (i.e. OSC-KPLS) has been proposed as a multivariate regression approach in our previous radiation metabolomics research[23].

Amino acids, being the basic units of the proteins, are cell signalling molecules, which regulate gene expression and the protein phosphorylation cascade. After

animals are irradiated, the changes in protein synthesis and degradation inevitably lead to amino acid metabolic changes in the body. The citrulline concentration in plasma has been assessed in patients accidentally irradiated as an indicator of the gastrointestinal damage. [24-26] In our previous report, plasma amino acid target analysis based on high performance liquid chromatography electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) was applied to estimate the acute radiation injury early triage. [23] Compared to serum and plasma, urine has the great advantage of being collected easily in a non-invasive manner. Therefore, urine analysis is readily accepted and promoted in clinical testing.

In this paper, an HPLC-ESI-MS/MS method was applied to investigate the effect of acute ionizing radiation on amino acid levels of rat urine at radiation damage early stages. The classification accuracy in various radiation injury levels during 72 h post-exposure was investigated and potential radiation injury biomarkers were selected. In addition, the involved perturbed metabolic pathways in response to radiation damage was explored in detail.

Experimental

Reagents

The unlabelled amino acids：L-aspartic acid (L-Asp), glycine (Gly), L-serine (L-Ser), L-lysine (L-Lys), L-alanine (L-Ala), L-tyrosine (L-Tyr), L-methionine (L-Met), L-glutamic acid (L-Glu), L-phenylalanine (L-Phe), L-asparagine (L-Asn), DL-3-aminoisobutyric acid (β-AIB), L-glutamine (L-Gln), L-proline (L-Pro),

L-histidine (L-His), trans-4-hydroxy-L-proline(Hyp), L-tryptophan(L-Trp), L-citrulline (L-Cit), L-threonine (L-Thr), L-cystine (L-Cy2), L-Arginine (L-Arg), L-norvaline (L-Nov), L-isoleucine (L-Ile), L-valine (L-Val), L-Cysteine (L-Cys), L-Leucine (L-Leu), L-ornithine (L-Orn), creatinine (Cre) and tridecafluoroheptanoic acid (TDFHA) were from Sigma-Aldrich. TDFHA was used as an ion-pairing reagent. The Stable isotope-labelled amino acids (including Ala-d5, Phe-d5, Lys-d4, Leu-d3 and Trp-d5) were obtained from Cambridge Isotope laboratories, Inc. (Andover, MA). Formic acid, acetonitrile and all the solvents were HPLC grade (TEDIA, USA), ammonia was analytical grade from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and methanol was from Merck. All solutions were prepared using LC-MS Ultra High Purity water.

Amino acid stock solutions including internal standards (IS) were prepared in 0.1% hydrochloric acid (Cy2, Tyr, Trp, Trp-d5, Asp) or water (His, Arg, Phe, Ala, Gln, β-AIB, Hyp, Asn, Glu, Thr, Leu, Val, Pro, Ile, Gly, Ser, Lys, Cit, Nov, Orn, Cys, Ala-d5, Phe-d5, Lys-d4, Leu-d3) and stored at -80 □. For analysis, the stock solutions were diluted to appropriate concentrations with water. The mixture of internal standards consisted of 5 μmol·L^{-1} Trp-d5, 50 μmol·L^{-1} Phe-d5, 100 μmol·L^{-1} Lys-d4, 50 μmol·L^{-1} Leu-d3, 100 μmol·L^{-1} Ala-d5 in water. The TDFHA stock solution was prepared in water and stored at -20□, and then was diluted to 0.5 mmol·L-1 with water before LC-MS/MS analysis.

Animals, Irradiations and Urine Collection

Male Sprague Dawley（SD）rats (180-200 g, Soochow University Laboratory Animal Center, China) were randomized into two groups including sham-irradiated (i.e. control, n=13) and irradiated. The irradiated rats were given total body irradiation with 2 Gy, 4 Gy, 6 Gy and 8 Gy X-rays by a 6 MV medical linear accelerator (Siemens, KD22) at a dose rate 2 $Gy \cdot min^{-1}$. The source surface distance (SSD) was 100cm. The lethal dose $LD_{50/60}$ for human and rats is 3-4.5 Gy and 6.75 Gy, respectively. [27] Accordingly, these doses are considered to be equivalent to 1.1, 2.2, 3.3 and 4.4 Gy, respectively, in humans. All animals were freely accessible to food and water until blood samples were collected. Urine samples from rat housed individually in metabolic cages were collected at 5h, 24h, 48h and 72h after radiation in the afternoon 12:00-15:00. Control rats were used for simultaneous control urine sample collection. There were 8-10 sample numbers in each dose group. The quality control sample (QC) was prepared by pooling and mixing 100 μL from each sample. All urine samples were stored at −80°C until analyzed. All experiments were conducted according to the Chinese regulations for animal experimentation [Commission on Science and Technology, State Bureau of Technical Supervision, No 593, 11 December 1997].

Sample preparation with solid-phase extraction (SPE)

The 6 mL strong cation-exchange (SCX) cartridges containing 500 mg of ion exchange sorbent (Chrom-Matrix Bio-Technology) were used to extract urinary amino acids according our previous report. [23]In brief, the ion-exchange SPE cartridges

were conditioned with 5 mL of methanol, and then with 5 mL of 0.1 mol·L-1acetic acid (pH=2.8). Subsequently, 100μL of urine with spiked with a 150μL mixture of IS was mixed with 100μL 0.1 mol·L^{-1} acetic acid. The entire sample was then loaded onto the SCX-SPE cartridge and drawn through by gravity. After loading, the cartridge was orderly washed with 5 mL of 0.1 mol·L^{-1} acetic acid and 5 mL of methanol. Finally, the analytes were eluted into a new test tube using 5 mL of 5% ammonium hydroxide in methanol. All the eluants were evaporated to dryness under nitrogen at 35 ℃ and then were stored at -20 ℃. Before analysis, the extracted samples were reconstituted with 200 uL of initial mobile phase (acetonitrile- 0.5 mmol·L^{-1} TDFHA, 1: 9, v/v) and 3 μL was injected into the LC-MS system.

High-performance liquid chromatography and Tandem mass spectrometry

The HPLC separation was performed on an XBridge C18 column, 5 μm, 2.1×100 mm with a guard column, 2.1×10 mm of the same packaging material from Waters or Agilent XDB-C18 2.1×100 mm columns with 3.5 μm particle sizes. The mobile phase A was 100% acetonitrile, and mobile phase B was 0.5 mmol·L^{-1} TDFHA in Ultra High Purity water. The total flow rate was 0.2 ml·min^{-1}. The column temperature was kept at 26 ℃. The gradient elution was adopted in our previous report. [23]

Mass spectrometry experiments and optimization of the method were conducted on a QTRAP 2000 LC/MS/MS system from Applied Biosystems/MDS Sciex (USA) equipped with a turbo ionspray source. The mass spectrometer was used in the multiple reaction monitoring (MRM) in the ESI-positive mode. The mass

spectrometric parameters and the quantification of urinary amino acids and creatinine were described previously.[23] And then the concentration of each amino acid was normalized to the endogenous creatinine levels (i.e μmol·mmol^{-1} creatinine).

Data Analysis

Univariate statistics were performed with SPSS using two-way analysis of variance (ANOVA) (α=0. 05) with Bonferrroni correction for multiple comparisons to investigate the differences among the control and irradiated groups with different exposure levels at different points. Differences among groups were considered to be statistically significant if p<0.05. In this work, genetic algorithm (GA) was incorporated with KPLS and used to select features that contributed most to the separation of the data set for different groups.[23] Data visualization based on non-linear PLS model and GA was carried out using SIMCA-P 11.5 demo version (Umetrics AB, Umeå, Sweden) and Matlab (version 7.0.0.19920, R14) softwares.

Results and Discussion

Amino acids metabolic changes in rat urinary samples at 5 h after radiation exposure

The effects of ionizing radiation on urinary amino acid levels were listed in Table S1. After radiation exposure, the changes of amino acid levels in the urine were complex: some amino acids such as β-AIB and Thr significantly increased, some ones including Tyr, Asp and Lys were markedly decreased, but none of them displayed distinct

dose-response relationship (Table S1). Since multivariate statistics analysis may show improved separation performances in more complex situations where many metabolites are responsible for the discrimination between case and control samples, [28] then multivariate analysis model was applied to classified control and irradiated groups. In this study, OSC-KPLS was chosen to establish a multivariable data model for radiation injury triage research.

Initially, 25 amino acids as variables were used to build model, which describe 30.8% of the variation in x (R2X=0.308), 70.2% of the variation in the response y R^2Y=0.702) and predict 62.5% of the variation in the response y (Q^2Y=0.625). R^2Y represents the goodness of fit and how well it is possible to mathematically reproduce the data of the training set, while Q^2Y reveals the predictive ability of the model. It has been reported that well-performed variable selection can greatly improve the predictive ability of the model. [29] On the contrary, irrelevant and redundant features or variables increase the dimensionality of the problem make the model difficult to interpret and even lead to model overfitting. [30,31] Recently, GA was shown to be efficient in feature selection in spectroscopic data.[32,33] Therefore, GA was attempted to feature selection before model building. Six significant variables including Trp, Thr, Asp, Lys, Cit and Orn (Table S5) were selected from the amino acid metabolic data. And then the six amino acids were used to build the OSC-KPLS model.

Fig.S1a displayed the 3D representation score plot between the healthy controls and the irradiated groups. Compared with before feature selection, the values of R^2X (0.397), R^2Y (0.879) and Q^2Y (0.841) are highly improved. To validate the model,

permutation tests with 100 iterations were performed. As shown in Fig. S1c, the validation plot presented that the value of permuted R^2-intercept (0.111, <0.4) and the value of Q^2-intercept (-0.306, <0.05), which suggested the model is valid. [34] The higher R^2/Q^2 values also demonstrated variable selection can make the model have a greater predictive ability.

The separation trend from the control group to different irradiated groups suggested that ionizing radiation could lead to amino acids metabolic disorders and the degree of disorders was positively related to radiation doses. There was clear separation between the control and irradiated group. This point indicated it is possible to distinguish exposed vs. non-exposed individuals, which are important for allocating medical resources in mass-casualty. The overlaps appeared between the neighboring irradiated groups showed that the information from urinary six amino acids was still insufficient to distinguish the radiation injury degree within 2-8 Gy at 5h after exposure.

The comparison between the predicted values and observed ones was shown in Fig.S1b. Except of the control and 4Gy irradiated group, the predicted doses of other irradiated groups deviated seriously from the observed ones. According to acute radiation injury classification criteria in our earlier report, [23] the accuracy of classification is listed in Table 1. Except of the higher accuracy of control (92.3%) and moderate (81.8%), the accuracy of the mild, severe and very severe was only 60.0%. The main cause of the lower accuracy is that overlap area is serious among the neighboring irradiated groups. Yet, it is satisfactory to distinguish exposed vs.

non-exposed animals at 5 h after exposure because of higher classification accuracy for the control group (92.3%).

Amino acids metabolic changes in rat urinary samples at 24 h and 48h after radiation exposure

The effect of radiation exposure on the urinary amino acids was listed in Table S2. Similarly, the comparison of the separation effect before and after variable selection can be inferred from the parameters including the values of R^2Y, Q^2Y, R^2-intercept and Q^2 –intercept at 24 h following exposure. Before variable selection, the values of R^2Y, Q^2Y, R^2-intercept and Q^2-intercept were 0.874, 0.787, 0.379 and -0.276, respectively. On the contrary, after variable selection, the values of R^2Y, Q^2Y, R^2-intercept and Q^2-intercept were 0.895, 0.86, 0.203 and -0.298, respectively. The higher R^2Y, Q^2Y and lower R^2-intercept and Q^2-intercept suggested that the appropriate feature selection can generate better classification and enhance the stability of the model[33]. Then, the features selected from GA algorithm included His, Phe, Gln, Hyp, Leu, Asp, Cit and Cys ((Table S5) were used to develop the KPLS model at 24 h post-exposure. Compared to 5 h after exposure, except of clear separation between 2 Gy and 4 Gy, other neighboring dose groups did not show obvious improved separation (Fig.S2a). The comparison between the predicted values and observed ones also indicated the consistent results: except that the predicted dose of the 2 Gy was relatively concentrated and close to the observed ones, the predicted ones of other dose groups deviated seriously from the observed ones (Fig.S2b). As a

result, the classification accuracy of the mild was raised to 100%, while the correct classification rates of the moderate and the very severe only 60.0% (Table1). Compared to 5 h post-irradiation, the classification accuracy for the control remained 92.3%, which is important for the separation irradiated and non-irradiated in a nuclear accident. The values of R^2-intercept (0.203) and Q^2-intercept (-0.298) from 100 permutation tests indicted the validation of the model.

In consideration of better classification after variable selection, the eight features including His, Ala, Hyp, Thr, Ile, Gly, Cit and Orn (Table S5) selected by the GA algorithm were used to build the model in order to separate different groups at 48 h post-exposure (Fig.S3a). Compared with two early time points, the separation of the high dose groups was greatly improved at 72 h following exposure. Fig. 3b displayed the comparison between the predicted radiation doses and observed ones. The predicted values in the most groups were close to the observed ones, which produced a better classification at this time point: 84.6% accuracy for the non-irradiated, 100.0 % classification accuracy for the mild, 80% accuracy for the moderate and the severe, 70% accuracy for the very severe (Table 1). The model parameters including R^2Y (0.91), Q^2Y (0.883), R^2-intercept (0.187, <0.4) and Q^2-intercept (-0.299, <0.05) showed the model had better interpretative ability, predictive ability and stability.

Amino acids metabolic changes in rat urinary samples at 72 h after radiation exposure

Compared with preceding time point after exposure, more amino acids exerted obvious changes in the irradiated groups at 72 h following exposure, especially in the high dose groups (Table S5). Furthermore, the numbers of features with evident variations in the different irradiated groups was greatly increased. For example, there was a significant elevation in the urinary excretion of Ser in the 6 Gy group than that of in the 2 Gy and 4 Gy irradiated groups. In addition, Cit showed obvious dose-response relationship at this time point. Because of the better classification performance, the selected six variables containing His, Ala, Thr, Gly, Cit and Nov (Table S5) by GA instead of 25 amino acids in urine were used to separate different groups by building KPLS model at 72 h post-exposure (Fig. 1a). Better separation in high dose groups displayed (such as 6 Gy and 8 Gy dose groups). Fig. 1b displayed the comparison between the predicted radiation doses and observed values. Most of the predicted values (such as of 2 Gy, 6 Gy and 8 Gy) were close to the observed ones. Table 1 listed classification results at this time point: 76.9% accuracy for the non-irradiated control, 100.0% classification accuracy for the mild and the severe, 70.0% accuracy for the moderate, 90.0% correct classification for the very severe.

Comparisons to the four time points after radiation exposure, the diagnosis for the non-irradiated control was lower at 72 h post-exposure than that of earlier time point. In contrast, the diagnosis for the higher dose groups became higher at this time point. The reasons may be associated with the acute radiation sickness progresses: the animals with mild radiation damage recovered to some certain degree and ones with serious radiation damage became worse with time lapse post exposure. These

results could also be reflected by the facts that less amino acids presented marked variations in the low dose groups and more ones showed remarkable changes in the high dose groups. The related model parameters covering R^2Y (0.936), Q^2Y (0.888), R^2-intercept (0.105, <0.4) and Q^2 –intercept (-0.321, <0.05) revealed the model had excellent interpretative ability, predictive ability and stability.

The time-response relationship induced by radiation exposure

A two-way ANOVA was undertaken in order to assess the change of urinary amino acid levels from two aspects including radiation doses and time (Table S6). There was a significant interaction ($p < 0.05$) between radiation doses and time for some urinary amino acids. Nearly all urinary amino acids presented time dependent trends except of Trp, Ser and Cys. The time-dependency at urinary amino acid levels also were demonstrated PLS 3D representation score plots (Fig.S1-S3, Fig1a) by better separation for most of dose groups with the post-irradiation time lapse longer.

Furthermore, some amino acids presented regularly changes in the irradiated groups with time post-exposure, such as β-AIB, Asp and Cit (Fig.S4). The urinary excretion β-AIB and Cit in the irradiated groups was down-regulated at the four time points. Yet, Asp was gradually up-regulated during 72 h post-irradiation. Because the collecting of urine samples is noninvasive, then dynamic analysis urine sample from animals at different times after radiation exposure becomes feasible and thus the cost of experimental animals could be greatly reduced.

Potential radiation injury biomarkers and involving metabolic pathways

It is generally known, protein molecules play a great important role in the biology. As the basic units of proteins, the dynamic study changes of urinary amino acids after radiation exposure revealed the development of acute radiation sickness, which were helpful for triage and diagnosis the illness. In this work, a combination of GA analysis and univariate statistic method was used to select the potential radiation injury biomarkers. Considering the urinary secretion of many amino acids were time dependent, the respective features which are mostly responsible for the separation of the data set for the control and different irradiated groups were firstly selected at four time points. And then *P*-values for radiation doses of these features were checked (Table S8), those variable with significant differences ($P < 0.05$) are chosen as potential radiation injury biomarkers: Trp, His, Phe, Ala, Gln, Hyp, Thr, Gly, Lys, Cit, Nov, Orn and Cys. Subsequently, the perturbed metabolic pathway associated with these potential radiation injury biomarkers were mapped on KEGG and the involved perturbed metabolic pathways in response to radiation damage were explored in detail in the following sections.

Urea cycle metabolic pathway. As a non-protein α-amino acid and a nitrogen end product of small bowel enterocyte metabolism, [35] serum or plasma Cit has been served as a biological indicator of gastrointestinal damage in several radiation accidents. [34-36]In previous reports, the decrease of plasma Cit has been proved to be related to radiation-induced intestinal injury. [23,35-37] Similarly, the urinary excretion of Cit was

also significantly declined in the irradiated groups, especially at 72 h after radiation exposure (Fig.2c). The better correlativity between the urinary Cit level and the radiation doses was confirmed by the p-value in the Table S6, which also indicted the urinary excretion of Cit was associated with radiation injury. Additionally, the urinary Arg and Orn levels showed significant coincidence decline in irradiated groups as well as Cit during 48 h following exposure (Table S1-S3). As the urea cycle intermediates, the coincident decrease in the levels of urinary Arg, Orn and Cit implied impaired urea cycle in response to radiation exposure. The urea cycle is the final common pathway for the excretion of waste nitrogen in mammals and is the primary detoxication pathway for ammonia.[38] Ionizing radiation could lead to the ratio of urea to ammonia sharply drop and thus give rise to hyperammonemia, [39,40] which suggested urea cycle defect development and further confirmed our results. Since the urea cycle happened mostly in the liver, therefore, the consistent reduction of the three urinary amino acids levels including Arg, Orn and Cit may reflect the radiation induced liver damage to some extent.

Glycine, serine and threonine metabolism. Gly is a major amino acid in mammals, which can be synthesized from Ser, Thr, choline and Hyp. [41] By converting into acetylCoA and then entering into the tricarboxylic acid (TCA) cycle, Gly, Ser and Thr have similar metabolic pathway. Ionizing radiation slowed down TCA cycle by down regulation of energy metabolite citrate. [14] The elevated urinary excretion of the three amino acid levels except of 24 h post-exposure (TableS1-S4) indicated the reduction

of catabolism them into TCA cycle and disorder of energy metabolism. [20] As a significant biological metabolic pathway in the body, TCA not only involves glucose aerobic oxidation, but also involves the major pathways for lipid and amino acid metabolisms. Thus radiation-induced inhibition of TCA cycle would inevitably lead to organ damage.

Being a sulfur-containing amino acid, Cys is synthesized from serine through different pathways in different organism groups and metabolized to pyruvate, glutathione or taurin. [42] Several animal experiments have shown that high doses of ionizing radiation lead to enhanced leakage of taurine from damaged cells into the extracellular fluid, followed by elevated urinary excretion.[16,43,44] Intensive taurine excretion was shown to be increased from the first hours to two days in response to ionizing radiation.[16,45] Due to the sensitivity of our instrument, urinary taurine was not detected in our study. However, the increased excretion of urinary Cys during 48 h post-exposure in our experiment (TableS1-S3) was in accordance with enhanced increments of urinary taurine in irradiated animals and indicative of more oxidative in response to radiation exposure.

Alanine, aspartate and glutamine metabolism and related metabolic pathway. Glycolysis is the process of converting glucose into pyruvate and generating small amounts of ATP (energy) and NADH (reducing power). Being a precursor of gluconeogenesis, Ala play an important role in the glycolysis to satisfy the increased energy demand. The plasma level of Ala was up-regulated in our previous study,

[23]which was consisted with Khan report. [19] In contrast, urinary Ala was decreased in the irradiated groups in the present study. Interestingly, the level of urinary Lys also displayed opposite change compared with that of plasma Lys after radiation exposure. During 48 h post-exposure, plasma Lys showed a significant elevation in irradiated groups, [20, 23] while urinary Lys presented an evident decrease in response to radiation. Yet, the mechanism of the phenomenon has been unclear. One of the several contributing factors responsible for these observations could be related to radiation-induced renal dysfunction including glomerular filtration and tubular reabsorption for the two amino acids.

When compared to that of the control group, the urinary Gln was up-regulated in most of the irradiated groups during 72 h post-exposure (TableS1-S4). As the most abundant free amino acid in the body, Gln has important physical roles, such as regulation protein turnover through cellular mTOR signaling, gene expression and immune function, a major fuel for rapidly proliferating cells and a required substrate for three enzymes involved in the de novo synthesis of purine, pyrimidine, amino sugars, and proteins. [46,47] It has been demonstrated Gln administration can protect against radiation damage of intestine, pancreas and esophagitis. [48,49] Additionally, Gln participates in the TCA cycle by metabolizing to oxoglutarate. Radiation exposure increased the release of Gln from the enhanced protein breakdown in skeletal muscle [50] and slowed down TCA cycle[19, 14], which may both contribute to the accumulation of Gln. Since Gln is an important precursor of purine and pyrimidine, thus the elevation of thymidine, 2'-deoxyuridine, 2'-deoxyxanthosine and xanthine levels in

the urine in response to γ irradiation [16,17] is also likely responsible for the increased excretion of urinary Gln.

The involved perturbed metabolic pathways in response to radiation injury were shown Fig.2.

Aside from the metabolic changes in the amino acid levels, some researchers have investigated the oxidative stress and inflammation biomarkers. Cellular exposure to ionizing radiation leads to oxidizing events through generation of reactive oxygen species (ROS). [51] These ROS induce oxidative damage to cellular membranes that are composed of lipids. [15] Khan et al. observed that serum lipids and lipoprotein were increased at day 5 after irradiation. [19] Additionally, the elevated plasma phospholipids and potential phospholipids biomarkers selection were also reported in our previous report. [52] All of these results suggested ionizing radiation could lead to membrane lipid metabolic disorder derived from oxidative stress. Furthermore, macrophages produce a wide range of cytokines and growth factors after radiation injury. Cytokines can cause dramatic expression of acute-phase proteins (CRP) and serum amyloid A (SAA). Thus, inflammatory biomarkers including CRP, IL-6 and SAA were investigated as a complementary approach to conventional biodosimetry for early assessment of radiation exposures in nonhuman primate total-body irradiation model. [53] Not only oxidative stress but also inflammatory responses has been observed to be responsible for mediating and participating the bystander effects of ionizing radiation in a variety of model systems. [54] As important organic substances, amino acids are inextricably linked to these biomarkers and bystander effects. For example, Ser is a

precursor for the synthesis of both phosphatidylserine and sphingolipids, [55] Gly and 5-Hydroxytryptamine (the metabolite of Trp) participate the modulation of bystander effects following ionizing radiation exposure. [56] Since it only measures selected known metabolites, targeted metabolomics analysis is restricted by the metabolite coverage. Accordingly, the untargeted metabolomics analysis for the early triage of radiation injury would be performed in our further study.

To our knowledge, it is the first report that utilization urinary amino acids to investigate acute radiation exposure injury early triage and select potential radiation injury biomarkers. Since radiation damage can be regulated through a number of metabolic pathways, investigation of the whole metabolites rather than several target metabolites would enable us to select more appropriate biomarkers to get better triage and to understand radiation-induced underlying pathophysiological status more comprehensively. Thereby, further study is to explore more metabolites found in urine to probe the effects of radiation exposure and perform more satisfactory radiation injury early triage.

Conclusion

The effect of acute ionizing radiation on urinary amino acids at early stage post-irradiation based on HPLC-ESI-MS/MS target analysis was investigated. Using a nonlinear KPLS model, features selected by the GA algorithm was applied to radiation injury triage. The potential radiation injury biomarkers and involved metabolic pathways were explored. To our knowledge, it is the first report to explore

the application urinary amino acids for early acute radiation injury triage. For most of radiation damage levels, the classification accuracy at 72 h post-exposure was superior to the earlier time points. This pilot study indicates noninvasive urinary biomarkers based on HPLC -MS/MS technique has great potential in understanding the pathogenesis of radiation injury. Moreover, the radiation injury biomarkers at metabolites levels may provide an efficient means of radiation dose assessment and rapid triage mass casualties in nuclear accident.

Acknowledgements

The study has been supported by the foundation (No.31000385) from the National Natural Science Foundation of China, Science and technology project in Suzhou (No.SYS201316), post-doctoral research funding of Jiangsu Province (No.0901052C) and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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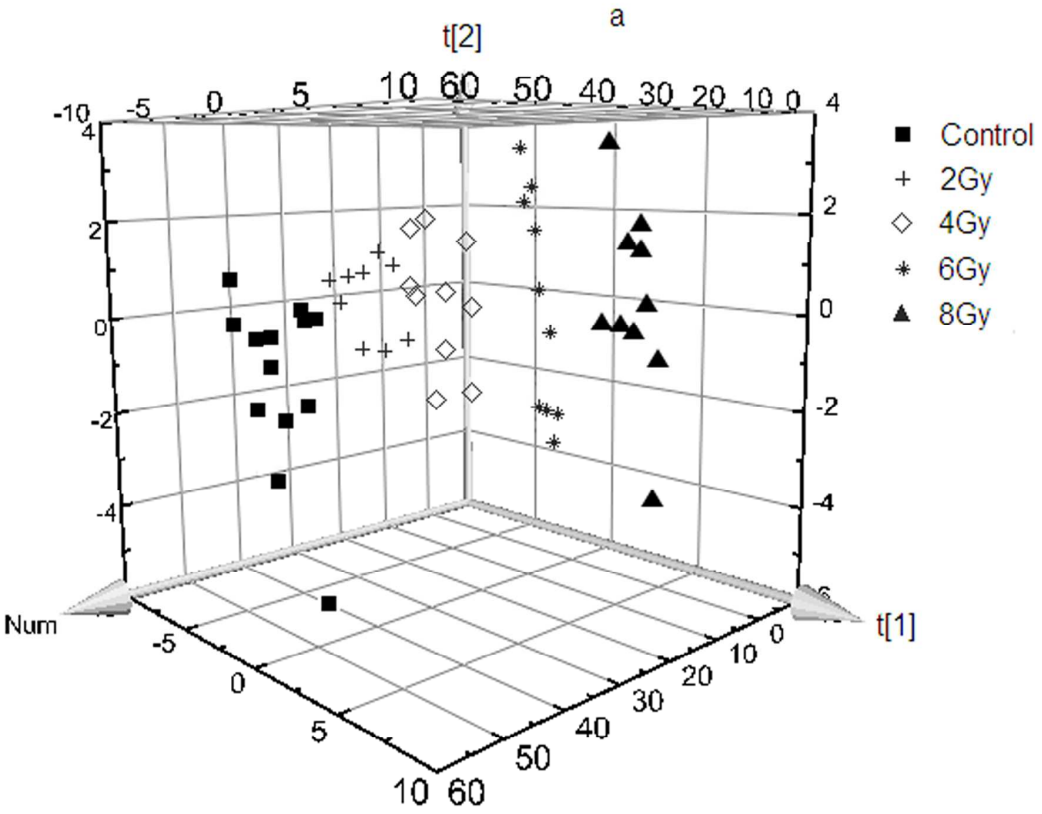
Table 1 Classification of radiation injury at different time points (5 h, 24 h, 48 h and 72 h) after radiation exposure by non-linear KPLS and potential biomarkers of radiation injury.

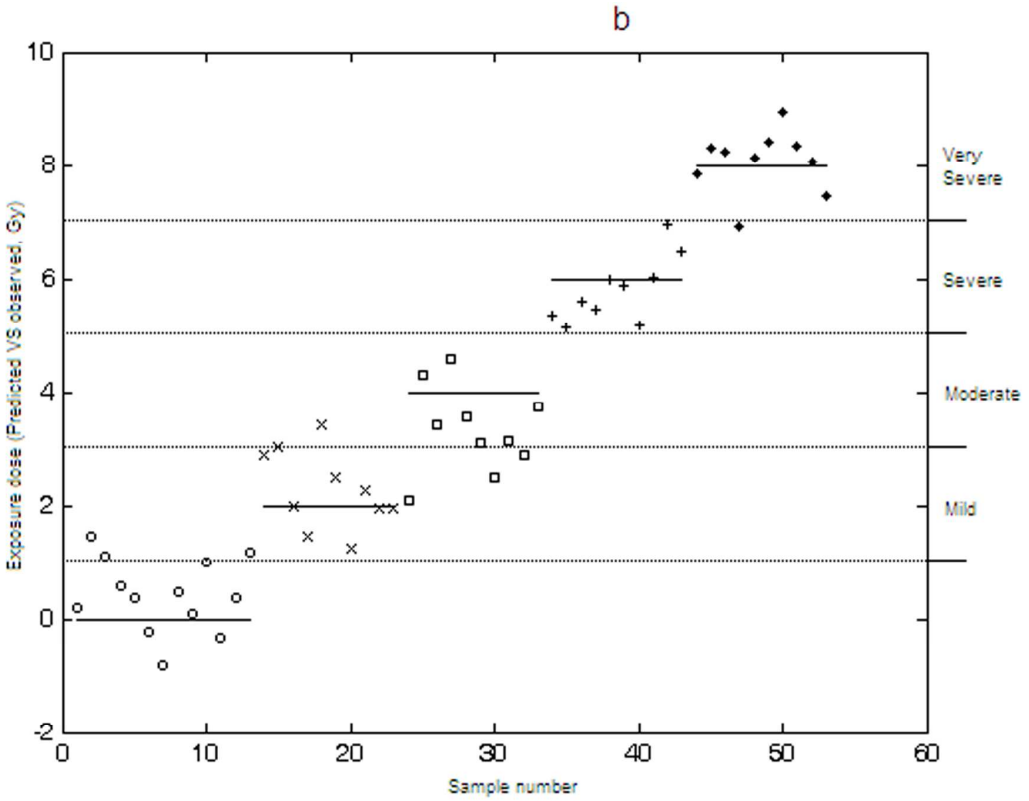
Triage	Control	Mild (1-3 Gy)	Moderate (3-5 Gy)	Severe (5-7 Gy)	Very Severe (> 7Gy)
Accuracy of classification at 5 h post radiation	92.3%	60%	81.8%	60.0%	60.0%
Accuracy of classification at 24 h post radiation	92.3%	100.0%	60.0%	75.0%	60.0%
Accuracy of classification at 48 h post radiation	84.6%	100.0%	80.0%	80.0%	70.0%
Accuracy of classification at 72 h post radiation	76.9%	100.0%	70.0%	100.0%	90.0%
Potential biomarkers of radiation injury	Trp, His, Phe, Ala, Gln, Hyp, Thr, Gly, Lys, Cit, Nov, Orn, Cys				

Figure Legends

Fig. 1 (a) Non-linear KPLS 3D representation score plot. (b) Comparison between the predicted radiation doses and observed values and (c) validation plot obtained from 100 permutation tests based on 72 h urinary amino acids after radiation exposure.

Fig.2 The integrated metabolic pathways related to radiation injury-induced biochemical perturbation.





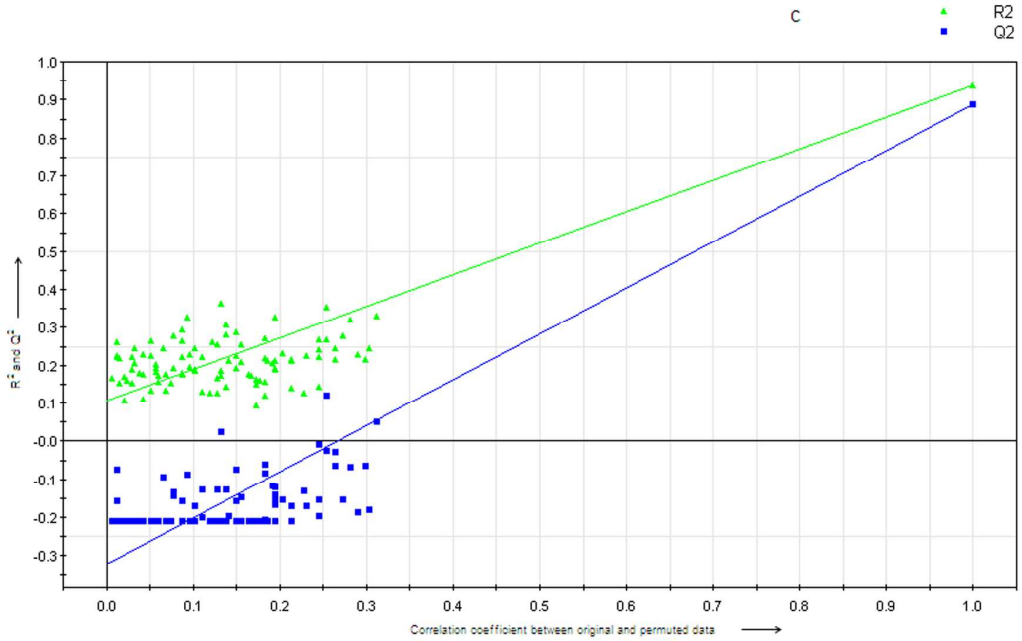


Fig.1

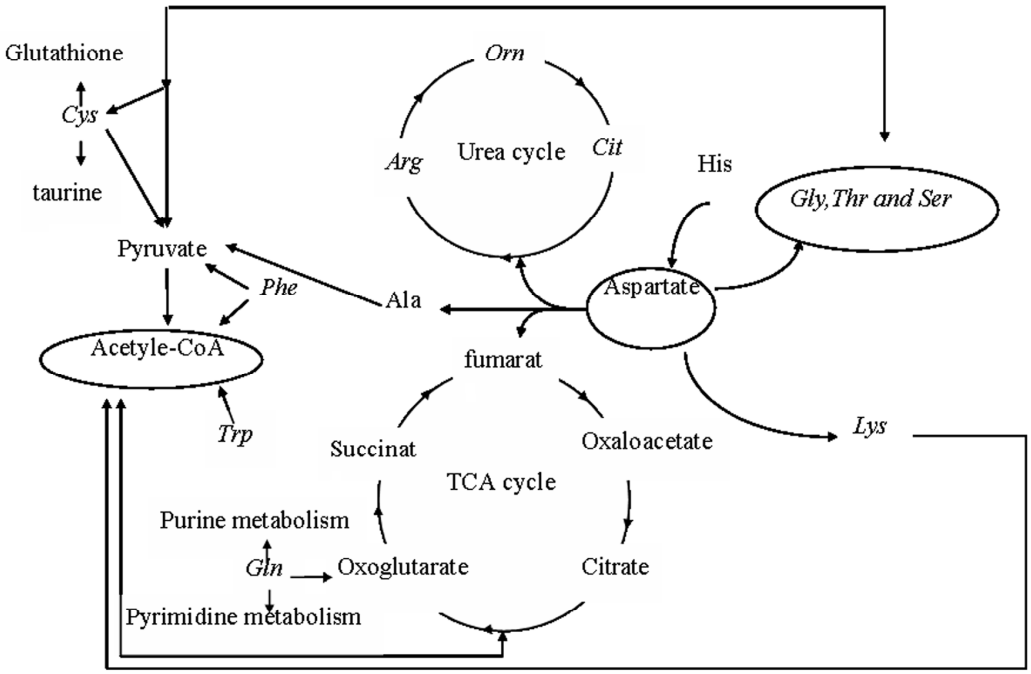


Fig.2