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Original Article

Serum metabolic changes in rats after conventional external beam radiotherapy by gas chromatography-mass spectrometry

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Abstract: Conventional external beam radiotherapy has been widely used in various clinical malignant and pain management applications. In this study, we developed a serum metabolomic method based on gas chromatography-mass spectrometry (GC-MS) to evaluate the effect of conventional external beam radiation on rats. Thirty rats were randomly divided to radiation group (600 lx, 800 lx) and control group. Radiation group were under radiation (600 lx, 800 lx) for 1 h. Blood samples were collected from the rats from the control group and radiation group at first, second and third days, respectively. Partial least squares-discriminate analysis (PLS-DA) revealed that radiation induced metabolic perturbations. Compared to the control group, the level of propanoic acid of the 600 lx radiation group increased; the level of d-Glucose of the 800 lx radiation group decreased at the first day. Compared to the control group, the level of propanoic acid and ethanedioic acid of the 600 lx radiation group increased at the second day. Compared to the control group, the level of propanoic acid of the 600 lx radiation group increased; the level of d-Glucose of the 800 lx radiation group decreased at the first day. The results indicate that metabolomic methods based on GC-MS may be useful to elucidate effect of radiation on rat through the exploration of biomarkers (propanoic acid, d-Glucose, ethanedioic acid).

Keywords: Metabolomics, GC/MS, radiation, rat

Introduction

Radiotherapy is the treatment of disease, esp cancer, by means of alpha or beta particles emitted from an implanted or ingested radioisotope, or by means of a beam of high-energy radiation [1-3]. In recent years, radiotherapy has been widely used in various clinical malignant and pain management applications [4, 5]. However, its inevitable and invisible damage to our bodies cannot be ignored, especially the radiation induced liver disease (RILD), which is mainly fatal complications secondary to radiotherapy [6-8]. Therefore, more and more efficient methods are applied to assess the injury severity as well as to take proper measures.

Analytical sensitivity is increasing with the use of new noninvasive methods. One of these is metabolomics, which appears useful for finding specific biomarkers of radiation [9-11]. Metabolomics is an emerging field with great potential for radiation biodosimetry for the fact that blood cells and serum have proven to be abundant sources of human radiation biomarkers [12-15]. To date, few studies interiorly use metabonomics method in radiation damage-related researches. In this study, we have harnessed the gas chromatography-mass spectrometry and various multivariate data analyses to uncover metabolomic responses in irradiated mice.

Material and methods

Chemicals and animals

Trimethylchlorosilane (TMCS) and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) we-

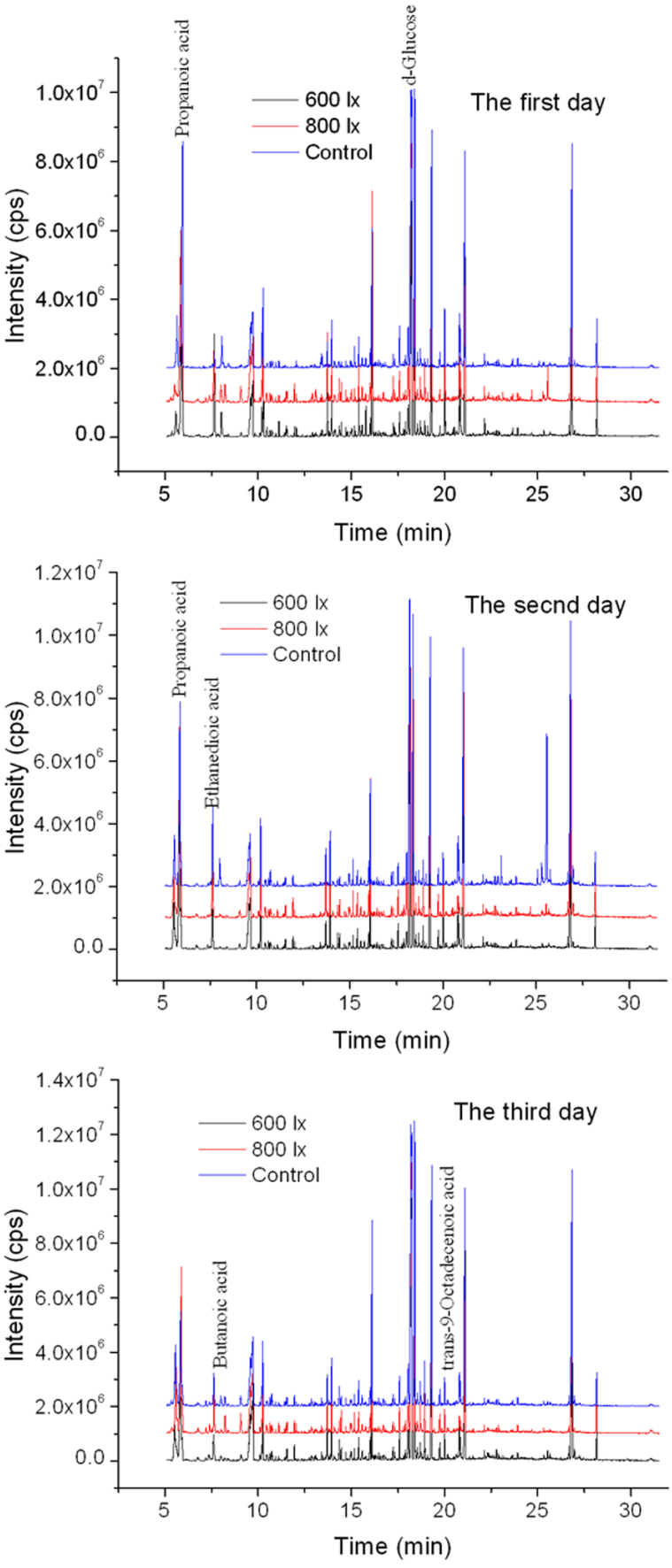

Figure 1. Typical GC-MS total ion chromatogram of rat serum after radiation at the first day, the second day and the third day.

re purchased from Sigma-Aldrich (Shanghai, China). HPLC-grade n-heptane and acetonitrile were purchased from Tedia Reagent Company (Shanghai, China). Pyridine and methylhydroxylamine hydrochloride were purchased from Aladdin Industrial, Inc. (Shanghai, China). Sprague-Dawley rats (male, 220±20 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd.

Instrumentation and conditions

Agilent 6890N-5975B GC/MS, HP-5MS (0.25 mm×30 m×0.25 mm), were from Agilent Company (Santa Clara, California, USA). The temperature was then gradually increased to 260°C at a rate of 10°C/min, and then kept at 260°C for 10 minutes. The GC oven was initially set at 80°C and was kept at this temperature for 5 minutes. Mass detection was conducted first in EI mode with electron energy of 70 eV, then in full-scan mode with m/z 50-550, and finally, by splitless mode injection [16, 17].

Sample preparation

The 250 μL of acetonitrile was added to 100 μL of serum, kept in an ice-bath for 15 min, and then were centrifuged at 10000 g for 10 minutes at 4°C. The 150 μL of the supernatant was transferred to a GC vial and evaporated to dryness under a stream of nitrogen gas. Methoximation was carried out at 70°C for 24 h after 50 μL of methylhydroxyl-

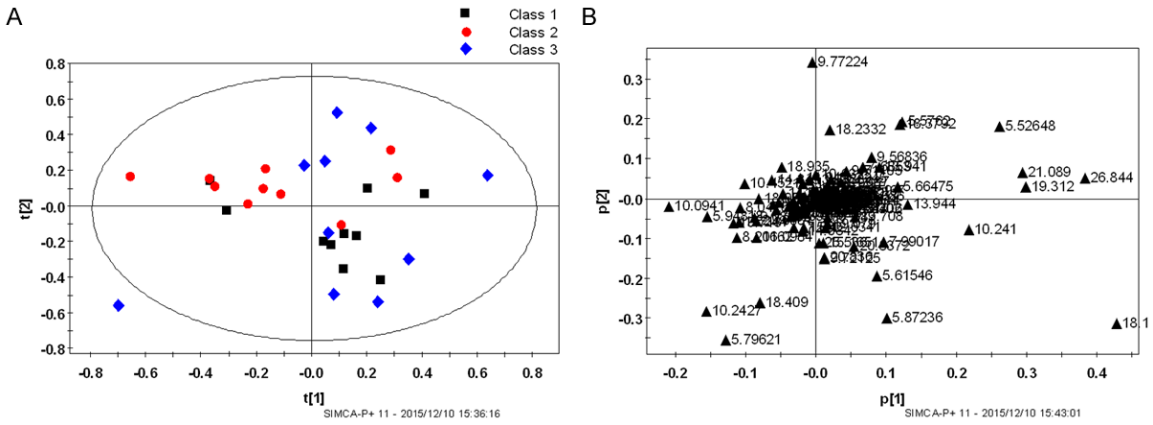

Figure 2. PCA score results of rat serum samples (A), after radiation (600 lx, Class 1; 800 lx, Class 2), Control (Class 3) at the first day; the corresponding load diagram (B).

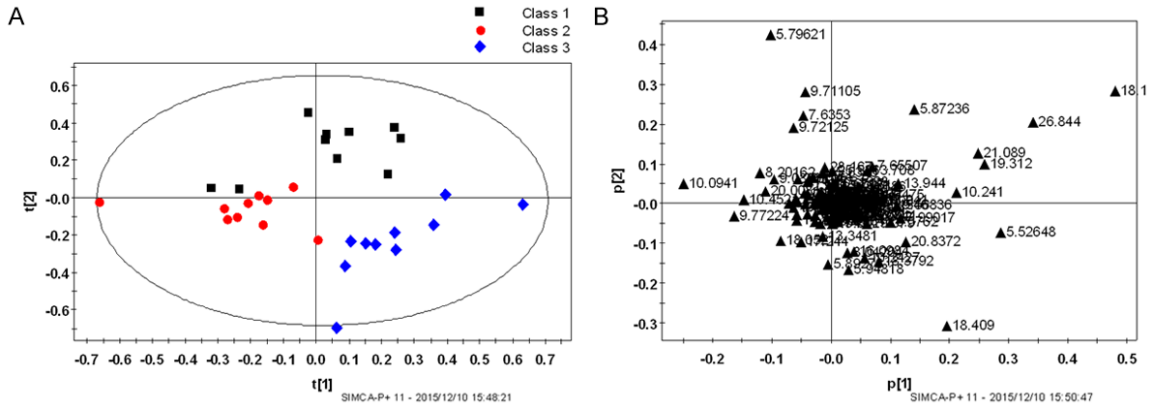

Figure 3. PLS-DA score results of rat serum samples (A), after radiation (600 lx, Class 1; 800 lx, Class 2), Control (Class 3) at the first day; the corresponding load diagram (B).

amine hydrochloride (15 mg/mL in pyridine) was added. The 50 μL MSTFA (with 1% TMCS as the catalyst) was added and kept at 70°C for another hour, and then vortexed after adding 150 μL n-heptane [17].

Metabolomics study

Rats were housed under a natural light-dark cycle conditions with controlled temperature (22°C). All thirty rats were housed at Laboratory Animal Research Center of Wenzhou Medical University. All experimental procedures were approved ethically by the Administration Committee of Experimental Animals of Wenzhou Medical University.

Thirty rats (220±20 g) were randomly divided to radiation group (600 lx, 800 lx) and control group. Radiation group were under radiation (600 lx, 800 lx) for 1 h. Blood samples were collected from the rats from the control group and radiation group at first, second and third days, respectively. The blood samples were collected and then centrifuged at 8000 g for 10 min at 4°C. The serum was stored at -80°C until measurement.

Data analysis

The GC/MS data was exported into Microsoft Excel, with the peaks normalized to the total sum of spectrum prior to multivariate analyses. The resulting data was processed through principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) using SIMCA-P 11.5 software (Umetrics, Umea, Sweden).

Statistical analysis

Statistical analysis was carried out using SPSS software (Version 18.0, SPSS). Independent samples T-test was applied in order to detect

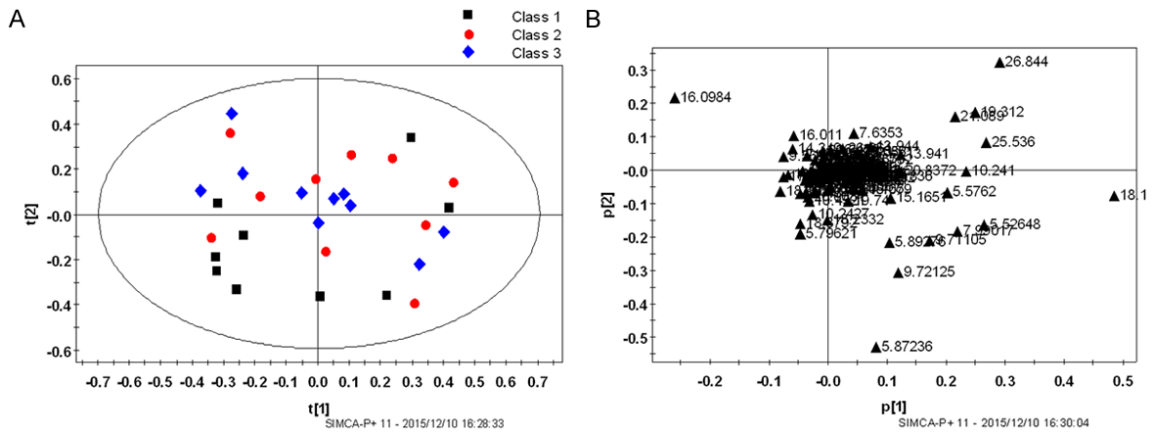

Figure 4. PCA score results of rat serum samples (A), after radiation (600 lx, Class 1; 800 lx, Class 2), Control (Class 3) at the second day; the corresponding load diagram (B).

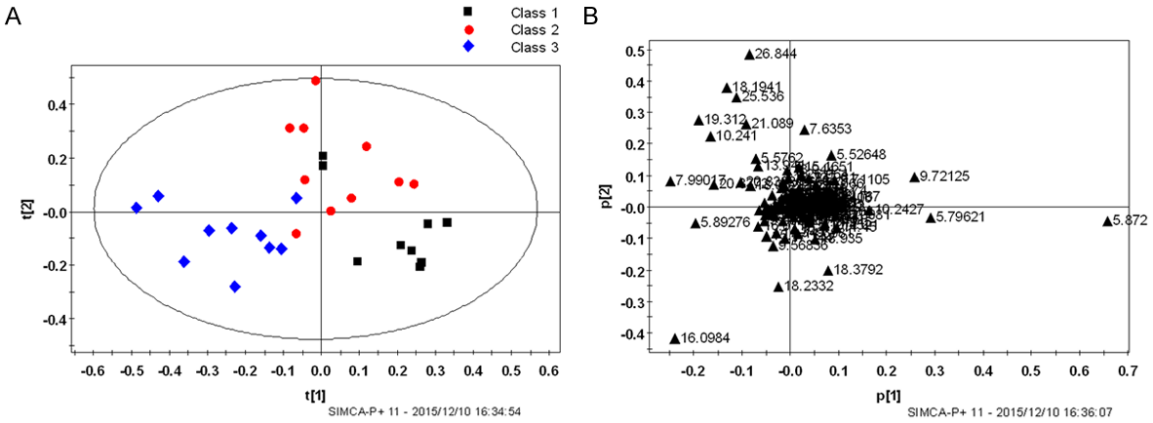

Figure 5. PLS-DA score results of rat serum samples (A), after radiation (600 lx, Class 1; 800 lx, Class 2), Control (Class 3) at the second day; the corresponding load diagram (B).

significant differences in all metabolites between two groups. A *P* value of <0.05 was considered statistically significant.

Results and discussion

Metabolomics study

Metabolomics is a newly emerging omics approach to the investigation of metabolic phenotype changes induced by environmental or endogenous factors [18-23]. It has shown promising results in healthcare fields, especially in disease diagnosis and drug-toxicity assessment, as reviewed recently [24, 25].

Figure 1 provides the typical metabolic profiles of serum at first, second and third day acquired through GC-MS technique. Metabolic profile data pretreatment resulted in a final dataset consisting of eighty metabolic features from GC-MS analyses. The endogenous metabolites in the serum were identified according to NIST 2005 mass spectrometry database.

In order to explore the metabolic profile changes of rats in radiation group (600 lx, 800 lx), we compared the GC-MS spectrum of PCA of the radiation group (600 lx, 800 lx) with the rats in the control group (**Figures 2A**, **4A** and **6A**), the corresponding load diagram was shown in **Figures 2B**, **4B** and **6B**. The PLS-DA of the radiation group (600 lx, 800 lx) with the rats in the control group (**Figures 3A**, **5A** and **7A**), the corresponding load diagram was shown in **Figures 3B**, **5B** and **7B**. **Figures 3A**, **5A** and **7A** PLS-DA score chart showed that the first principal components of the rats in the radiation group (600

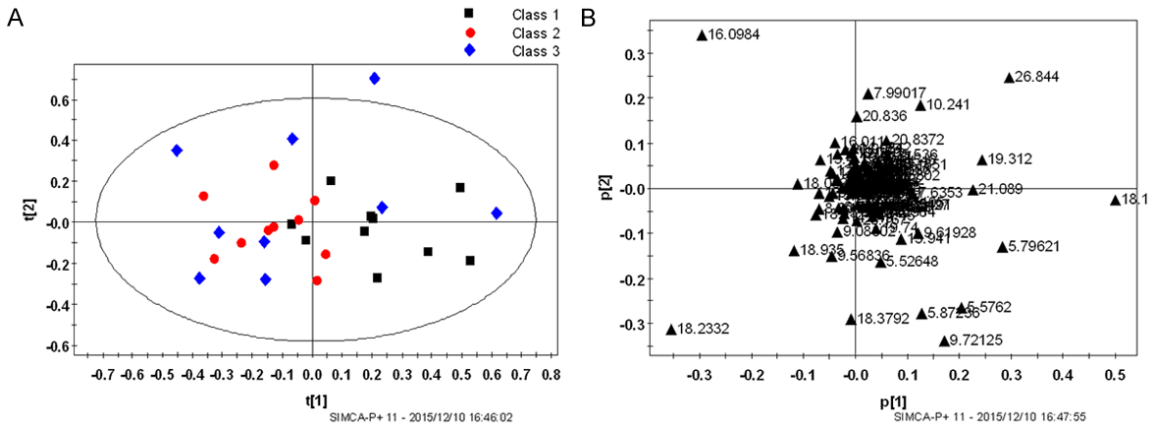

Figure 6. PCA score results of rat serum samples (A), after radiation (600 lx, Class 1; 800 lx, Class 2), Control (Class 3) at the third day; the corresponding load diagram (B).

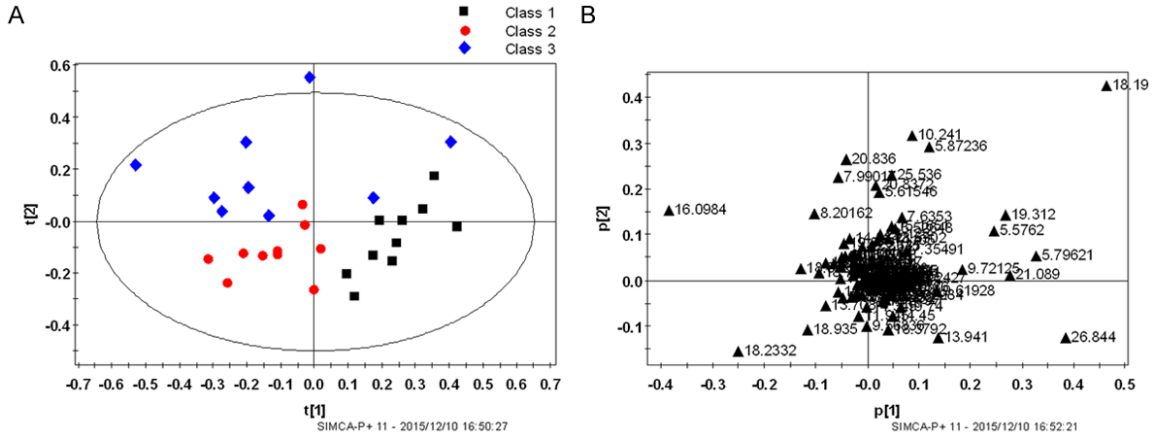

Figure 7. PLS-DA score results of rat serum samples (A), after radiation (600 lx, Class 1; 800 lx, Class 2), Control (Class 3) at the third day; the corresponding load diagram (B).

lx, 800 lx) were distinguished from the rats in the control group, the results of PLS-DA were better than PCA.

Changes in metabolite

Metabolomics comprises the measurement of endogenous metabolites, including amino acids, nucleic acid precursors, lipids, and degradation products of chemical intermediates in catabolism and biosynthesis. The advantage of metabolomics is that it provides the most functional measure of cellular status and can help to describe an organism's phenotype [26-28].

In this study, the changes of metabolites between radiation groups and their control group were shown in **Tables 1-3**. Compared to the control group, the level of propanoic acid of the 600 lx radiation group increased; the level of d-Glucose of the 800 lx radiation group decreased at the first day, **Table 1**. Compared to the control group, the level of propanoic acid and ethanedioic acid of the 600 lx radiation group increased at the second day, **Table 2**. Compared to the control group, the level of propanoic acid of the 600 lx radiation group increased; the level of d-Glucose of the 800 lx radiation group decreased at the first day, **Table 3**. These finding may be useful for new evidences in radiation study. Additional prospective studies will be required to better understand these observations.

Conclusion

These biomarkers (propanoic acid, d-Glucose, ethanedioic acid) could be useful for further

Table 1. Summary of the changes in relative levels of metabolites in rat serum after radiation at the first day

NO.	Renten time/min	Metabolite	VIP	Group	
				600 lx	800 lx
1	18.1941	d-Glucose	4.06616	-	↓*
2	18.409	L-Tyrosine	3.11377	-	-
3	5.87236	Propanoic acid	2.86904	↑*	-
4	7.6353	Ethanedioic acid	2.66764	-	-
5	9.71105	Urea	2.50205	-	-
6	26.844	Benzoic acid	2.38154	-	-
7	8.20162	Glycine	2.06363	-	-
8	20.8372	trans-9-Octadecenoic acid	2.01641	-	-

Note: Variable importance in the projection (VIP) was acquired from the PLS-DA model with a threshold of 2.0. Marks indicate the direction of the change, i.e. ↓ for decrease, ↑ for increase, - for no change. Compared control group with radiation group (600, 800 lx), *P<0.05, as indicated by the statistical analysis T-test.

Table 2. Summary of the changes in relative levels of metabolites in rat serum after radiation at the second day

NO.	Renten time/min	Metabolite	VIP	Group	
				600 lx	800 lx
1	5.87236	Propanoic acid	6.28194	-	↑**
2	16.0984	L-Cysteine	3.17248	-	-
3	7.6353	Ethanedioic acid	3.09429	-	↑**
4	25.536	Glycine	2.54632	-	-
5	7.99017	Butanoic acid	2.54243	-	-
6	26.844	Benzoic acid	2.21736	-	-

Note: Variable importance in the projection (VIP) was acquired from the PLS-DA model with a threshold of 2.0. Marks indicate the direction of the change, i.e. ↓ for decrease, ↑ for increase, - for no change. Compared control group with radiation group (600, 800 lx), **P<0.01, as indicated by the statistical analysis T-test.

Table 3. Summary of the changes in relative levels of metabolites in rat serum after radiation at the third day

NO.	Renten time/min	Metabolite	VIP	Dose group	
				Low	High
1	26.844	Benzoic acid	4.01279	-	-
2	18.1941	d-Glucose	3.80095	-	-
3	16.0984	L-Cysteine	3.54475	-	-
4	5.87236	Propanoic acid	3.20581	-	-
5	20.8372	trans-9-Octadecenoic acid	2.92908	-	↓*
6	19.312	Hexadecanoic acid	2.55216	-	-
7	21.089	Octadecanoic acid	2.45395	-	-
8	25.536	Glycine	2.44574	-	-
9	7.99017	Butanoic acid	2.16151	↓*	↓*

Note: Variable importance in the projection (VIP) was acquired from the PLS-DA model with a threshold of 2.0. Marks indicate the direction of the change, i.e. ↓ for decrease, ↑ for increase, - for no change. Compared control group with radiation group (600, 800 lx), *P<0.05, as indicated by the statistical analysis T-test.

radiation. We demonstrated that metabolomic methods based on GC/MS could provide a useful tool for exploring biomarkers in radiation study.

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Disclosure of conflict of interest

None.

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