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The effect of feeding state on the level of detections of plasma metabolites in rats after irradiation

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► ***Original article***

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ABSTRACT

***Background*:** The existence of correlates between radiation and plasma metabolites in rats might be affected by feeding conditions. ***Materials and Methods*:** The rats were kept without food and water for a certain time before the blood was harvested on the seventh day after X-ray irradiation at doses of 0 and 8 Gy. The plasma metabolites were tested using Enzyme-Linked Immunosorbent Assay (ELISA). ***Results*:** Our results showed that abrosia for 2 h before blood harvesting could increase the level of detections of both interleukin-6 (IL-6) and glycine (Gly) in rats. Furthermore, abrosia and meanwhile water deprivation for 2-4 h increased better the level of detections of IL-6 and Gly in rats. ***Conclusion*:** The level of detections of biomarkers in the blood may be more authentic and can better reflect the changes in the experimental animals after stress when they are treated by both abrosia and water deprivation for 2 h before blood harvesting.

INTRODUCTION

Along with the extensive application of atomic energy and nuclear medicine, there is growing concern over how radiation will affect the environment and human health. To evaluate radiological hazards, diagnose and treat various types of radiation-related damage, novel methods that can estimate biological exposure in a time-saving way are urgently needed [1]. Conventional biological methods of estimating radiation dosage include chromosome aberration analysis [2-6], premature chromosome condensation assay [7, 8], micronucleus assay [9-11], somatic cell mutation detection [12], *etc*. Lately, researchers at Columbia University established Rapid Automated Biodosimetry Tool to measure the level of γH2AX of leukocytes as a marker for DNA damage [13]; however, these methods have some drawbacks to some extent, for example, time-consuming processes, complicated procedures, high cost, *etc*. Consequently, seeking novel ionizing radiation associated biological markers and widely-applicable testing methods are tasks of top priority, which will bring about significant influences on both rescue efficiency and therapeutic effect. Following genomics and proteomics, metabolonomics has become an emerging research hotspot [14-16]. Detecting changes in the amounts of amino acid metabolites or cytokines in the blood (urine) samples of radiation victims directly with highly sensitive instruments saves time, while being more sensitive, reliable, and minimally invasive, and more widely applicable [17, 18]. Biological markers can not only help to study pathogenesis from a molecular perspective but also have their unique advantages in terms of evaluating accuracy and sensitivity for low-level damage in early -stage, thus providing early warning and auxiliary diagnosis [19]. Collecting blood samples for early diagnosis, physical examination, and prognosis analysis has been well known and widely applied in clinics for human medicine, in which some examinations require the patient to have an empty stomach in the morning to avoid interference arising from dietary metabolism [20-24].

Although there have been reports on a dose estimation method established by measuring the content change of metabolites after radiation, a search of the literature found that the feeding conditions of animals before blood harvesting remain unclear or simply were not investigated [18, 25]. To judge whether feeding conditions before hemospasia affect the serum biomarker levels of experimental animals after radiation, we did cut off their food and/ or water supply before blood harvesting. Then the levels of IL-6 (interleukin-6, inflammation factor) and Gly (glycine, amino acid metabolites) were measured for analysis and assessment. The aim of our current work is to provide an experimental standard in

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animal blood harvesting for the measurement of biomarkers in serum or plasma, to establish an ideal radiation dosage biological model for radiation protection or diagnose the disease with more authentic, more accurate detection data.

MATERIALS AND METHODS

Irradiation experiment

The irradiation in this study was carried out by the X-Rad 320 irradiator (USA) at the research platform of radiation protection and emergency technology in Southern Zhejiang, Wenzhou Medical University. The dose rate was 2 Gy/min.

Animal grouping and treatment

Animal experiments conducted in current experiment were approved by Wenzhou Medical University Institutional Animal Use and Care Committee. Sixty male SD rats (at an age of seven weeks) were purchased from Zhejiang Vital River Experimental Animal Technology Co. Ltd (Charles River Lab. China). The rats were randomly divided into two groups (n = 30) and were irradiated with 0 and 8 Gy.

Before blood samples were collected on the seventh day after irradiation, 60 rats were divided into twelve groups (five rats per group) following different feeding treatments (table 1).

Table 1. Grouping and treatment of rats.

Feeding treatment	Unirradiated control group (0 Gy)	Irradiated group (8 Gy)
Free diet (continuous food and water supply) before hemospasia	A0	A8
Without food for 2 h, but continuous water supply before hemospasia	B0	B8
Without food for 4 h, but continuous water supply before hemospasia	C0	C8
Without food for 8 h, but continuous water supply before hemospasia	D0	D8
Without food and water for 2 h before hemospasia	E0	E8
Without food and water for 4 h before hemospasia	F0	F8

The blood samples of rats in each group were harvested, using the tail-cutting method, into the blood collection vessels containing anticoagulant, and centrifuged at 3000 rpm for 5 min. The plasma was collected and stored at -80 °C for subsequent use.

Measurement of serum metabolites

The rat IL-6 enzyme-linked immunosorbent (ELISA) kit was purchased from Shanghai Shenggong Co., Ltd; Rat Gly ELISA kits were purchased from Wuhan Moshak Biotechnology Co., Ltd. The BioTek 800 (BioTek Company of the United States) microplate meter was used to measure the OD value of serum metabolites.

The levels of IL-6 and Gly were measured by ELISA according to the manufacturer's instructions (*i.e.* we take out the kit and allow equilibration to room temperature, dilute the working fluid and standard fluid according to the instructions; 100 μL of standard or test samples were added to each reaction well, and plates were then incubated at 37 ℃ for 90 min. The liquid was discarded, dumped and dried, and 100 μL of biotin-labelled IL-6 antibody working solution was added to each reaction well; the plate was then blocked and incubated at 37 ℃ for 60 min; after discarding the liquid, we shook the liquid dry, added 350 μL of wash liquid to each reaction well, soaked it for 1-2 min, and shook it dry with the wash liquid, which was repeated four times. 100 μL HRP of labelled streptavidin working solution was added to the reaction well, then blocked and incubated for 30 min at 37 ℃ . The plate was re-washed five more times and 90 μL of color developer was added to the reaction well, incubated in the dark at 37 °C for 15 min before adding 50 μL termination solution. (OD values were measured at 450 nm with a BioTek 800 microplate meter). The comparison between groups was conducted according to the OD values.

Statistics

Results are presented as mean ± s.d (n = 5). Statistical analyses were performed using Prism software (GraphPad Software 9). The statistical significance (P values) in mean values of two-sample comparison was determined with Student's t-test. A value of $P < 0.05$ was considered statistically significant (*).

RESULTS

Effect of abrosia on the level of detection of metabolites in rat plasma

Abrosia for 2 h increased the level of detection of IL-6

Figure 1 shows the relative level of IL-6 in plasma of rats 7 days after irradiation. As illustrated in figure 1A, after abrosia for 2-4 h, the level of IL-6 in irradiation groups was higher than those of non-irradiated specimens although there was no significant difference between them. While the relative contents of IL-6 in both irradiated and non-irradiation groups after abrosia for 2 h increase in contrast with free diet groups (0 h group). Figure 1B shows that the level of IL-6 in the unirradiated groups (0 Gy) abrosia for 2 h, and in 8-Gy irradiation groups after being treated without food for 2-4 h tended to increase, compared with those of free diet groups, respectively. These results indicate that abrosia for short-time (2 h) may slightly activate inflammatory factor generation.

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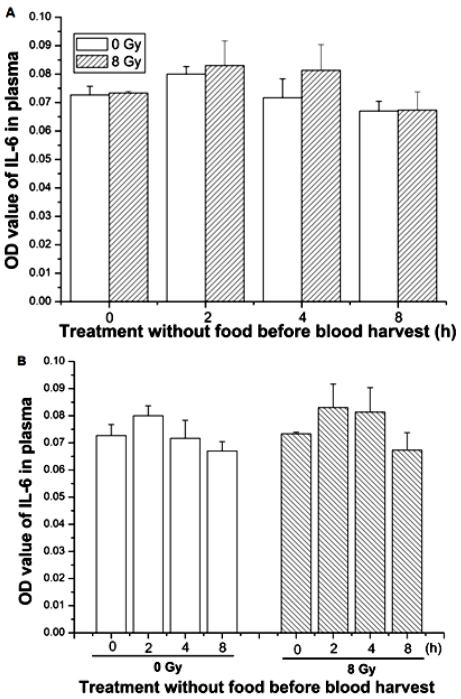

Figure 1. Effect of abrosia before blood harvesting on the level of detection of IL-6 in plasma of rats. **(A)** Comparison between unirradiated and irradiated groups under the condition of same feeding treatment. **(B)** Comparison among different abrosia treatments under the same level of irradiation.

Abrosia for 2 h increased the measured level of Gly

Chromatographic analysis shows the contents of nine kinds of amino acids increased including Gly in the serum of rats exposed to γ-rays [26]. Here, Gly was selected as a representative to estimate the effect of abrosia on the detection of amino acid metabolites in blood samples using the ELISA method.

Figure 2 shows the Gly levels of plasma in irradiation groups increased compared with those of non-irradiated groups respectively. Furthermore, the abrosia for 2 h in the irradiated group significantly increased the level of Gly, compared to the non-irradiated specimens (figures 2A & 2B). However, there was no significant increase when all of the abrosia groups were compared with the free diet group within the unirradiated groups (figure 2B). These results suggest that short-term abrosia may increase the level of detection of Gly in rats at 7 d after irradiation.

Effect of treatment without food and water on the level of detection of metabolites in rat plasma

Abrosia and water deprivation for 2-4 h increased the level of detection of IL-6

Given that too long a water-deprivation test was likely to have an unpredictable influence on the physiological states of rats, only three time points were set, namely 0, 2 and 4 h. Figure 3 demonstrates the effect of treatment without food and water on the level of detection of IL-6 in the plasma of rats 7 d after irradiation. The levels of IL-6 in irradiation groups were higher than those of the non-irradiated groups when these rats were deprived for food and water for 2-4 h, and there was a significant increase between the irradiated and unirradiated groups in the 4-h treatment (figure 3A).

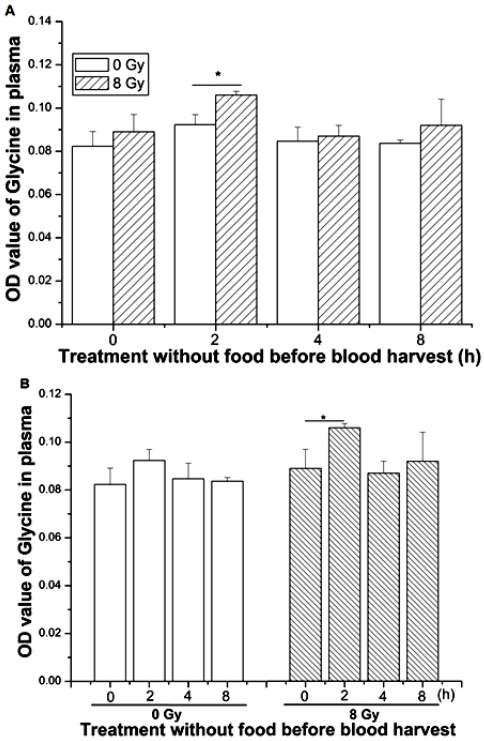

Figure 2. Effect of abrosia before blood harvesting on the level of detection of Gly in plasma of rats. **(A)** Comparison between unirradiated and irradiated groups under the same feeding treatment. **(B)** Comparison among different abrosia treatments under the same irradiation. * $P < 0.05$.

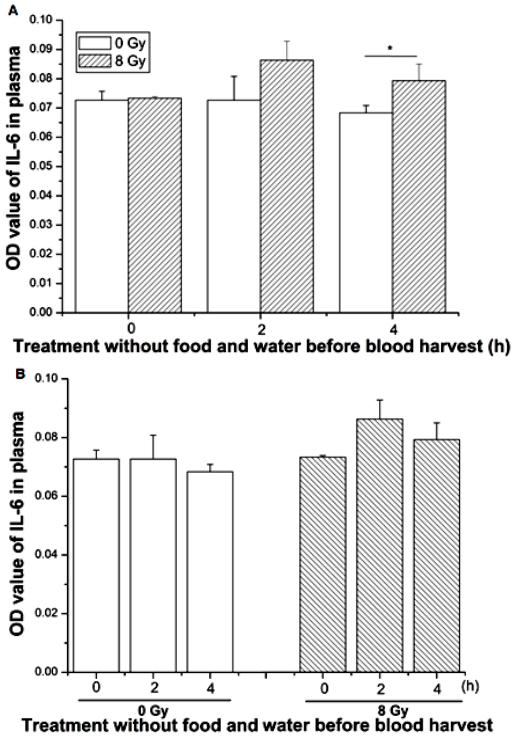

Figure 3. Effect of food and water deprivation before blood harvesting on the level of detection of IL-6 in plasma of rats. **(A)** Comparison between unirradiated and irradiated groups under the same feeding treatment. **(B)** Comparison among different abrosia and water-deprivation treatments under the same irradiation. * $P < 0.05$.

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Abrosia and water deprivation for 2 h increased the level of detection of Gly

Figure 4 illustrates the changes in Gly level in the plasma of rats after a certain period of abrosia and water deprivation before blood harvest. Compared with the non-irradiation control groups, the levels of Gly in irradiated specimens increased when the rats were deprived of food and water for 0 and 2 h. Furthermore, there was a significant difference between the irradiated and non-irradiated groups after being treated without food and water for 2 h (figure 4A). Furthermore, the levels of Gly in the groups treated without food and water for both 2 and 4 h were higher than those of groups with a free diet either in 0 or 8-Gy irradiation groups (figure 4B); however, the level in the unirradiated group was a little higher than that in the irradiated group under the food and water deprivation for 4 h (figure 4A). Thus, this result suggests that food and water deprivation for 2 h was of benefit to improving the level of detection of serum metabolites.

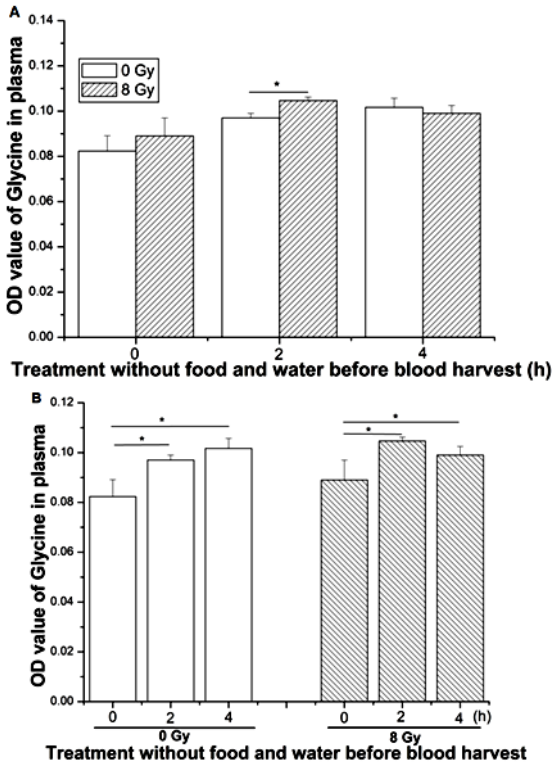

Figure 4. Effect of food and water deprivation before blood harvesting on the level of detection of Gly in plasma of rats. **(A)** Comparison between unirradiated and irradiated groups under the same feeding treatment. **(B)** Comparison among different abrosia and water-deprivation treatments under the same irradiation. * P < 0.05.

DISCUSSION

After being stimulated by irradiation, the organism will produce a series of oxidative stress reactions, catalyzing the changes of the small molecule metabolites accordingly in the bodily fluid [27, 28]. Moreover, taking the body fluid (blood or urine) as a biomarker source has certain advantages in finding non-invasive indicators for radiation damage [19]. Studies relevant in metabolomics indicate that various small molecule metabolites, including amino acids, lipids and inflammatory factors, are of great importance in analysis of radiation biological effects [29-31]. Although there are parts of previous studies for using metabolomics to screen radiation dose as the markers, there remains a paucity of available radiation biomarkers [15, 16, 32].

Given that there are usually abrosia requirements for taking blood samples from patients in a clinical setting, it was supposed that feeding conditions would also affect the plasma metabolite levels of the experimental animals after irradiation. Therefore, in this study, SD rats were under different feeding conditions controlled before blood harvest, and then the plasma levels of IL-6 and Gly were measured. IL-6, produced mainly by lymphocytes, is a multifunctional cytokine with a wide range of biological activities and plays an important role in the body cytokine network [33]. Our results showed that IL-6 levels were generally higher in the irradiated group compared to the non-irradiated group. After 2 h of abrosia, the relative content of IL-6 was increased in both the irradiated and non-irradiated groups compared to the free-diet group. Thus, abrosia can change the plasma IL-6 levels in animals. Moreover, the irradiated group presented higher IL-6 levels compared to the non-irradiated group, while there was no statistical difference therein. Combined with the water deprivation treatment for 2–4 h, it was found that the irradiated rats had significantly higher plasma IL-6 levels than those of the non-irradiated group.

UV stress was found to impair IL-6 / JAK2 / STAT3 signaling in cells and activate the inflammatory mediators IL-6 and TNF-α, inducing apoptosis [34]. Dreyfuss *et al.* found that the placental growth factor, IL-6, and TNF-α significantly increase in irradiated heart tissue and plasma of mice compared to unirradiated controls at second and eighth weeks, and decreased near to control levels at four weeks post-radiotherapy [35]. A study using cell model showed that the IL-6 is one of the valid evaluation indicators, in which it was higher in the 6-Gy irradiation group than that in the 0-Gy group after irradiation [36]. Our results showed that the difference between irradiated and non-irradiated groups was not significant. Combined with the results of Dreyfuss, we considered that IL-6 levels may fluctuate, first increasing after irradiation, then decreasing at 7 d, then increasing again.

After exploring the inflammatory factors represented by IL-6, we turned our attention to Gly. As an amino acid-like substance, Gly is also involved in multiple metabolic pathways [37, 38]. Radiation causes an increase in the number of oxygen radicals [39], while Gly can relieve oxidative stress damage by regulating two enzymes, catalase and superoxide dismutase 1 [40, 41]. In UM-SCC-74B cells,

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the major alterations after irradiation were related to serine and Gly metabolism, purine metabolism, and nicotinic acid and nicotinamide metabolism [42]. Furthermore, Liu *et al.* combined gas chromatography/time-of-flight mass spectrometry with principal component analysis to evaluate changes in serum metabolites levels in rats, and found that all nine metabolites (including Gly) could serve as potential biomarkers for the diagnosis of radiation injury [43]. In current experimental results, we found that the Gly levels in plasma were elevated in the irradiated group compared to the non-irradiated group. Through the comparison of different abrosia time treatments with free diet, Gly levels were significantly increased in the irradiated group subject to abrosia for 2 h, while the difference between the unirradiated groups was not statistically significant. Thus, it is reasonable to speculate that diet may partially mask the effect of irradiation stimulation on Gly metabolism in rat plasma. Meanwhile, the same results were obtained in abrosia and water deprivation for 2-h treatments, and Gly levels were higher in all the groups with treatments than in the free diet groups. Therefore, abrosia and water deprivation for 2 h may improve detection of the levels of serum metabolites.

In our study, how feeding conditions before blood harvesting effect on the level of detection of certain metabolites in rat plasma was explored. Compared with conventional animal studies which concentrated more on the effect of irradiation, we also attach importance to the easily-neglected aspect, which may provide a new idea for experimental standardization. To clarify the effect of feeding conditions on the levels of metabolites in rat plasma, a series of experiments were conducted. The limitation of our study is that we only measured the changes of IL-6 and Gly in plasma, which constrained our findings from being generally applicable to other body fluids such as tissue fluid and other plasma metabolites, thus further research into various body fluids and other types of plasma metabolites is warranted.

CONCLUSION

Above all, based on our experimental results, it can be concluded that abrosia and water deprivation for 2 h before blood harvesting is a better way of detecting the level of biomarker in serum of plasma, and can really reflect the effect of stress such as irradiation on living creatures.

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Conflict of interest: The authors declare no competing interests.

Ethical consideration: The animal experiments conducted in this study were approved by the Wenzhou Medical University Institutional Animal Use and Care Committee.

Author contributions: B.H., R.L., and Y.W. contributed to the conception and design of the study. J.C., Y.W., and C.F. performed the investigation. R.L., J.C., and B.H. wrote the manuscript. All authors contributed to the article and approved the submitted version.

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