



## Elucidation of gastrointestinal dysfunction in response to irradiation using metabolomics

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### ABSTRACT

Gastrointestinal toxicity is frequently observed secondary to accidental or therapeutic radiation exposure. However, the variation in the intestinal metabolites after abdominal radiation exposure remains ambiguous. In the present study, C57BL/6 mice were exposed to 0, 2, and 20 Gy irradiation dose. The head and chest of each mouse were covered with a lead shield before x-ray irradiation. 24 h post-irradiation treatment, intestinal tissue of each mouse was excised and prepared for metabolites measurement using gas chromatography-mass spectrometry (GC-MS). Our comprehensive analysis of metabolites in the intestinal tissues detected 44 metabolites after irradiation, including amino acids, carbohydrates, organic acids, and sugars. Amino acid levels in the intestinal tissue gradually rose, dependent on the radiation dose, perhaps as an indication of oxidative stress. Our findings raise the possibility that amino acid metabolism may be a potential target for the development of treatments to alleviate or mitigate the harmful effects of oxidative stress-related gastrointestinal toxicity due to radiation exposure.

### 1. Introduction

Radiotherapy is well known as the mainstay treatment for several types of malignancies [1]. Approximately half of the cancer patients undergo radiation therapy and it plays a role in curing 40% of these cases [2]. Recent technological advances in the field of radiation therapy has boosted its impact and ability to target cancerous tissues. However, many patients still suffer from side effects [3]. Irradiation of pelvic tumors in particular damages the nearby healthy tissues involved in digestion [4]. The small intestine is particularly vulnerable to incidental

irradiation during the targeting of tumors in the pelvis. Nearly all patients exposed to radiation for pelvic or abdominal cancers exhibit gastrointestinal tract symptoms [5]. Previous studies elucidated the response of the small intestine to small doses of ionizing radiation (8 Gy or lower); the treatment caused visibly apoptosis in the intestinal crypts, followed by a shortening of the villi length within 5–7 days. The mice subsequently achieved full recovery with low or no persistent injury to intestinal stem cells. However, doses above 15 Gy of total body irradiation led to complete death of mice within two weeks, with complete damage of most intestinal crypts and severe loss of the epithelium [6–8].

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**Table 1**  
Profile of intestinal metabolites at 24 h post-irradiation with 0, 2, and 20 Gy.

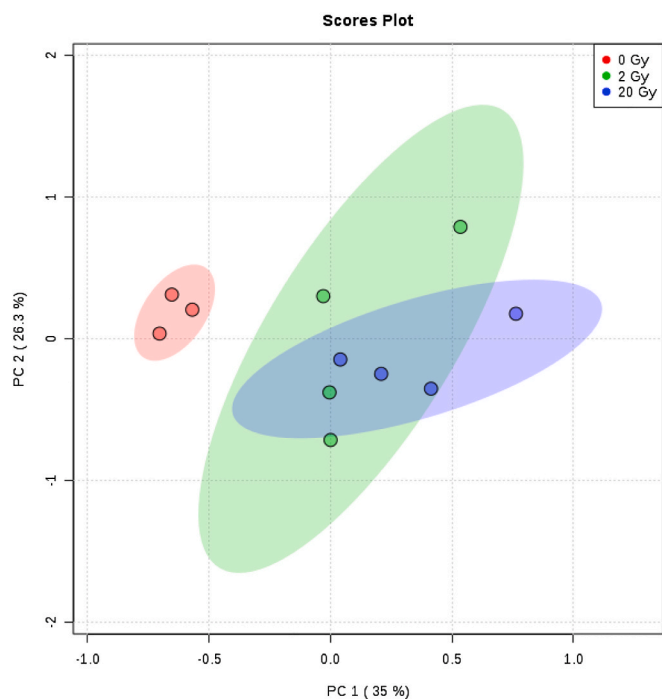
No.	Metabolite	ANOVA		Tukey-Kramer HSD			Class
		f value	p-value	0Gy vs 2Gy	0Gy vs 20Gy	2Gy vs 20Gy	
1	L-Serine	27.018	0.001 <		**	*	Amino acid
2	L-Isoleucine	26.475	0.001 <		**	**	Amino acid
3	L-Leucine	23.095	0.001 <		**	*	Amino acid
4	L-Methionine	20.784	0.001		**	*	Amino acid
5	L-Valine	20.731	0.001		**	*	Amino acid
6	L-Threonine	12.553	0.003		*	*	Amino acid
7	Glycine	10.116	0.006	*			Amino acid
8	L-Alanine	9.222	0.008		*		Amino acid
9	Cadaverine	8.310	0.011				Biogenic amine
10	L-Proline	8.043	0.012		*		Amino acid
11	L-Phenylalanine	6.119	0.024				Amino acid
12	Glycerol	5.511	0.031				Lipid metabolite
13	Creatinine	5.382	0.033				Protein waste product
14	Pyroglutamic acid	5.234	0.035				Amino acid
15	DL-β-Hydroxybutyric acid	5.074	0.038				Ketone body
16	L-Aspartic acid	4.999	0.039				Amino acid
17	GABA	4.263	0.055				Protein intermediate
18	Stearic acid	4.128	0.059				Fatty acid
19	Adenosine	4.031	0.062				Nucleoside
20	Oxalic acid	3.764	0.070				Carbohydrate intermediate
21	2,3-Bisphospho-glyceric acid	3.707	0.073				Carbohydrate intermediate
22	D-Arabinose 5-phosphate	3.689	0.073				Carbohydrate intermediate
23	Pyruvic acid	3.570	0.078				Carbohydrate intermediate
24	Citric acid	3.549	0.079				Carbohydrate intermediate
25	Ethanolamine	3.039	0.104				Lipid intermediate
26	DL-Malic acid	2.927	0.111				Carbohydrate intermediate
27	L-Sorbose	2.779	0.121				Monosaccharide
28	Taurine	2.223	0.171				Protein intermediate
29	Urea	2.018	0.195				Protein waste product
30	D-Glucuronic acid	1.505	0.279				Carbohydrate intermediate
31	Succinic acid	1.453	0.289				Carbohydrate intermediate
32	O-Phosphoethanolamine	1.391	0.303				Lipid intermediate
33	Ascorbic acid	1.259	0.335				Vitamin
34	D-Ribose	1.202	0.350				Monosaccharide
35	Glyceric acid	1.082	0.384				Carbohydrate intermediate
36	Hypotaurine	1.008	0.407				Protein intermediate
37	Inositol	0.892	0.447				Vitamin
38	L-Glucose	0.725	0.514				Monosaccharide
39	D-Galactose	0.724	0.514				Monosaccharide
40	Fumaric acid	0.722	0.515				Carbohydrate intermediate
41	Inosine	0.578	0.583				Nucleoside
42	Pyrophosphoric acid	0.523	0.612				Energy carrier product
43	L-Glutamic acid	0.223	0.805				Amino acid
44	L-Lactic acid	0.176	0.842				Carbohydrate waste product

HSD = Honestly Significant Difference. \* $p < 0.05$  and \*\* $p < 0.01$  (one-way ANOVA followed by post-hoc Tukey-Kramer HSD test).

The cellular exposure to ionizing radiation can directly disrupt the atomic structures through the direct energy deposition onto biomolecules or indirectly via generating the reactive oxygen species (ROS), this causes damage to the nucleic acids, proteins, and lipids [9]. Heat shock proteins (HSPs) family, particularly Hsp70 and Hsp90, displays a cytoprotective role against the pathological conditions such as hyperthermia, oxidative stress, ischemia, and bacterial infection via enabling the cellular protection against protein denaturation, accelerating of protein folding, and degradation of misfolded proteins [10]. HSPs are overexpressed after the cellular exposure to ionizing radiation as a response to ROS production, thus suggesting their role in the regulation of cell cycle, DNA repairs, and apoptotic cascades [11].

Metabolomics is a promising emerging technology that can analyze small metabolites (50–150 Da) in biological specimens and that is being applied to various pathological cases and therapies [12]. Superior to transcriptomics and proteomics, metabolomics registers the end-response to therapy in terms of changes at the gene and protein expression levels, providing insight into the ongoing biological processes after radiation exposure [13]. Metabolic profiling in plasma suggests potential new radiation injury metabolites related to tryptophan and pyrimidine pathways that might be utilized to diagnose whole-body radiation injury [14]. Another mouse total body irradiation

model was established to determine the metabolites that were expressed in the jejunum and circulating plasma after the exposure to sub-lethal and lethal radiation dose. Several amino acids were obviously elevated in both jejunum and plasma at 24 h post-radiation exposure indicating prospective insight into the initial inflammatory reaction during Radiation-induced small bowel disease [15]. Putative gastrointestinal markers were reported after acute exposure to low doses of radiation. Tryptophan was detected upregulated while Glutamate, Cysteine-Glycine dipeptide and methionine were also found to be downregulated [16]. Previous study showed elevation in phenylalanine and glutathione with reduction in aspartate level in the intestinal tissue after long term whole body exposure to gamma irradiation [17]. Amino acids are crucial for protein synthesis, and they are considered dynamic regulators of multiple metabolic pathways [18]. Particularly in the gastrointestinal system, amino acids participate in supporting intestinal mucosa integrity and maintain its function [19,20]. Dysregulation of intestinal amino acid metabolism post-radiation exposure may identify a panel of biomarkers used as a diagnostic, prognostic, and drug development tool. Although few studies have discussed the metabolic changes in intestinal tissue injury following whole body radiation exposure, none investigated these changes after local irradiation to the intestine that is commonly applying when using radiation therapy to treat patients with



**Fig. 1.** Principal component analysis (PCA) showed a comparison of non-irradiated (0 Gy) and irradiated groups (2 and 20 Gy). Colored circles representing 95% confidence intervals between different groups.

pelvic malignancies.

The primary goal of the present study is to identify the intestinal-specific metabolic markers during abdominal exposure to conventional (2 Gy) or potentially destructive (20 Gy) doses of ionizing radiation. That will be beneficial to find out a means to inform on injury status, expect injury outcome, and potential for expediting medical countermeasure development. We showed that radiation exposure induces distinct changes in the metabolic profile of the intestine, using a murine model.

## 2. Materials and methods

### 2.1. Experimental animals

Animals were handled under the guidance of the Committee of the Ethics of Animal Experiments of Kobe University (Permission number: P160403). All surgical approaches were performed under isoflurane anesthesia and every effort was made to minimize suffering. The mice were 10-week-old adult male C57BL/6J mice weighing 20–25 g. They were housed in standard cages at a temperature of  $20 \pm 5$  °C under a 12-h day/night cycle in pathogen-free conditions and were allowed access to food and water ad libitum. Mice were randomly assigned to treatment groups.

### 2.2. X-ray irradiation

X-ray irradiation was carried out using a MBR-1505R2 instrument (Hitachi, Tokyo, Japan) at a voltage of 150 kV and a current of 5 mA with a 1-mm-thick aluminum filter (0.5 Gy/min at the target) for *in vivo* studies. The anesthetic Somnopentyl (0.1 mg/g body weight) was intraperitoneally administered prior to radiation exposure. A lead shield covering the head and chest of each mouse was used prior to x-ray irradiation [21].

### 2.3. Sample collection and preparation

After 24 h of irradiation treatment, 6 cm of mouse intestinal tissue, starting from the lowest part of the stomach, was excised and collected. The weight of each sample was approximately 10 mg. Intestinal samples were frozen in liquid nitrogen and stored at  $-80$  °C for comparative metabolomics. Low molecular weight metabolites were extracted from intestinal tissue according to previously mentioned methods [22–24]. Intestinal samples were mixed with 1.0 mL of a solvent mixture (MeOH: H<sub>2</sub>O:CHCl<sub>3</sub> = 2.5:1:1). 10  $\mu$ L of 0.5 mg/mL 2-isopropylmalic acid dissolved in distilled water was added as an internal standard, followed by sonication for 20 s. The solution was incubated for 30 min at 37 °C and centrifuged for 3 min at 4 °C at 15,000 rpm. 500  $\mu$ L of CHCl<sub>3</sub> was poured to 1.0 mL of the supernatant followed by lyophilisation using a freeze dryer. The lyophilized samples were dissolved in 40  $\mu$ L of 20 mg/mL methoxyamine in pyridine and incubated for 90 min at 30 °C. The samples were derivatized with 20  $\mu$ L of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) for 30 min at 37 °C.

### 2.4. Gas chromatography-mass spectrometry

The gas chromatography-mass spectrometry (GC-MS) analysis of the intestinal samples was implemented using a GCMS-QP2010 Ultra (Shimadzu Co., Kyoto, Japan) according to previously described research [22,25]. A fused silica capillary column (CP-SIL 8 CB low bleed/MS; 30 m 0.25 mm inner diameter, film thickness: 0.25  $\mu$ m; Agilent Co., Palo Alto, CA, USA) was utilized. The temperature of the column was kept at 80 °C for 2 min, then elevated at 15 °C/min to 330 °C. The temperatures of the transfer line and ion source were 250 °C and 200 °C, respectively. An 85–500 *m/z* mass was exposed to 20 scans per second using the Advanced Scanning Speed Protocol (ASSP, Shimadzu Co., Kyoto, Japan). Data were analyzed using MS-DIAL software [22,26] and normalized to the tissue weight.

### 2.5. ROS measurement

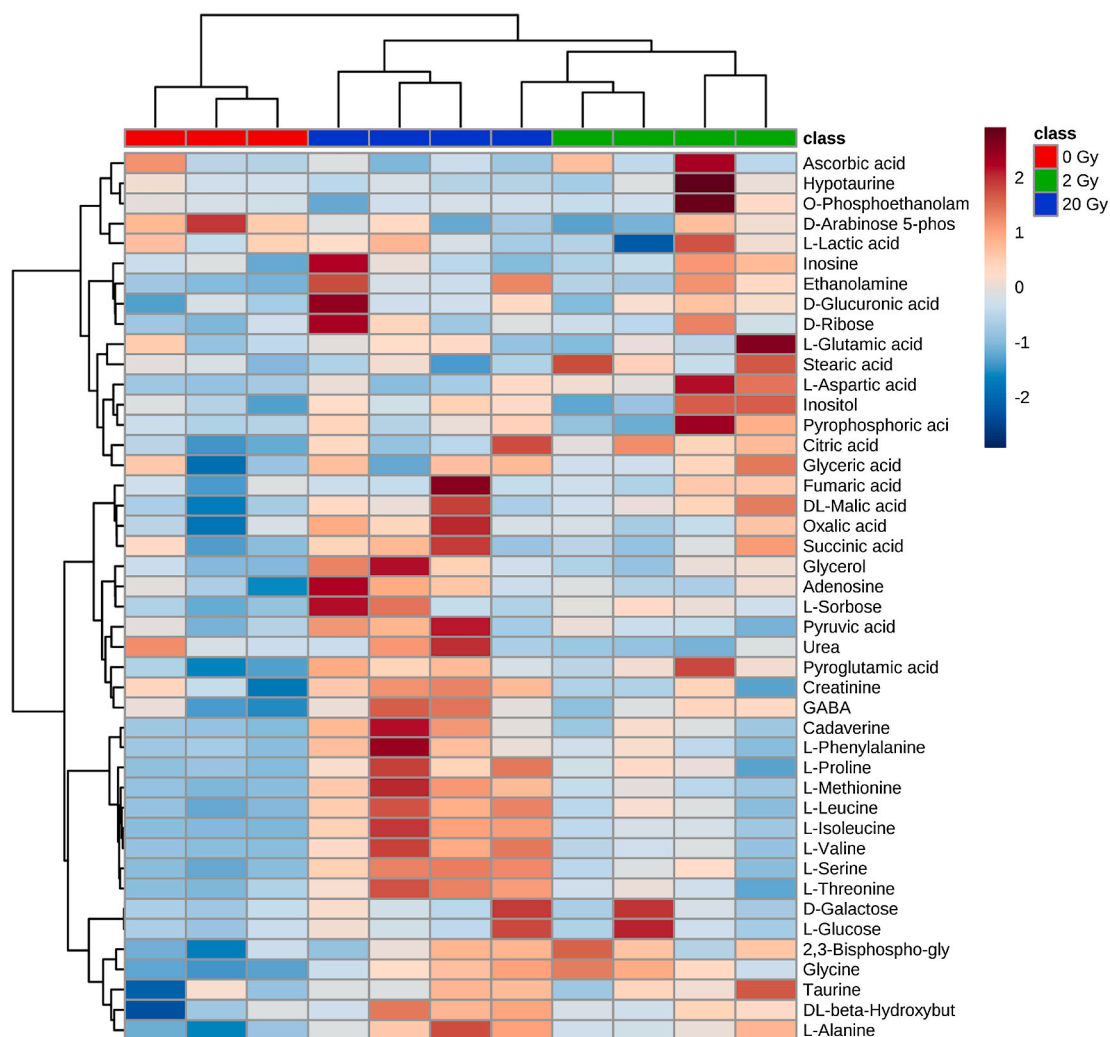
Dihydroethidium (DHE) was measured in the intestinal tissue as previously described [21]. In brief, DHE was dissolved in dimethyl sulfoxide and diluted with PBS immediately before use. One hour prior to irradiation, 200  $\mu$ L DHE (30 mg/kg) was injected intraperitoneally. Intestinal tissue was collected 24 h after irradiation and rapidly frozen at  $-80$  °C. Frozen sections were prepared and ROS was assessed by BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

### 2.6. Immunohistochemistry, and immunofluorescence

The intestinal tissue was removed and cut into sections in 5 mm thickness, and immediately fixed in 4% paraformaldehyde in PBS. 5  $\mu$ m sections were cut and stained with hematoxylin and eosin (HE) for histological examination. For immunohistochemistry, sections were stained using the peroxidase-labeled, peroxidase, anti-peroxidase (PAP) antibody method (Dako REAL peroxidase blocking solution S2023, Glostrup, Denmark) with an anti-PCNA antibody (1:100, Santa Cruz Biotechnology, INC, sc-56), anti-HSP70 (1:100, Cell Signaling, #4872), and anti-HSP90 (1:100, Santa Cruz Biotechnology, INC, sc-7947). Mayer's hematoxylin stain was used for nuclei staining (Muto Pure Chemicals Co., Tokyo, Japan). For immunofluorescence, Anti-caspase-3 (1:100, Cell Signaling, #9664) was purchased. Stained slides were assessed using BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

### 2.7. Data processing and statistical analysis

MetaboAnalyst 4.0 (<http://www.metaboanalyst.ca>) was used for metabolite analysis [27–29]. Principal component analysis (PCA) and Hierarchical clustering analysis were applied to efficiently demonstrate



**Fig. 2.** Hierarchical clustering analysis of 44 metabolite levels in the intestinal tissue of irradiated and non-irradiated groups generated with the data utilizing logarithmic transformation and a column-wise normalization. The colors from blue to red indicate the relative contents of metabolites. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

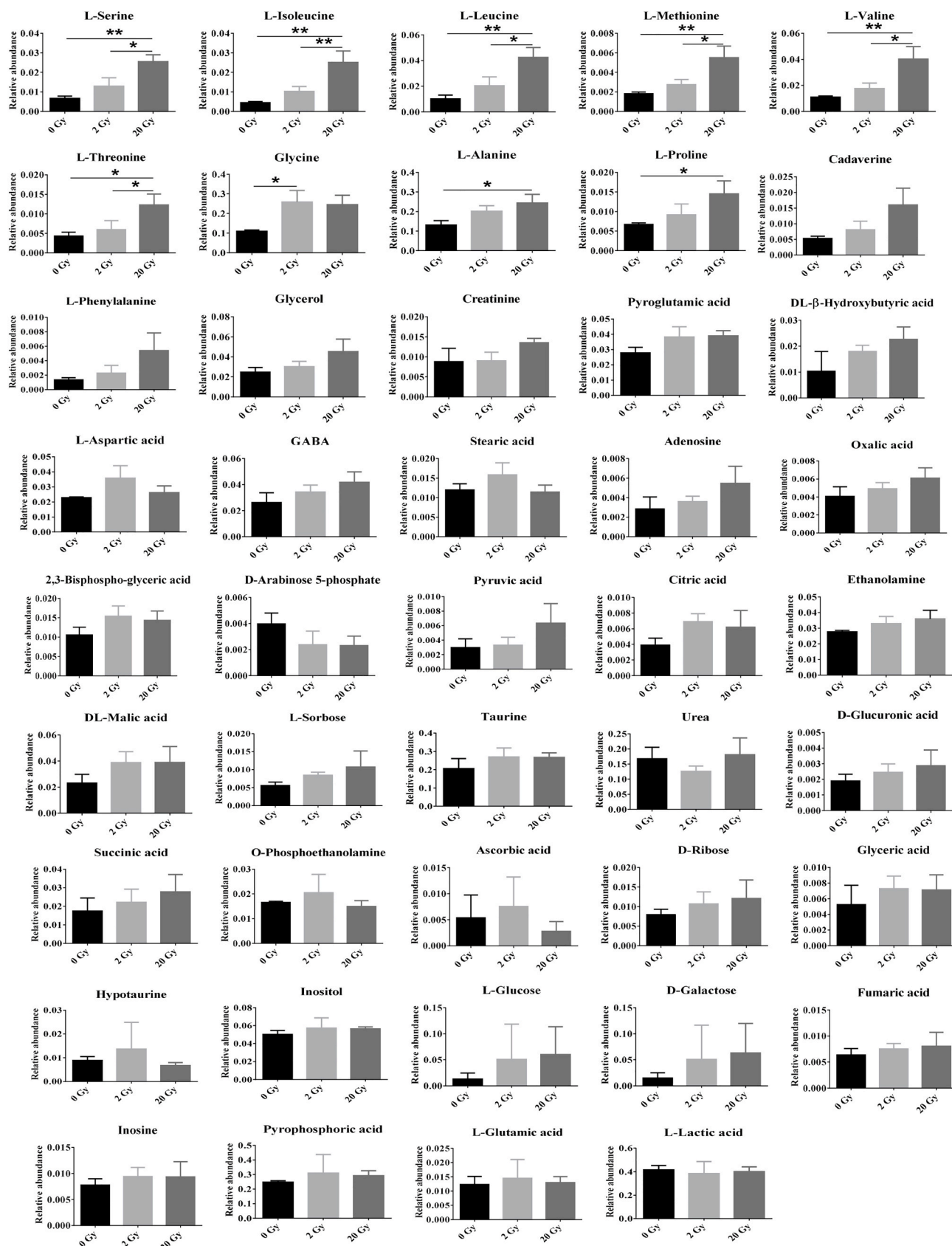
the variance between irradiated and non-irradiated groups. Data were analyzed statistically using multiple comparison one-way ANOVA with Tukey-Kramer as a post-hoc.  $p$ -values less than 0.05 were considered statistically significant.

### 3. Results

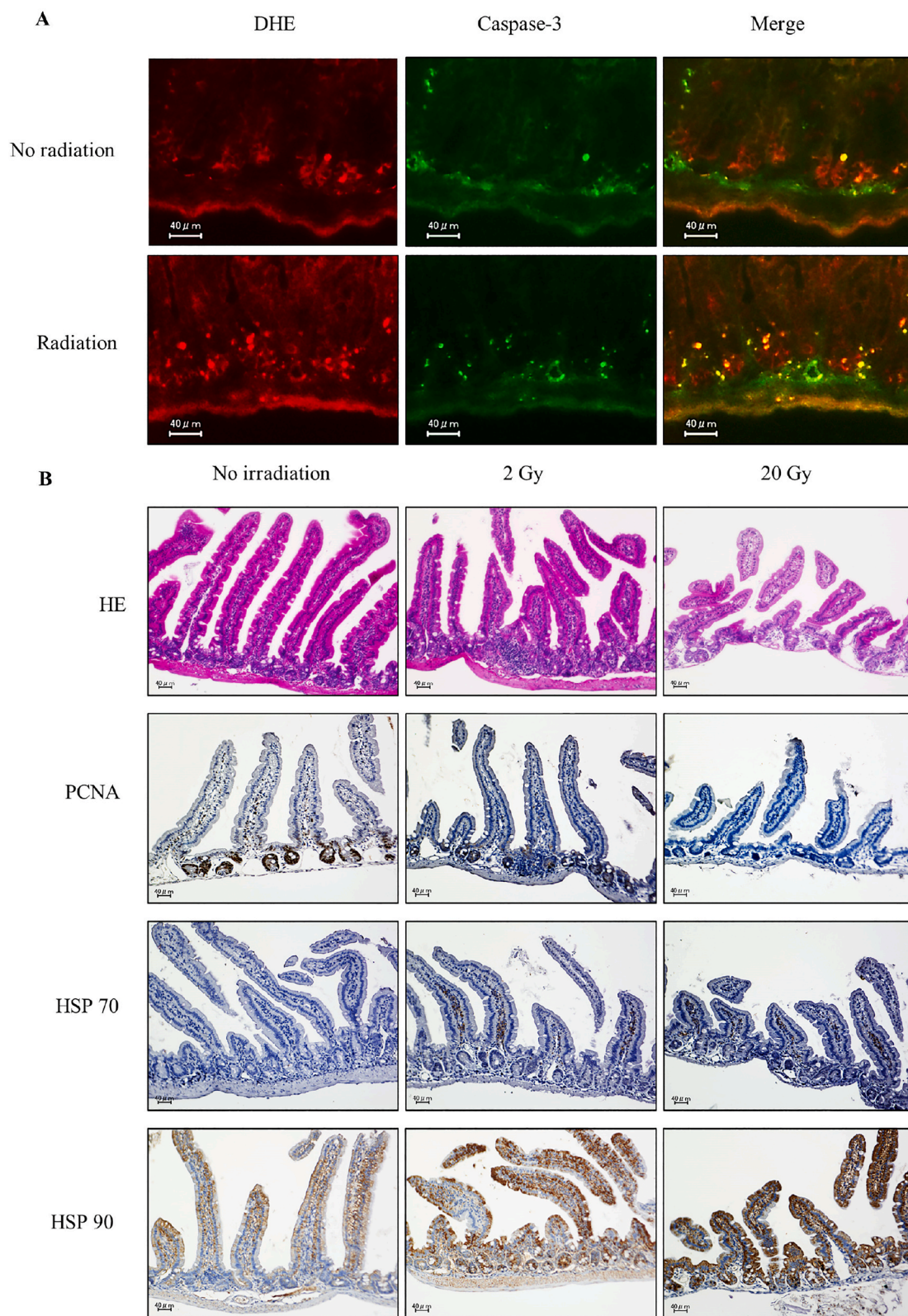
To examine the metabolic profile associated with radiation exposure, we used a comprehensive metabolome analysis with GC-MS to analyze the relative abundance of metabolites in the intestines. We detected 44 metabolites overall in both control and radiation groups. These metabolites were classified into carbohydrate, lipid, and protein intermediates (Table 1 and Table S1). PCA segregated the data into three groups according to the principal component 1 (PC1) value (35.0% of variance; Fig. 1), suggesting that PC1 captured radiation dose-dependent variance in the data. Hierarchical clustering for each treatment group demonstrated distinct clustering of the three groups (Fig. 2). The key differentiating metabolites were amino acids. We identified nine amino acids that significantly differed between the control and radiation groups, with a  $p$ -value < 0.05 (Fig. 3 and Table S1). The levels of serine, isoleucine, leucine, methionine, valine, threonine, alanine, and proline were significantly increased in a dose-dependent manner. The level of glycine was significantly elevated in response to the low dose of radiation. These results suggest that radiation exposure of the intestines

affects amino acid metabolism.

We further investigated the effect of low (2 Gy) and high (20 Gy) radiation dose on ROS-induced intestinal injury. We analyzed DHE, a ROS marker, and caspase-3, an apoptotic marker, 24 h post-radiation exposure. ROS-generating cells as well as apoptotic cells were widely detected after the exposure to the high radiation dose that localized specifically throughout the crypts and lamina propria (Fig. 4A). Histopathological analysis showed markedly shortening in the intestinal villi length after the exposure to radiation, as a dose-dependent response. High radiation dose (20 Gy) showed severe destruction in the intestinal villi and crypts (Fig. 4B). In addition, the intestinal proliferating cells, detected by proliferating cell nuclear antigen (PCNA), were markedly decreased in the crypts after the exposure to low radiation dose, whereas no proliferating cells were detected after the exposure to the toxic dose (20 Gy, Fig. 4B). Finally, we checked HSP 70 and HSP 90, stress markers, in the intestinal tissue as a response to the ROS-induced radiation. Both HSP 70 and HSP 90 expression was visibly increased after radiation exposure, as a dose dependent response. HSP 90 was partially expressed in the non-irradiated intestinal villi and its expression was extensively elevated and distributed in both the crypt and villi as a response to the different radiation doses (Fig. 4B), indicating the role of HSPs in protecting the intestinal tissue from the radiation damage.



**Fig. 3.** Barplot visualization of 44 metabolites relative abundance in the intestinal tissue after radiation exposure. Nine amino acids showed a significant difference between the control and radiation groups. X-axis shows radiation dose. Y-axis shows relative abundance of metabolites. Statistical significance was determined by one way ANOVA, \* $p < 0.05$  and \*\* $p < 0.01$ . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 4.** Representative image shows the intestinal tissue response to low (2 Gy) and high (20 Gy) radiation dose. This response was observed at 24 h after radiation exposure by detection of DHE and caspase-3 (A), intestinal villi length, PCNA, HSP 70, and HSP 90 (B). Scale bar: 40  $\mu$ m.

**4. Discussion**

The present study identified a gradual increase in multiple amino acids in the intestinal tissue including, serine, isoleucine, leucine, methionine, valine, threonine, alanine, and proline, which may be

associated with oxidative stress, as a dose-dependent response to radiation. In contrast, the upregulation of glycine level was associated with a low dose of radiation. Interestingly, glycine and L-alanine were upregulated most in the intestinal tissue, concerning other previously mentioned amino acids. Glycine represents 11.5% of the total amino

acid content in the human body and 20% of the total amino acid nitrogen in body proteins is derived from glycine. Additionally, glycine plays a key role in the protection of the intestine from radiation injury and mesenteric ischemia, immune response, and survival of living beings [30]. Similar to glycine, alanine constitutes a high percentage of the amino acids in the total proteins and other branched amino acids such as valine, leucine, and isoleucine can yield alanine during amino acid catabolism. Alanine is a glucogenic  $\alpha$ -amino acid can be converted to pyruvate, an intermediate in sugar metabolism, by a transamination reaction and it is a nitrogenous end product of the glutamine, glutamate, and aspartate that is a basic constituent of crucial proteins biosynthesis and immune response that have a significant advantage in the intestinal inflammation [31,32]. This explains why glycine and alanine were highly upregulated compare to other identified amino acids.

It remains unclear why radiation increases the levels of amino acids in the intestine and how elevated amino acids exert protective effects against radiation. We suggested that radiation causes ROS-dependent damage to the intestinal tissue that promotes autophagy process, which results in an increase in amino acids. Increasing in amino acids may be a response to protein degradation via autophagic-lysosomal proteases. These amino acids can form gluconeogenic and ketogenic precursors, which are used as energy sources for the synthesis of survival proteins, consistent with previous findings [33–36]. Autophagy maintains cytoplasmic homeostasis by eliminating intracellular misfolding or aggregation of proteins and damaged organelles [37]. A previous study showed that autophagy is induced from 1 day after irradiation at dose 10 and 20 Gy [38]. Furthermore, the activation of autophagy suppressed inflammation and the production of ROS by irradiation through the PI3K/AKT signaling pathway in normal intestinal epithelial cells [39]. Thus, it is possible that radiation activates autophagy, which helps eliminating oxidative stress damage.

Previous work showed that amino acids, including alanine and branched-chain amino acids (isoleucine, leucine, and valine) suppressed the oxidative stress-induced inflammatory response in intestinal epithelial cells [40]. The branched-chain amino acids in particular have potential as inducers of antioxidant enzymes, leading to active glutathione-S-transferase and catalase [41]. Another study indicated that an upregulated concentration of serine mitigated oxidative stress by mediating glutathione and methionine biosynthesis. Serine in particular combines with homocysteine to create cysteine, furnishing one carbon for homocysteine demethylation [42]. Glycine exerts an antioxidative effect to protect intestinal cells from oxidative stress by preserving intracellular glutathione levels and promoting GLYT1 expression, as well as reducing the activation of ERK, JNK, and p38 in the MAPK signaling pathway [43,44]. However, the molecular mechanisms and signaling pathways remain unclear; thus, further investigations are needed.

## 5. Conclusions

From this metabolomic profiles we concluded that exposure of intestinal tissue to 0, 2, and 20 Gy irradiation doses shows a gradual increase in the level of amino acids in a dose-dependent response to radiation. Ionizing radiation-induced oxidative damage may activate the autophagy pathway, resulting in the degradation of cellular proteins and production of excessive amino acids. These amino acids may suppress the oxidative stress-induced inflammatory response in intestinal epithelial cells suggested that amino acids level may be a potential marker of oxidative stress-related gastrointestinal toxicity due to radiation exposure.

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## Authors' contributions

All authors participated in the conception and design the study. Mohammed Salah, Saki Osuga, Makiko Nakahana, and Yasuyuki Shimizu are involved *in-vivo* study. Mohammed Salah, Saki Osuga, Yasuhiro Irino, Masakazu Shinohara, Ai Nakaoka, Kenji Yoshida, Yoshiaki Okamoto, and Ryohei Sasaki analyzed the data, Mohammed Salah, Naritoshi Mukumoto, Hiroaki Akasaka, Daisuke Miyawaki, and Takeaki Ishihara shared in the interpretation of this study. All authors read, revised, and approved the final manuscript.

## Declaration of competing interest

The authors declare that they have no competing interests.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2020.100789>.

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