Gut commensal derived-valeric acid protects against radiation injuries
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ABSTRACT
Background: Hematopoietic and intestinal systems side effects are frequently found in patients who suffered from accidental or medical radiation exposure. In this case, we investigated the effects of gut microbiota produced-valeric acid (VA) on radiation-induced injuries.
Methods: Mice were exposed to total body irradiation (TBI) or total abdominal irradiation (TAI) to mimic accidental or clinical scenarios. High-performance liquid chromatography (HPLC) was performed to assess short-chain fatty acids (SCFAs) in fecal pellets. Oral gavage with VA was used to mitigate radiation-induced toxicity. Gross examination was performed to assess tissue injuries of thymus, spleen and small intestine. High-throughput sequencing was used to characterize the gut microbiota profile. Isobaric tags for relative and absolute quantitation (iTRAQ) were performed to analyze the difference of protein profile. Hydrodynamic-based gene delivery assay was performed to silence KRT1 in vivo.
Results: VA exerted the most significant radioprotection among the SCFAs. In detail, VA replenishment elevated the survival rate of irradiated mice, protected hematogenic organs, improved gastrointestinal (GI) tract function and intestinal epithelial integrity in irradiated mice. High-throughput sequencing and iTRAQ showed that oral gavage of VA restored the enteric bacteria taxonomic proportions, reprogrammed the small intestinal protein profile of mice following TAI exposure. Importantly, keratin 1 (KRT1) played a pivotal role in the radioprotection of VA.
Conclusions: Our findings provide new insights into gut microbiota-produced VA and underpin that VA might be employed as a therapeutic option to mitigate radiation injury in pre-clinical settings.

Introduction
Cancer is an urgent front-burner public health problem and is the second leading cause of death worldwide. Radiation therapy is used in at least 70% of cancer patients and contributed to 25% of cancer survivors. Under the rapid development of image guidance and radiation delivery techniques, radiotherapy has been developed from basically two-dimensional to three- or even four-dimensional computed tomography (CT) imaging, while the radiation toxicity remains the overwhelmingly most important barrier to patients with malignancies. During radiation therapy of cancer patients, the hematopoietic and gastrointestinal system are under the risk of short-term or long-term complications, which seriously affect the quality of life and even lead to substantial mortality. Since there are few therapeutic options available, it is sorely in need of novel and effective therapeutic approaches to mitigate radiation-induced hematopoietic and intestinal injuries.

Mammalian gastrointestinal (GI) tract harbors a huge amount of commensal microbiota, comprising more than 1000 diverse strains. Mounting evidence implicated the intestinal microbiota not only participates in food degradation and energy intake but also serves as a critical contributor to the regulation of host health and immune response. This may be partially achieved by releasing short-chain fatty acids (SCFAs), which are the most abundant bacterial metabolites derived from the fermentation of indigestible fiber-rich diets by specific colonic bacteria, including formic acid (FA), acetic acid (AA), propionic acid (PA), butyric acid (BA), isobutyric acid (IBA), valeric acid (VA) and isovaleric acid.

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The deficiency of SCFAs may affect the development of metabolic syndrome, appetite control, and neurological diseases. For instance, IVA was witnessed to be associated with the development of depression. In addition, VA was identified as a potential therapeutic target for a variety of disease pathologies, including cancer and colitis. To date, whether VA alleviates radiation-induced injury (typically characterized by inflammation) remains unknown.

Keratins (KRTs) have yet been discovered consist of more than 20 members. A major role fulfilled by KRTs is to protect epithelial cells from mechanical and non-mechanical stresses. Additional functions of KRTs manifest including the regulation of cell signaling and stress responses. Dysfunction of KRT may cause various diseases. For example, epidermolysis bullosa simplex (EBS) is related to the mutation of KRT5 and KRT14. KRT8/KRT18 variants could be a risk factor for liver fibrosis progression. In addition, diffuse expression of KRT7 and KRT19 in the end-stage kidney could increase the risk of tumor development. Importantly, clinical and laboratory researches have demonstrated the significant role for KRTs in the protection of the epithelial integrity in the small intestine, and the down-regulation of KRT1 is correlated with the progression of inflammatory bowel disease (IBD).

In this study, we aimed to investigate the effects of VA on radiation injuries. Our observations demonstrated that VA administration exhibited beneficial effects against radiation-induced hematopoietic and intestinal injuries via the reduction of inflammation and preservation of the intestinal bacterial structure in irradiated animals. Importantly, KRT1 plays a pivotal role in protection against radiation injury in vivo and in vitro. Taken together, our findings identify that VA may be employed as a therapeutic option to mitigate the complications associated with radiotherapy in pre-clinical settings.

Materials and methods

Animals

Six- to 8-week-old male and female C57BL/6J mice were purchased from HFK Bioscience (Beijing, China), and housed in a specific-pathogen-free (SPF) animal facility at the Institute of Radiation Medicine (IRM), the Chinese Academy of Medical Sciences (CAMS). Mice were fed under standard conditions with ambient temperature at 22 ± 2°C, relative air humidity 40–70%, a 12 h/12 h light/dark cycle and continuous access to a rodent chow and sterile water. The mice in this study were co-housed in a big cage before irradiation. After irradiation, six mice were separated into a cage randomly. The mice in the same cohort were blended and re-separated every 2 d throughout the whole experiments. All mice were treated according to the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Irradiation study

A Gammacell® 40 Exactor (Atomic Energy of Canada Lim, Chalk Rive, Canada) was used for all experiments. Mice were treated with a single dose of 12Gy or 15Gy γ-ray total abdominal irradiation (TAI) using a specific steel chamber, or a single dose of 4Gy or 7Gy total body irradiation (TBI) at a rate of 1.0Gy/min, radiation dose was monitored by a dose rate meter. After irradiation, the mice were sent back to the animal facility for daily observation and treatment. Mice were sacrificed and tissue samples were collected after the 21-d course of the experiment. As to in vitro irradiation, HIEC-6 cell line at 70% to 80% confluence was treated with a single dose of 4Gy γ-ray. Sham irradiation involved placing cell culture plates at a similar temperature for the length of irradiation.

Valeric acid (VA) administration

Sodium valeric acid (Tokyo Chemical Industry, Japan) was treated to mice through oral route every day after irradiation until euthanasia or added to cells. Sterile water was used as a control for the experiments in vitro and in vivo.
**Measure of spleens and thymuses**

Following euthanasia at 15 d of 4Gy TBI, mouse spleens and thymuses were excised and measured.

**Short-chain fatty acids (SCFAs) measurements**

Quantification of SCFAs was performed by high-performance liquid chromatography (HPLC) in supernatants of fecal samples reconstituted in PBS. Briefly, 1 ml of fecal solution was acidified with 1/10 volume of H$_2$SO$_4$ (0.01M) and passed through a condenser to isolate volatile compounds within a sample. Following filtration through 0.45 μm membrane, an equal volume of samples was loaded onto the HPLC (Thermo Fisher U3000 liquid chromatograph). Chromatographic column: C18 AQ (4.6*250 mm), sulfuric acid (0.01 M) was used as the mobile phase, and the levels of SCFAs were determined by external standard calibration method.

**Periodic acid–schiff (PAS) staining**

Mice were euthanasia after 21 d of TAI, the small intestines of mice were harvested and fixed in Carnoy for 24 h at room temperature. After dehydration, tissue samples were embedded in paraffin; then, sectioned at 5 μm thickness and dipped in periodic acid and hematoxylin according to the standard protocols.

**Fluorescein isothiocyanate (FITC)–dextran permeability experiments**

After 21 d of TAI, mice were fasted for 14 h and administrated with FITC-dextran (Sigma-Aldrich, Spain) (60 mg per 100 g body weight) in a volume of 0.2 ml. Blood was collected by cardiac puncture at 4 h after FITC–dextran administration and centrifuged at 1000 rpm for 15 min at room temperature to obtain the serum. Fluorescence intensity of each serum sample (DTX 880 Multimode Detector, USA) was measured.

**Immunohistochemistry staining (IHC)**

Following euthanasia, pieces of intestine were fixed in 4% buffered formalin overnight at room temperature and then embedded in paraffin. Tissues were sectioned at 5 μm thickness, and blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C and incubated with FITC-conjugated goat anti-rabbit IgG antibody for 1 h at room temperature, then stained by Diaminobenzidine (DAB) Staining Kit (ZSGB Bio, China), followed by hematoxylin nuclear counterstaining. The primary antibody of rabbit anti-KRT1 (Abcam, Cambridge, USA) was used.

**Enzyme-linked immunosorbent assay (ELISA)**

Frozen intestine samples were ground up, respectively, and reconstituted in PBS to a final concentration of 0.1 g/ml, followed by centrifuging for 10 min at 14,000 g and 4°C. Plasma was collected using ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Centrifuge for 15 min at 1000 g and 4°C. Protein level was measured from the clear supernatant using ELISA kit (Solarbio, China) according to the manufacturer’s protocol. Optical density was read at 450 nm (Rayto, China).

**Measurement of malondialdehyde (MDA)**

The level of MDA in the small intestine was determined using Micro MDA Assay Kit (Solarbio, China) according to the manufacturer’s instructions. Levels of MDA were measured and calculated by the following formula, according to the manufacturer’s instructions:

The levels of MDA (nM) = 25.8 × (A532 − A600)

**Quantification of fecal lipocalin (LCN2)**

Frozen fecal samples were re-suspended in PBS containing 0.1% Tween 20 to a final concentration of 0.2 g/ml, then vortexed to produce a homogenous fecal suspension, followed by centrifuging for 10 min at 14,000 g and 4°C. LCN2 level was measured from the clear supernatant using Mouse LCN2 ELISA kit (Solarbio, China) according to the manufacturer’s protocol. Optical density was read at 450 nm (Rayto, China).

**Bacterial diversity analysis**

Stool samples were freshly collected from five mice in different cages and stored at −80°C until use.
Fresh feces were collected before and after 6 d of radiation, as well as 12 d after radiation, respectively, for downstream analysis. DNA was extracted from the stool samples using the Power fecal® DNA Isolation Kit (MoBio Carlsbad, USA) before radiation or after 6 or 12 d of radiation. The 16S ribosomal RNA (rRNA) V4 gene amplification and sequencing were done using the Illumina MiSeq technology. Sequence analyses were performed by UParse software (UParse v7.0.101, http://drive5.com/uparse/). Sequences with ≥97% similarity were assigned to the same OTUs. The representative sequence for each OTU was screened for further annotation. For each representative sequence, the Silva123 Database was used based on the RDP classifier (Version 2.2, http://sourceforge.net/projects/rdp-classifier/) algorithm to annotate taxonomic information. The primers are listed in Supplementary Table 1.

**Polymerase chain reaction (PCR)**

Total RNA was separated from the tissues using Trizol (Invitrogen, USA) according to the manufacturer’s protocol. Reverse transcription was performed using poly (A) – tailed total RNA and reverse transcription primer with ImPro-II Reverse Transcriptase (Promega, USA) according to the manufacturer’s protocols. PCR was performed using DreamTaq™ Hot Start Green PCR Master Mix (Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. The quantitative real-time polymerase chain reaction (qRT-PCR) was performed according to the instructions of Fast Start Universal SYBR Green Master (Rox) (Roche Diagnostics GmbH Mannheim, Germany). The primers used in this study were listed in Supplementary Table 2. Gapdh was used as the housekeeping gene.

**Peptide fractionation and identification by mass spectrum (MS)/MS**

The small intestinal protein was extracted according to the manufacturer’s protocol. The tryptic peptides were labeled by the 8-plex isobaric tags for relative and absolute quantitation (iTRAQ) reagents (AB Sciex, CA). Then, the labeled peptides were pooled together for further identification. The mixed peptides were used to analyze at Novogene (Beijing, China).

**Plasmid constructs**

Sh-KRT1 sequences were forecasted by BLOCKiT™ RNAi Designer, and then inserted into the pRNA-U6.1/Neo vector at BamHI and Hind III sites to obtain the plasmid. The plasmid was synthetized by GENEWIZ® (Suzhou, China). The sequencing of sh-KRT1 is shown in Supplementary table 3.

**Plasmid DNA injection techniques**

Male mice were anesthetized to hold it immobile. Then, 10% of the body weight of sh-KRT1 plasmid DNA was diluted with sterile saline and was injected via the retro-orbital sinus into mice approximately within 8 s. Plasmid DNA injection was performed before 2 d of radiation.

**Cell culture**

The human enterocyte HIEC-6 cell line was cultured in RPMI-1640 medium (Gibco, CA) supplemented with 10% fetal bromide serum (Gibco, CA) at 37°C in a 100% humidified atmosphere of 5% CO₂.

**Cell transfection**

For cell transfection, the cells were cultured in a 6-well plate for 24 h and then were transfected with siRNA. All transfections were performed using polyethylenimine (PEI) according to the manufacturer’s protocol. si-KRT1 and si-AML1 were synthesized by RiboBio (Guangzhou, China). The sequences of siRNA are listed in Supplementary Table 3.

**Cell counting kit-8 (CCK-8) assay**

Cell proliferation was measured by CCK-8 assay according to the manufacturer’s guidelines. Ten µl of CCK-8 reagents was added after 0, 24, 48 or 72 h of irradiation to assess cell proliferation.
Colony formation assays

For colony formation analysis, 48 h after irradiation, 500 viable irradiated (transfected) cells were placed in 6-well plates and maintained in complete medium for 2 weeks. Then, colonies were fixed with methanol and stained with methylene blue.

Western blotting analysis

Total protein lysates were obtained from the tissues using RIPA (Solarbio, China) buffer at 4°C for 30 min and centrifuged at 12,000 g and 4°C for 15 min. Tissue lysates were separated by 12% SDS-PAGE, and then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk and incubated with primary antibodies. The rabbit anti-KRT1 monoclonal antibody was obtained from Abcam (Cambridge, USA). Fluorescein conjugated affinity-pure goat anti-rabbit IgG (H + L) was obtained from Proteintech (Proteintech Group, USA).

Induction of dextran sodium sulfate (DSS)-induced colitis

To induce acute colitis in mice, 1.5% (w/v) DSS (36000–50000 Da, Yeasen biotech Co., Ltd, China) was dissolved in sterile water and provided to male C57BL/6J mice for 15 d. For VA supplementation, 0.3 mg/ml VA was orally administrated to mice daily for 15 d throughout DSS treatment. As a comparison, control mice were drinking sterile water and gavaged with water and bred in the same facility.

Statistical analysis

Each experiment was repeated at least three times. Data were assessed normal distribution using the Kolmogorov–Smirnov test. Significance was assessed by comparing the mean values (6 standard deviation; SD) using Student’s t-test between each two cohorts as follows: * P < .05, ** P < .01, *** P < .001. Statistical difference of 16s rRNA high-throughput sequencing was assessed by Tukey’s HSD. Two-way ANOVA was performed for body weight analysis. Results with P < .05 were considered statistically significant. The statistical tests were clarified in related legends.

Results

Gut microbiota-derived VA ameliorates radiation caused hematopoietic damage

Our previous study has proved that fecal microbiota transplantation (FMT) plays a protective role in combating radiation-induced toxicity.22 We next detected the abundance of SCFAs, the major metabolites from the fermentation of fiber by intestinal bacteria, in the feces of mice. FMT elevated the levels of SCFAs in feces decreased by total body irradiation (TBI) (Figure 1A and Fig. S1A-C), but colony formation assays negated the radioprotective functions of acetic acid (AA), propionic acid (PA) to human enterocyte HIEC-6 cells (Fig. S1D-F). Butyric acid (BA) showed radioprotection to the cells slightly. However, investigations on BA experience a renaissance recently, as another key member of SCFAs, the physiologic and pathologic roles of valeric acid (VA) remains poorly understood. Thus, we focused on VA to unravel whether it exerts therapeutic effects in FMT-improved radiation injury. Using a mouse model of TBI, we observed that high doses of VA had improved the survival rate of irradiated mice (Figure 1B). Next, we measured the levels of VA in feces to test the difference of high and low doses of VA on TBI-induced injury. Results showed that a low level of VA treatment failed to remedy the decrease in the excrementitious VA, and a high level of VA treatment elevated that of VA to a higher level but still lower than the natural level (Fig. S1G). We also observed...
a significant decrease in malondialdehyde (MDA) in irradiated mice with VA treatment (Figure 1G). These results implied that enteric microbioproduced VA prevents radiation-caused hematopoietic injury.

**VA restores GI tract function and epithelial integrity after irradiation**

Given the GI system is highly sensitive to ionizing radiation, we next determined the effects of VA on radiation enteropathy. We performed 12Gy total abdominal irradiation (TAI) and administered mice with water (“Con” and “TAI” groups) or VA (“TAI+VA” group). Results showed that compared to a radiation-challenged group, VA replenishment facilitated body weight recovery (Figure 2A). Importantly, VA prevented radiation-caused intestinal inflammation and dysfunction, as indicated by lengthening colon (Figure 2B and Fig. S2A) and increasing goblet cells (Figure 2C and Fig. S2B). In parallel, VA treatment significantly heightened the relative expression of the genes involved in the maintenance of intestinal integrity (Figure 2D and Fig. S2C,D). In order to measure the permeability of intestines, mice were treated with FITC-dextran in
Figure 2. VA ameliorates GI tract injury and inflammation after TAI.
(A) The body weight of mice in the three groups was measured after 12 Gy TAI. Significant differences are shown relative to the “TAI” group using two-way group ANOVA (*P < .05). (B) Colon tissues of mice in the three groups were shown at 21 d after 12 Gy TAI. (C) The morphology of the small intestine of mice in the three groups was shown by AB-PAS staining. Goblet cells are indicated by arrows. (D) The expression level of Glut1 in small intestine tissues of mice in the three groups was examined by qRT-PCR. Significant differences are shown relative to the “TAI” group: ***P < .001; Student’s t-test between each two cohorts, n = 12 per group. (E) The FITC–dextran in PB of mice in the three different groups was assessed at 21 d after irradiation exposure. Significant differences are shown relative to “TAI” group: **P < .01, ***P < .001; Student’s t-test between each two cohorts, n = 12 per group. (F-H) The concentrations of TNF-α (F), IL-6 (G) and MDA (H) in small intestines were examined by ELISA. Significant differences are shown relative to the “TAI” group: *P < .05, **P < .01, ***P < .001; Student’s t-test between each two cohorts, n = 12 per group. (I) Fecal level of the inflammatory marker LCN2 was examined by ELISA. Statistically significant differences are shown relative to the “TAI” group: ***P < .001; Student’s t-test between each two cohorts, n = 12 per group.
oral route. As expected, compared to the TAI group, VA treatment significantly reduced the levels of FITC-dextran in PB, indicating that VA might ameliorate irradiation-mediated destruction of the intestinal structure and function (Figure 2E). ELISA (Figure 2F,G) and qRT-PCR (Fig. S2E-G) analysis further validated that oral gavage of VA significantly reduced intestinal inflammation caused by TAI. Consistent with the aforementioned results, irradiated mice carried lower concentrations of MDA in plasma and LCN2 in feces following VA replenishment (Figure 2H,I), indicating that oral gavage of VA might ameliorate GI tract toxicity caused by radiation exposure.

**Oral gavage of VA retains gut bacterial composition pattern after TAI**

Gut microbiota has been reported to modulate the host’s radiosensitivity, while VA is a major metabolite produced by the gut bacteria during polysaccharides fermentation. Thus, we performed 16S rRNA high-throughput sequencing to elucidate the effects of VA on bacterial taxonomic proportion following the irradiation challenge. Chao1, ACE and weighted unifrac analysis exhibited no significant difference of α- and β-diversity among the mice before irradiation (Figure 3A-C), indicating the experimental mice share a similar baseline of gut bacteria initially. The α-diversity of intestinal bacteria was decreased slightly on day 6 after irradiation, while VA replenishment reduced the alterations (Figure 3D,E). Weighted unifrac analysis revealed that whether VA treatment or not, β-diversity changed after TAI exposure (Figure 3F). Principal coordinates analysis (PCoA) reflected that TAI elicited separations of gut microbiota composition from mice; however, VA treatment narrowed the shifts as indicated by the cluster is closer to the control cohort than TAI-alone group (Figure 3G,H). We also applied Linear discriminant analysis (LDA) and LDA effect size (LEfSe) analysis to identify the differentially abundant operational taxonomic units (OTUs) in mice treated with VA or vehicle before and after exposed to irradiation, as well as the significantly different taxa in mice after 6 d of irradiation combined with VA treatment (Figure 3I,J). To elaborate, the species sorted by LDA score were Akkermansia, Parabacteroides, Enterobacteriaceae, Blautia, and Erysipelatoclostridium after TAI, while Parasutterella and Ruminococcaceae showed the highest LDA score in irradiated mice combined with VA treatment (Fig. S3A). At the family level further, significant elevation of the relative frequency of Desulfovibrionaceae and reduction in Lactobacillaceae induced by TAI were not observed in VA-treated group (Figure 3K).

The dynamic change of gut microbiota in mice under the same treatment was also assessed during 12 d after TAI. Chao1 and ACE diversity index displayed un-alteration (Fig. S4A-F). Compared to non-irradiated animals (Fig. S5A), the intestinal bacterial flora profile substantially shifted after TAI exposure at day 12 (Fig. S5B) and was tuned by VA replenishment (Fig. S5C). PCoA further represented the role of VA in educating the gut microbiota profile (Fig. SSD-F). Specifically, mice harbored increased abundances of Anaerotruncus, Coprococcus_1 and Erysipelatoclostridium after TAI (Fig. S5G). However, the oral gavage of VA stabilized the abundance of bacteria from above genera (Fig. S5H). Taken together, our observations demonstrated that VA treatment preserves the intestinal microbiota composition in mice exposed to radiation.

**VA administration reprograms protein profile of small intestine tissues after irradiation**

To further explore the mechanism underlining VA alleviating radiation-caused injury, we examined the protein profile of small intestine tissues from TAI-exposed mice with or without VA administration by iTRAQ. We identified the reliable proteins that met the criteria of $P \leq 0.05$ and fold changes less than 0.83 or greater than 1.20. Of these, we obtained 183 significantly differentially expressed proteins between “control” and “TAI” group and 28 between “TAI” and “TAI+VA” group (Supplementary Table 4). The scatter plot was drawn to demonstrate the variation of protein expression between “control” and “TAI” group (Figure 4A, purple dots) or TAI followed by water or VA replenishment groups (Figure 4A, cerulean dots). The functions of differentially expressed proteins were annotated and predicted by analysis of Gene Ontology (GO) based on three parts: biological process, cellular component and molecular function. GO analysis of biological
process showed that differentially expressed proteins after TAI exposure were significantly related to organonitrogen compound, amide and peptide metabolic and biosynthetic process (Fig. S6A). However, VA supplement further changed the spectrum of protein expression in the small intestine majoring in the

Figure 3. Oral gavage with VA retains the intestinal bacterial composition pattern impaired by TAI. Mice were treated with water ("Con" and "TAI" groups) or VA ("TAI+VA" group), respectively. (A,D) The chao1 diversity index of intestinal bacteria in mice was examined by 16S rRNA high-throughput sequencing before or after 6 d of 12Gy TAI exposure ("TAI" and "TAI+VA" groups) or sham-irradiation ("Con" group). Statistically significant differences are indicated: non-significance (NS); TukeyHSD, n = 9, 8 and 8 for "Con", "TAI" and "TAI+VA" groups, respectively. (B,E) The ACE index of intestinal bacteria in mice was examined by 16S rRNA high-throughput sequencing before or after 6 d of 12Gy TAI exposure or sham-irradiation. Statistically significant differences are indicated: non-significance (NS); TukeyHSD, n = 9, 8 and 8 for "Con", "TAI" and "TAI+VA" groups, respectively. (C,F) The β diversity of intestinal bacteria in mice before or after 6 d of 12Gy TAI exposure or sham-irradiation was examined by 16S rRNA sequencing. The top and bottom boundaries of each box indicate the 75th and 25th quartile values, respectively, and lines within each box represent the 50th quartile (median) values. Ends of whiskers mark the lowest and highest diversity values in each instance. Statistically significant differences are shown relative to the "Con" group: * P < .05; TukeyHSD, n = 9, 8 and 8 for "Con", "TAI" and "TAI+VA" groups, respectively. (G,H) The principal coordinates analysis (PCoA) of intestinal bacteria in mice before (I) or at 6 d (J) after 12Gy TAI was examined by 16S rRNA high-throughput sequencing. n = 9, 8 and 8 for "Con", "TAI" and "TAI+VA" groups, respectively. (I,J) Linear discriminant analysis effect size (LEfSe) of fecal microbiomes of mice before (I) or at 6 d (J) after 12Gy TAI was shown by cladogram. The circle of radiation from the inside to the outside represents the classification level from phylum to species. Each small circle at different classification levels represents a taxonomic rank at that level, and the diameter of the small circle is proportional to the relative abundance. The species with no significant difference were colored yellow, and the differential species biomarker followed the group for coloring. Prefixes represent abbreviations for taxonomic rank of each taxon, with phylum (p), class (c), order (o), family (f) and genus (g). Taxa in cladogram are statistically significant (P < .05). (K) The alteration of intestinal bacterial patterns at the family level in mice at 6 d after 12Gy TAI was examined by 16S rRNA high-throughput sequencing. The heatmap is color-based on row Z-scores. The mice with the highest and lowest bacterial level are in red and blue, respectively.
Figure 4. VA reprograms protein expression perturbed by TAI.

(A) Volcano plots showed differential expression of proteins between “TAI” group and “Con” group (purple spots) or “TAI+VA” group and “TAI” group (blue spots). (B-D) The differentially expressed proteins with the top 20 enrichment score between “TAI” group and “TAI+VA” group were categorized based on Biological Progress (B), Cellular Component (C) and Molecular Function (D). The horizontal axis is the enrichment score for the GO terms, and the vertical axis is the GO terms. Statistically significant differences are indicated using the Fisher Exact Test. (E) Heat map of 20 differentially expressed proteins between “TAI” group and “TAI+VA” group. Each column represents one sample; each row represents one probe set. The red indicated up-regulated proteins and the green indicated down-regulated proteins. (F) The expression level of KRT1 in small intestine tissues of mice in the three groups was examined by qRT-PCR. Significant differences are shown relative to the “TAI” group: * P < .05, *** P < .001; Student’s t-test between each two cohorts, n = 12 per group. (G) The level of KRT1 in the small intestine of mice was measured by immunohistochemistry. (H) A diagram represented the interactions between KRT1 and barrier functional genes based on the string database. (I,J) The expression levels of Cldn5 (I) and Oc1n (J) in small intestine tissues of mice in the three groups were examined by qRT-PCR. Significant differences are shown relative to the “TAI” group: *** P < .001; Student’s t-test between each two cohorts, n = 12 per group.
fertilization process (Figure 4B). For cell component, the differential proteins were associated with intracellular organelle, ribonucleoprotein complex, and ribosome (Fig. S6B). As to the molecular function terms of differentially expressed proteins, these proteins were mainly attributed to structural molecule activity (Fig. S6C), whereas cytoskeletal part were the most representative term between “TAI+VA” and “TAI” group in cellular component, which were annotated with structural molecule activity (Figure 4C,D). Heat maps of 20 significantly differentially expressed proteins in the intestines of irradiated mice compared to non-irradiated animals and TAI-exposed mice with or without VA treatment were exhibited to illustrate the distinguishable protein expression profile of the small intestine samples. We found decreased Keratin, type II cytoskeletal 1 (KRT1) expression in mice exposed to radiation (Fig. S6D). And this down-regulation was eradicated when the irradiated mice received VA treatment (Figure 4E). Quantitative RT-PCR and immunohistochemical (IHC) further validated the results (Figure 4F,G). Given intestine permeability is controlled by several barrier functional proteins,28 we screened key genes of tight-junction integrity interacting with KRT1 and performed a diagram using the string database (https://string-db.org/cgi/input.pl, Figure 4H). Then, we investigated the mRNA levels of those tight-junction markers in small intestines and observed that VA treatment dramatically increased the mRNA expressions of Claudin-5 (Cldn5), Occludin (Ocln), Catenin β1 (Ctnnb1), Desmocollin3 (Dsc3) and ZO-1 in abdominal irradiated mice (Figure 4I,J and Fig. S6E-G). Together, our observations suggested that VA seems to protect against radiation injury involving KRT1.

**VA protects against radiation injury via KRT1**

To further interrogate the role of KRT1 in VA-mediated radioprotection, retro-orbital sinus hydrodynamic injections of pRNA-U6.1/Neo carried shRNA targeting KRT1 were performed to down-regulated the expression of KRT1 in mice (Fig. S7A).29 Intriguingly, the injected mice were unresponsive to VA replenishment after TAI, representing as weight loss and colitis (Figure 5A,B and Fig. S7B), impaired gut integrity and increased intestinal inflammation compared to non-transfected mice (Figure 5C,D and Fig. S7C-F), suggests that KRT1 might play a key role in the protection effects of VA on radiation enteropathy. We also assessed the radioprotection of VA in vitro. As shown in figure S8A-C, lower concentrations (0.1 and 0.2 mM) of VA had no effect on HIEC-6 cell growth, while 0.4 mM of VA exerted cytotoxicity. When HIEC-6 cells were exposed to radiation, VA treatment accelerated radiation-inhibited proliferation of HIEC-6 cells in a dose-dependent fashion (Figure 5E,F and Fig. S8D-F). Western blotting was performed to test the expression of KRT1 in the cells. Consistent with the results in animal research, KRT1 proteins were down-regulated in irradiated cells, while VA treatment elevated the expression of KRT1 in HIEC-6 cells after irradiation (Figure 5G). Next, specific siRNA was used to silence the expression of KRT1 in HIEC-6 cells (Fig. S8G). CCK-8 and colony formation assays showed that KRT1 silencing aggravated inhibition of HIEC-6 cell proliferation following irradiation, and the cells were also unresponsive to VA replenishment (Figure 5H and Fig. S8H,I), suggesting that the radioprotection of VA at least partly depends on KRT1. The protein levels of KRT1 in cells were examined (Figure 5I). AML1 has been proved as a transcription factor to activate KRT1 expression.30 To further explore the mechanism by which VA up-regulates KRT1, we silenced AML1 in HIEC-6 cells using specific siRNA (Fig. S8J). As expected, the deletion of AML1 resulted in the down-regulation of KRT1 even with VA treatment, confirmed that AML1 might be involved in VA-enhanced KRT1 expression (Figure 5J,K). Together, these results suggested that KRT1 might play an important role in VA-mediated radiation toxicity.

**VA alleviates radiation enteritis of female mice**

The investigations using experimental animals with single-sex limit clinical trials transformation. Thus, we repeated the experiments using female C57BL/6J mice. Specifically, the oral gavage of VA improved the survival rate of female mice after 15Gy TAI exposure (Figure 6A). VA replenishment also mitigated GI tract injuries after 12Gy TAI, representing as reduced colon inflammation (as detected by the longer colon) (Figure 6B,C), increased numbers of goblet cells (Figure 6D,E), enhanced intestinal integrity and decreased expression of pro-inflammatory cytokines (Figure 6F-L). Together, our observations
Figure 5. VA protects against radiation injury involving KRT1.

(A-D) As to the “shKRT1+ TAI+VA” group, mice were retro-orbital sinus hydrodynamic injected with pRNA-U6.1/Neo carried shRNA targeting KRT1 to down-regulate the expression of KRT1 before 2 d of irradiation and were gavaged with VA after radiation. (A) The body weight of mice in the three groups was measured after 12 Gy TAI. Significant differences are shown relative to the “TAI+VA” group using two-way group ANOVA (* P < .05). (B) The length of colon tissues of mice in the three groups was measured at 15 d after irradiation exposure. (C) The expression level of Glut1 in small intestine tissues of mice in the three groups was examined by qRT-PCR. Significant differences are shown relative to the “TAI+VA” group: * P < .05; Student’s t-test between each two cohorts, n = 12 per group. (D) The expression level of TNF-α in small intestine tissues of mice in the three groups was examined by qRT-PCR. Significant differences are shown relative to “TAI+VA” group: ** P < .01; *** P < .001; Student’s t-test between each two cohorts, n = 12 per group. (E,F) Colony formation assay was performed to assess the growth of HIEC-6 cells treated with vehicle or different concentrations of VA after IR. Significant differences are shown relative to the “IR+vehicle” group: *** P < .001; Student’s t-test between each two cohorts, n = 7 per group. (G) The expression levels of KRT1 protein in HIEC-6 cells were measured by Western blotting. (H) CCK-8 assay was performed to assess the growth of HIEC-6 cells with or without KRT1 silencing and treated with VA or vehicle after IR or not. (I) The expression levels of KRT1 were measured by Western blotting. (J,K) RT-PCR (J) and Western blotting (K) were performed to assess the expression of KRT1 in HIEC-6 cells transfected with or without AML1 siRNA before IR and treated with VA or vehicle after IR.
Figure 6. VA ameliorates GI tract injury and inflammation of irradiated-female mice.

(A) Kaplan-Meier survival analysis of irradiated mice with or without VA treatment was performed. \( P < .05 \), \( n = 18 \) per group. (B, C) The length of colon tissues of mice in the three groups was measured at 21 d after TAI. Statistically significant differences are shown relative to the “TAI” group: * \( P < .05 \); Student’s t-test between each two cohorts, \( n = 12 \) per group. (D) The morphology of the small intestine of mice in the three groups was shown by AB-PAS staining. Goblet cells are indicated by arrows. (E) The number of goblet cells per crypt was counted in the small intestine of mice in the three groups. Statistically significant differences are shown relative to the “TAI” group: *** \( P < .001 \); Student’s t-test between each two cohorts, \( n = 5 \) per group. (F) The FITC-dextran in PB of mice in three different groups was assessed at 21 d after irradiation exposure. Significant differences are shown relative to “TAI” group: * \( P < .05 \), *** \( P < .001 \); Student’s t-test between each two cohorts, \( n = 12 \) per group. (G-I) The expression levels of Glut1 (G), MDR (H) and Pgk1 (I) in small intestine tissues of mice in the three groups were examined by qRT-PCR. Significant differences are shown relative to “TAI” group: * \( P < .05 \), ** \( P < .01 \); Student’s t-test between each two cohorts, \( n = 12 \) per group. (J-L) The expression levels of TNF-\( \alpha \) (J), IL-6 (K) and Nrf2 (L) in small intestine tissues of mice in the three groups were examined by qRT-PCR. Significant differences are shown relative to the “TAI” group: * \( P < .05 \), ** \( P < .01 \), *** \( P < .001 \); Student’s t-test between each two cohorts, \( n = 12 \) per group.
demonstrated that VA fights against radiation-induced intestinal toxicity of both male and female mice.

VA protects mice from developing dextran sodium sulfate (DSS)-induced colitis

To evaluate whether the enteric protection of VA was specific to radiation-induced intestinal injury, we assessed the therapeutic effects of VA on GI tract injuries using DSS-induced colitis mouse models. Throughout the course of DSS-induced colitis, we observed that VA-treated mice were protected from the DSS-colitis phenotype, as indicated by better weight recovery (Figure 7A), sustained colon length (Figure 7B,C), restored gut barrier function (Figure 7D-F) and decreased intestinal inflammation (Figure 7G-I). These results indicate that VA treatment confers protection against the development of DSS-induced colitis, and that this protective effect is neither specific nor limited to radiation enteritis model.

Discussion

Ionizing radiation (IR) is a complicated and often scary topic. Exposure to IR is in connection with both acute and chronic health problems. Nevertheless, the general public is constantly exposed to various sources of IR, like natural radiation, medical procedures, and nuclear accidents. Given that hematopoietic stem cells (HSCs) are sensitive to IR, radiation mortality caused by accidental or intentional exposure mainly imputes to HSCs damage, particularly as the radiation dose increases. Moreover, radiation is a nonspecific and widely applied therapeutic method for malignant neoplasms. However, with the increasing number of pelvis and abdomen cancer patients seeking treatment for GI syndrome, it is important to be concern about the effects of irradiation on GI. It has been reported that patients with renal cell carcinoma experienced colon ulceration or perforation within the radiation field after radiotherapy. With the developments in precise delivery techniques and radiation treatment planning, the incidence of severe radiation enteropathy diminished as time goes on. However, at least half of the patients receive pelvic or abdominal radiotherapy will undergo chronic intestinal dysfunction. Our previous study has shown that FMT could increase the survival rate and improve the intestinal epithelial integrity of irradiated mice. While, cause the complicated administration of FMT, finding effective and convenient methods to reduce the severity of radiation injury is an unmet need. In light of our previous study, we further interrogated the radioprotective roles of SCFAs, a family of classic gut microbe metabolites, and found both BA and VA performed as potential radiation protective candidates. However, compared to BA, VA showed more significant radioprotection. Thus, we focused on gut microbiota-derived VA and identified that VA might be employed as a therapeutic agent for the treatment of accidental or iatrogenic ionizing radiation-induced hematopoietic and intestinal injuries.

Human intestinal system must constantly maintain a delicate dynamic balance between microbiota and the host immune system, growing bodies of work have shown perturbation of this equilibrium across a wide spectrum of metabolism syndrome, atherosclerosis, and asthma and the like. As a major class of microbial metabolites, SCFAs, produced by gut bacteria from indigestible dietary fiber, are predominantly regarded as an important energy source. Recent advances in SCFAs have highlighted their protective effects on various systems in vivo and in vitro. AA could protect against intestinal inflammation in a mouse model. In addition, colon cancer patients represent a significant reduction in the number of butyric acid-producing bacteria in the gut, while the supplement of BA may ameliorate experimental colitis through G-protein-coupled receptor 109A (GPR109A), proposing SCFAs have a protective role in colitis. More specifically, some studies representing evidence of the interplay between VA and the reduction in the incidence of necrotic enteritis. In addition, as a histone deacetylase (HDAC) inhibitor, VA may serve as a potential therapeutic agent for neurodegeneration and cardiovascular disease. However, whether gut microbiota metabolites may ameliorate radiation toxicity and the underlying mechanism remains unknown. Our results detected that VA represented a new frontier in the treatment of the radiation-induced injury. The concentration of VA in feces from different groups was analyzed. Oral gavage of high level of VA elevated TAI-reduced fecal VA, but still lower than natural level, suggesting the exogenous VA might be absorbed (or utilized) by
Figure 7. VA treatment protects mice from developing DSS-induced colitis.

(A) The body weight of mice in the three groups was measured after 12Gy TAI. Significant differences are shown relative to the “DSS” group using two-way group ANOVA (* P < .05). (B, C) The length of colon tissues of mice in the three groups was measured after 15 d of DSS administration. Statistically significant differences are shown relative to the “DSS” group: ** P < .01; *** P < .001; Student’s t-test between each two cohorts, n = 12 per group. (D) The FITC–dextran in PB of mice in the three different groups was assessed after 15 d of DSS administration. Significant differences are shown relative to the “DSS” group: * P < .05; Student’s t-test between each two cohorts, n = 12 per group. (E, F) The levels of GLUT1 (E) and PGK1 (F) in the small intestine of mice in the three groups were assessed by ELISA after 15 d of DSS administration. Significant differences are shown relative to the “DSS” group: ** P < .01; *** P < .001; Student’s t-test between each two cohorts, n = 12 per group. (G-I) The levels of TNF-α (G), IL-6 (H) and NRF2 (I) in the small intestine of mice in the three groups were assessed by ELISA after 15 d of DSS administration. Significant differences are shown relative to the “DSS” group: ** P < .01; *** P < .001; Student’s t-test between each two cohorts, n = 12 per group.
hosts and gut microbes. In our previous study, we identified that gut microbiota was related to radiation-induced GI toxicity. Here, we observed that oral gavage of VA restructured the intestinal bacterial composition after TAI, which might contribute to the radioprotection of VA. In addition, VA was able to mitigate hazardous compound-caused enteritis as well. Male and female carry different microorganism configurations and gene expression profiles. Investigators are accustomed to relying on exclusively male animals in their researches. However, focus on a single sex threatens to limit the impact of research findings, as results may be relevant to only half of the population. Of note, VA performed radioprotection to both male and female mice, implying that VA emerges as a potential radiation protective agent in pre-clinical settings.

To gain insight into the biology mechanism underlying VA-moderated radiation injury, we determined the differential expression levels of proteins in small intestines by iTRAQ. Among these differentially expressed proteins, we focused on KRT1 to further study its role in the therapeutic effect of VA. KRTs, the most abundant sub-group of intermediate filament (IF) proteins in epithelial cells, assembled with microtubule (MT) and microfilament (MF) protect epithelial cells against micromechanical insults. Besides, such a cytoskeleton endows organism with the ability to regulate a range of pathophysiological processes including neurodegeneration and cancer. KRTs were prevailing served as intracellular scaffolds, there is also considerable evidence reported that KRT1 exerts crucial functions in an inflammatory network as well as the maintenance of skin integrity in keratinocytes. Here, we first detected that KRT1 was down-regulated in the small intestine after radiation exposure, while VA treatment increased the expression level of KRT1 at gene transcription and translation levels, suggesting that KRT1 may play a role in VA-induced amelioration of radiation injury. In support of this, we validated the hypothesis in vivo and in vitro to specifically down-regulated KRT1. As expected, silencing KRT1 blocked the beneficial effects of the VA administration on irradiated animals and cells. When AML1, the transcription factor that activates KRT1 expression, was knockdown, VA failed to up-regulated KRT1 gene and protein. Although we observed an elevation of KRT1 protein in silencing KRT1 cells treated with VA after radiation, which may have no physiological effect on cell growth. SCFAs are able to enter into the cells via transporters or indirectly through the activation of G-protein-coupled receptors (GPCRs). Thus, VA might activate AML1/KRT1 signaling through specific transporters or GPCRs. Taken together, our findings suggested that the protection effect of VA on radiation injury is partly involving KRT1.

In summary, there are intriguing preclinical data concerning that VA treatment is able to combat radiation-induced alteration of gut microbiota and protect against radiation insults. Moreover, we show for the first time that KRT1 plays a key role in attenuate radiation injury in addition to its role in protecting cell against mechanical stress. Clinically, VA could be used to ameliorate radiation-induced injury in patients undergoing accidental exposure or radiotherapy.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing interests. This article does not contain any studies with human subjects performed by any of the authors.

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Author contributions

The authors’ responsibilities were as follows: YL and MC designed and performed experiments, analyzed data, and wrote the paper. JLD, HWX, SQZ, and BW performed experiments. SJF oversaw the entire project.

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