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Intervention Effect and Mechanism of Blueberry Extract on Acute Radiation Intestinal Injury

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Abstract

Exposure to even a single high dose of radiation can lead to radiation-induced intestinal damage, and no clinically effective drugs are available for prevention or treatment of this damage. In this study, we investigated the interventional effects of blueberry extract on radiation-induced intestinal injury. We found that blueberry extract promotes the integrity of intestinal epithelium by increasing the number of Ki67- and Olfm4-positive cells, and by decreasing the number of TUNEL-positive cells. Moreover, pretreatment with blueberry extract also improves radiation-induced intestinal damage by reducing the levels of inflammatory cytokines, enhancing the activity of superoxide dismutase and glutathione levels, and total antioxidant capacity, as well as by promoting resistance to ferroptosis. In conclusion, intervention with blueberry extract can facilitate intestinal repair following radiation exposure and ameliorate radiation-induced intestinal damage.

Keywords: Radiation-induced acute intestinal injury; Blueberry extract; Anti-inflammatory; Antioxidant; Ferroptosis

Abbreviations

ARS: AcuteRadiation Syndrome; **BE:** Blueberry Extracts; **GSH:** Glutathione; **IR:** Ionizing Radiation; **ISC:** Intestinal stem cells; **MDA:** Malondialdehyde; **ROS:** Reactive Oxy-

gen Species; **SOD:** SuperoxideExcretion Enzyme; **T-AOC**: TotalAntioxidants Capacity; **SOD:** SuperoxideDismutase

Introduction

Ionizing radiation releases energy by atoms in the form of particles (α or β) or electromagnetic waves (x or γ)1 separating atoms or molecules. Since the discovery of x-rays by Röntgen in 1895, the global exploit of nuclear technology and the development of nuclear business has continued to grow. Nuclear and its associated technologies are widely used in military, medical, scientific research, energy and other applications. These uses are associated with increased opportunities for human to be exposed to nuclear radiation. Radiation can bring benefits to human, but it also can be associated with the negative effects of acute or chronic radiation exposure. Such exposure has been associated with the atomic bombings of Hiroshima and Nagasaki in Japan during World War II, accidents at nuclear power plants, including Chernobyl in the former Soviet Union in 1986 and Fukushima in Japan in 2011, and even radiotherapy for cancer patients. Therapeutic exposure is of particular importance, as the global cancer burden is expected to reach 28.4 million cases by 2040, a 47 percent increase from 20202, and approximately 70% of cancer patients receive radiotherapy3. Accidental or purposeful exposure radiation, either in high acute doses or in lower chronic doses, can significant injuries to normal tissue, and exposure to a high dose of radiation over a short period of time can lead to acute radiation syndrome (ARS)1. The small intestine, as a tissue that renews itself rapidly, is particularly prone to radiation damage4. Radiation exposure can cause the death of a large number of intestinal epithelial stem cells and thus can disrupt intestinal epithelial integrity. Between 60 and 80% of patients receiving radiation for abdominal and pelvic tumors, including rectal, prostate, bladder, cervical, testicular, and uterine cancers, exhibit radiation-induced intestinal injury5. Because of the prevalence of radiation and the extent of its potential damage, the protection against radiation-associated injury is important to socioeconomic development and the overall health of societies. Amifostine is currently the only drug approved for radiation protection by the FDA6. The metabolites of amifostine can alleviate radiation-induced genome instability. However, due to its rapid metabolism and strong side effects, the clinical applications of this drug have been limited6, 7. The limited number of therapeutic options has significant public health implications as well as implications for national security and social development, and it is important to develop safe, effective, low-toxicity radiation protective agents for the prevention and treatment of acute radiation injury. Natural products derived from plants have recently gained interest in the field of radiation production. Plant-derived chemicals and extracts have been shown to have a wide-variety of relevant activities, including anti-inflammatory and antioxidant effects, and thus have attracted much attention in the drive to develop effective and low-toxic radiation protective agents8. A potential target of small-molecule therapeutics is ferroptosis, a type of programmed cell death that is characterized by aberrant iron metabolism. The specific features of ferroptosis are the depletion of glutathione (GSH) and the excess accumulation of lipid peroxide and iron9. Previous studies10, 11have indicated that ionizing radiation can induce ferroptosis, which is manifested by increasing levels of reactive oxygen species (ROS) and oxidative stress. The blueberry (Vaccinium corymbosum) has been categorized as one of the top five health foods by the Food and Agriculture Organization of the United Nations (FAO)12. Consumption of blueberries is associated with multiple beneficial effects, including anti-inflammatory, antioxidant, and antitumor activities, and they have been found to support immune system function, slow aging, and decrease the risk of cardiovascular and neurodegenerative diseases. These activities are due to the richness of blueberries in anthocyanins, proanthocyanins, flavonoids, vitamins, minerals, and other nutrients and phytochemical active ingredients13, 14. Blueberry extracts have been shown to prevent cyclophosphamide-induced heart and lung injury by activating cellular antioxidant systems and inhibiting inflammatory gene expression15, 16. Furthermore, blueberry extracts can also improve mild cognitive impairment17, reduce the risk of Alzheimer's disease, and exert a neuroprotective effect18,19. The broad range of these effects suggest that blueberry extracts might have additional protective effects that have yet to be discovered. Specifically, we hypothesized that blueberry extracts might protective against intestinal injury induced by acute radiation. In this study, to test this hypothesis, we performed studies in vitro and in vivo to quantify the effects of blueberry extracts in this context and to explore the potential mechanisms by which it influences radiation-induced intesti-

nal injury.

Materials and Methods

Animal model

Male C57BL/6J mice, aged 6 to 8 weeks, were acclimated for one week in a specific pathogen-free facility at the Institute of Radiation Medicine, Chinese Academy of Medical Sciences. Under standardized housing conditions, the mice were provided with a consistent diet and sterile water. Eighty-one mice were randomly divided into nine groups: a control group, a 6000 mg/kg blueberry extract group, a 7500 mg/kg blueberry extract group, an irradiation-only group sampled 2 d post-irradiation, an irradiation-only group sampled 3.5 d post-irradiation, a group treated with 6000 mg/kg blueberry extract sampled 2 d post-irradiation, a group treated with 7500 mg/kg blueberry extract sampled 2 d post-irradiation, a group treated with 6000 mg/kg blueberry extract sampled 3.5 d post-irradiation, and a group treated with 7500 mg/kg blueberry extract sampled 3.5 d post-irradiation. After 14 consecutive days of daily oral gavage with blueberry extract or normal saline, the mice were subjected to 17 Gy 137Cs γ-ray whole-abdominal irradiation. Mice were sacrificed and the jejunum was harvested at the noted time points.

Cell Lines and Cell Culture

The mouse intestinal epithelial cell line MODE-K was purchased from ATCC. MODE-K cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) at 37℃ with 5%CO2 and 95% humidity. The cells were passaged when they achieved a confluence of approximately 85%. To prepare blueberry extract solutions for use in in vitro experiments, a stock solution was created by dissolving 10 mg of the extract in 10 mL of sterile double-distilled H2O (ddH2O). This solution was sterilized by passage through a membrane filter (0.22μm pore size). Serial dilutions were prepared in water to achieve concentrations of 100μg/mL, 10μg/mL, and 1μg/mL of extract. These diluted solutions will then be utilized for in vitro cell experiments.

Hematoxylin and Eosin Staining, Immunohistochemical Staining, and Tunelstaining

Jejunum samples were fixed in 4% formaldehyde and embedded in paraffin, and sections (4μm) were cut for further analysis. The samples were subjected to staining using hematoxylin and eosin (HE). The lengths of 15 villi per mouse were determined using ImageJ, and the average villar length was calculated. The expression of Ki67 was analyzed using immunohistochemistry with an anti-Ki67 antibody (Abcam, Cambridge, UK). Thirty crypts per mouse were examined, and the total numbers of Ki67-positive cells were counted using ImageJ. Apoptotic cells were quantified using the One Step TUNELApoptosis Assay Kit (Beyotime, Beijing, China). TUNEL--positive cells were counted in 5 fields of view using ImageJ.

Western Blotting Analysis

Proteins from mouse jejunal tissues and MODE-K cells were extracted using the RIPA lysis method. The protein samples were separated by polyacrylamide gel electrophoresis and transferred onto 0.45μm PVDF membranes. After transfer, the membranes were blocked with 5% BSA for 2h at room temperature. Following the blocking step, the membranes were incubated overnight at 4 °C with antibodies against the following proteins: ZO-1 (Abcam, Cambridge, UK), occludin (Abcam, Cambridge, UK), Claudin-1(Abcam, Cambridge, UK), NRF2(Abcam, Cambridge, UK), XCT (Proteintech, Chicago, USA), TfR (Proteintech, Chicago, USA), GPX4(Abcam, Cambridge, UK), SLC3A2 (Proteintech, Chicago, USA), Fn-L (Proteintech, Chicago, USA), Fn-H (Proteintech, Chicago, USA), COX2 (Proteintech, Chicago, USA), FSP1 (Abcam, Cambridge, UK), and GAPDH (Proteintech, Chicago, USA). The membrane was thoroughly washed three times with TBST buffer and then incubated with the corresponding secondary antibodies for 2h at room temperature. The signal was developed using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, MA, USA), and membranes were imaged with the ChemiDoc MPImaging System (Bio-Rad, CA, USA).

Quantitative Reverse Transcription PCR (RT-Qpcr) Analysis

Total RNA was extracted RNA from mouse jejunum tissue samples with TRIzol reagent (Ambion, Texas, USA) according to manufacturer's instructions. The extracted RNA was reverse transcribed into cDNA using the PrimeScript RT reagent Kit (Takara, Japan). Quantitative PCR was performed using EvaGreen 2× qPCR Mastermix-No Dye (Abm, Australia). Gene expression was quantified using the 2-ΔΔCt method, with expression levels normalized to those of GAPDH. For

thw ZO-1 gene, the forward primer was 5'-GCCGCTAAGAGCACAGCAA-3' and the reverse primer was 5'-TCCCCACTCTGAAAATGAGGA- 3'. For the Occludin gene, the forward primer was 5'-TTGAAAGTCCACCTCCTTACAGA-3' and the reverse primer was 5'-CCGGATAAAAAGAGTACGCTGG-3'. For the Claudin-1 gene, the forward primer was 5'-GGGGACAACATCGTGACCG-3'and the reverse primer was 5'-AGGAGTCGAAGACTTTGCACT-3'. For the IL-1β gene, the forward primer was 5'-GCAACTGTTCCTGAACTCAACT-3' and the reverse primer was 5'-ATCTTTTGGGGTCCGTCAACT-3'. For the IL-6 gene, the forward primer was 5'-TAGTCCTTCCTACCCCAATTTCC-3' and the reverse primer was 5'-TTGGTCCTTAGCCACTCCTTC-3'. For the TNF-α gene, the forward primer was 5'-CCCTCACACTCAGATCATCTTCT-3' and the reverse primer was 5'-GCTACGACGTGGGCTACAG-3'. For the IL-8 gene, the forward primer was 'GTGATATTCGAGACCATTTACTG-3' and the reverse primer was 5'-GCCAACAGTAGCCTTCACCCAT-3'. For the IL-10 gene, the forward primer was 5'-GCAGACTCAATACACACTGCA-3' and the reverse primer was 5'-AATAAGCTCCAAGACCAAGG-3'. For the GAPDH gene, the forward primer was 5'AGGTCATCCCAGAGCTGAA-3' and the reverse primer was 5'-CTGCTTCACCACCTTCTTGA-3'.

Measurement of GSH, SOD, T-AOC, and MDA

Total antioxidant capacity (T-AOC) was determined according to levels of glutathione (GSH) and malondialdehyde (MDA) and superoxide dismutase (SOD) activity as antioxidant indexes. These parameters were quantified using biochemical kids, and all experimental steps were performed according to the manufacturers' instructions. The T-AOC and GSH assay kits were obtained from Biotechnology (Elabscience, China), the SOD assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and the MDA kit was obtained from Biyuntian Biotechnology (Shanghai, China).

Detection of Reactive Oxygen Species and Lipid Peroxidation by Flow Cytometry

10mg/mL of blueberry extract was prepared with sterilized water, then filtered through a membrane filter (0.22μm pore size), and finally the blueberry extract was diluted into 100μg/mL, 10μg/mL, or 1μg/mL. MODE-K cells were seeded in 12-well plates and treated with blueberry extract at concentrations of 100μg/mL, 10μg/mL, or 1μg/mL, or were left untreated. After the cells adhered, they were subjected to 8 Gy radiation. For ROS analyses, at 24 h post-irradiation, the cells were collected and incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) at 37 ℃ for 30 min. Flow cytometry was performed to determine the levels of ROS within the cells. For lipid peroxidation analyses, at 48 h post-irradiation, the cells were incubated with BODIPY™ C11 fluorescence probe at 37 ℃ for 30 minutes, and the levels of lipid peroxidation were measured using flow cytometry.

Statistical Analysis

GraphPad Prism (v 8.3, GraphPad Software, Inc.) was used for all statistical analyses. Data are presented as mean ± SD. The unpaired Student's t-test (two-tailed) was used to calculate the significance of differences between groups. A threshold of $p < 0.05$ was taken into account for statistical significance. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$.

Results

Blueberry Extract Improves Radiation-Induced Intestinal Injury

Within the small intestine of mice, IR has been found to induce the destruction of villi, atrophy of crypts, loss of crypt cells, damage to the mucosal base structure, and other pathological phenomena20. To examine the effects of blueberry extracts on acute radiation-induced intestinal injury, a mouse model of radiation-induced intestinal injury was established by exposing C57BL/6 mice to total abdominal irradiation (TAI) of 17 Gy from a 137Cs γ-ray source. Prior to irradiation, certain mice were subjected to a 2 weeks regimen of continuous intragastric administration of blueberry extracts. Jejunal tissue samples were subsequently harvested at 2 or 3.5 d post-irradiation (Fig. 1A). Compared to control mice, the small intestines of irradiated mice were significantly shorter; however, the intragastric administration of blueberry extract prior to irradiation resulted in a significant decrease of the amount of intestinal shortening caused by the irradiation (Fig. 1B and C). Pathological analyses using HE staining showed that irradiation caused structural damage to the jejunum, manifested by shortening of villi and the loss of crypts, but treatments with the blueberry extract ameliorated these pathological defects (Fig. 1D). We next specifically focused on villar length as an in vivo measure of irradiation-induced damage (Fig. 1E). We found that the average length of

the villi was significantly shortened in mice in a time-dependent manner at 2 and 3.5 d after irradiation as compared with mice in the control group, but mice treated with blueberry extract prior to irradiation exhibited an elevated villar length as compared to mice treated with irradiation alone. These results suggested that a high radiation dose caused acute intestinal injury in mice and that blueberry extracts had a protective effect with respect to this intestinal injury.

Blueberry Extract Protects Intestinal Barrier Function and Reduces Radiation-Induced Levels of Inflammatory Factors

The normal function of the intestinal barrier depends primarily on the integrity of its mechanical, biological, immune and chemical barriers, with the mechanical barrier playing a central role. The intestinal barrier separates host tissue from gut microbes, which is important for maintaining intestinal homeostasis21. Radiation has been shown to increase intestinal permeability, and it induces a series of inflammatory reactions22. Therefore, we used Western blot analyses to investigate the effects of irradiation and blueberry extract on the expression levels of proteins associated with the tight junctions that comprise the mechanical barrier. Compared with the control group, irradiated mice were associated with significantly decreased levels of ZO-1, Occludin, and Claudin-1 expression at 2 d and 3.5 d post-irradiation, but pretreatment with blueberry extract reversed this phenomenon (Fig. 2A). The results of RT-qPCR analyses of gene expression were consistent with those of Western blot analyses (Fig.2B). We also quantified the relative expression of several genes associated with inflammation by RT-qPCR (Fig. 2C). Compared with the control group, irradiation dramatically elevated the expression of genes encoding the inflammatory factors ofIL-6, IL-8, IL-10, IL-1β, and TNF-α in the mouse jejunum. However, pretreatment with blueberry extract group led to significant inhibition of the increase of the levels of mRNA associated with these inflammatory factors. These results indicate that blueberry extracts preserve molecular signatures associated with intestinal barrier function during irradiation, and they suggest a mechanism by which such treatment would improve radiation-induced intestinal inflammation.

Blueberry Extract Promotes the Proliferation of Intestinal Crypt Cells after Irradiation

The rapid self-renewal of intestinal epithelium cells every 3 to 5 d depends on the activity of intestinal crypt stem cells, in a process that is particularly important following radiation injury23-25. Therefore, in order to evaluate whether blueberry extract affects the proliferation of intestinal epithelial cells, we performed immunohistochemistry to detect the presence of KI67 and OLFM4, two markers of intestinal crypt cell proliferation and survival, 2 and 3.5 d after irradiation (Fig. 3A, C). Jejunum samples from mice 2 and 3.5 d after irradiation exhibited significantly decreased numbers of KI67-positive cells and OLFM4-positive cells relative to the control (Fig. 3B, D). Here, pretreatment with blueberry extract significantly enhanced the proliferation of intestinal crypt cells after irradiation, both at the 6000 mg/kg and 7500 mg/kg doses. Promotion of the proliferation of intestinal crypt cells would be expected to provide additional support for intestinal integrity after irradiation.

Blueberry Extract Lowers the Apoptotic Activity of Intestinal Cells in Irradiated Mice

To test the effect of blueberry extracts on radiation-induced cell death, we examined the rates of apoptosis in cells of the jejunum of mice at 2 and 3.5 d post-irradiation. As shown in Fig. 4A, the numbers of TUNEL-positive apoptotic cells per field were significantly increased in samples from irradiated mice as compared with samples from the control group. However, the numbers of apoptotic cells in samples from mice pre-treated with blueberry extract were lower at these time periods in irradiated mice (Fig. 4B). These results provide further evidence for the protective effect of blueberry extract on intestinal cells in irradiated mice.

Blueberry Extract Regulates Radiation Induced Intestinal Redox Imbalance

The level of intracellular ROS is significantly increased by radiation, which leads to oxidative stress and inhibition of the body's antioxidant defense system26. Therefore, we used flow cytometry to analyze the effect of blueberry extract on radiation-induced ROS increases in MODE-K cells. We found that irradiation indeed caused significant increases in ROS at 48h after irradiation and that this ROS accumulation was significantly lower in irradiated cells treated with blueberry extract than in vehicle-treated irradiated cells (Fig. 5A). Intragastric blueberry extract administration resulted in significant change in SOD activity and T-AOC compared to both at 2 and 3.5d post irradiation (Fig. 5B-C). These results indicated

that blueberry extract may protect against radiation-induced intestinal injury by ameliorating oxidative stress.

Blueberry Extract Promotes Resistance to Ferroptosis and Alleviates Radiation-Induced Intestinal Injury

Increasing evidence indicates that radiation-induced tissue injury is closely associated with ferroptosis. Ferroptosis is a newly discovered iron-dependent, non-apoptotic form of cell death27, which involves the disruption of lipid protection mechanisms and consequent extensive lipid peroxidation28. MDA is a pivotal end product of lipid peroxidation, and its accumulation in cells is closely related to ferroptosis. To investigate the potential effects of blueberry extract on radiation-induced ferroptosis, we measured the levels of the marker of lipid peroxidation MDA as well as direct products of lipid peroxidation in control or extract-treated MODE-K cells at 72h after irradiation. We found decreased levels of MDAboth in vivo and in vitro with blueberry extract pretreatment, and the increase of lipid peroxidation levels in MODE-K cells was significantly inhibited (Fig. 6A-C). GSH is closely associated with the cysteine-glutamate transporter. Studies have shown that glutathione depletion and glutathione peroxidase 4 (GPX4) inactivation are the main mechanisms of ferroptosis28. We analyzed the effect of the pretreatment of blueberry extract on the levels of GSH in intestinal tissues of mice (Fig. 6D). Compared with control conditions, radiation exposure was associated with significantly decreased GSH levels. However, pretreatment with blueberry extract prior to irradiation restored GSH levels. In addition, another potential target of blueberry extract's action was detected. We examined the levels of ferroptosis-related proteins by Western blot analyses. Compared with the control group, we found significant decreases of XCT, FSP1, Fn-L, Fn-H, SLC3A2, COX2, TfR, Nrf2, and GPX4 protein levels in the jejunum after irradiation, but pretreatment with blueberry extract markedly reversed this phenomenon (Fig. 6E-G). We next detected the accumulation of Fe2+ in MODE-K cells. And we found that irradiation increased the levels of Fe2+, consistent with increased ferroptosis activity. However, blueberry extract mitigated this increase in Fe2+ levels (Fig. 6H). Thus, the strong antioxidant effects of blueberry extract and the lowered Fe2+ levels in this in vitro model suggests that the extract might mitigate radiation-induced cellular damage in part by inhibiting ferroptosis.

Discussion

Therapeutic and accidental exposures to radiation can involve varying degrees of damage to biomolecules and cells. In particular, the gastrointestinal tract, being highly sensitive to radiation, can exhibit acute gastrointestinal syndrome when exposed to radiation doses of 6 to 15Gy. Irradiation of gastrointestinal cells results in the overproduction of ROS and reactive nitrogen species (RNS), thereby causing significant intestinal dysfunction1. Due to the severe side effects of various synthetic radio-protective drugs, research on radio-protective agents has begun to explore the radio-protective activities of natural plant compounds and functional foods. Specifically, accumulating evidence has indicated that some plant-based natural products can mitigate radiation injury. In this study, we investigated the protective effects of blueberry extract on radiation induced intestinal injury using in vivo and in vitro models. Our results demonstrated that blueberry extract significantly alleviated radiation induced intestinal injury by reducing oxidative stress, inhibiting inflammation, and promoting ferroptosis resistance. These findings suggest blueberry extract could serve as a potential therapeutic agent for radiation enteropathy. Blueberry extract is known for its potent antioxidant properties. Our findings revealed that blueberry extract significantly diminished the levels of ROS while boosting the activities of SOD and T-AOC, thereby effectively mitigating oxidative damage. In addition, maintaining intestinal barrier integrity is essential for preventing bacterial translocation and systemic infection. Our study found that blueberry extract enhanced the expression of tight junction proteins such as ZO-1, Occludin, and Claudin-1, thereby strengthening the intestinal barrier. Radiation induced intestinal injury is often accompanied by significant inflammation. We found that blueberry extract downregulated the expression of inflammatory cytokines such as IL-6, IL-8, and TNF- α. This indicates that blueberry modulates the inflammatory response, which is crucial for preventing excessive tissue damage and promoting recovery following radiation exposure. Above all, we also found that blueberry extract can mitigate radiation induced ferroptosis by upregulating the expression of ferroptosis-resistant proteins. Anthocyanin is naturally occurring bioactive substances in blueberry, which has antioxidant and anti-free radical effects. Hao et al29 showed that anthocyanin could effectively antagonize hematopoietic damage caused by 60Coγ, which was manifested as a decrease in the activity of peripheral white blood cells (WBC) and serum SOD in mice. In addi-

tion, Pang et al30 found that blueberry anthocyanin can promote the expression of SIRT1, inhibit the expression of FoxO1, enhance antioxidant capacity, inhibit oxidative stress, improve energy metabolism, and reduce cell death, thereby protecting the reproductive organ damage induced by microwave radiation in male rats. Prior researches on the role of blueberry extract in mitigating radiation induced intestinal injury have been less explored. Our study provides novel insights into the protective mechanisms of blueberry extract in the context of radiation enteropathy, highlighting its potential as a radioprotective agent. Compared to other radioprotective agents, blueberry extract has several advantages. It is a natural compound with minimal side effects, making it a promising candidate for protection against radiation induced intestinal injury. Additionally, blueberry extract's ability to promote ferroptosis resistance sets it apart from other agents that primarily focus on reducing oxidative stress and inflammation. Given the increasing incidence of radiation induced intestinal injury, our findings suggest that blueberry extract could be a valuable adjunctive therapy to mitigate intestinal complications. Although many natural plant compounds have shown significant potential in the prevention and treatment of radiation-induced intestinal injury, there is currently a lack of understanding of their mechanisms of action and limited clinical evidence to support their use. Furthermore, the effects of these compounds may differ between acute and chronic radiation injury. Therefore, conducting in-depth research on these natural products and developing and applying additional naturally derived radiation protectors is essential to fully develop their potential in the radioprotection against tissue injury.

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