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ORIGINAL ARTICLE

Hematopoietic protection and mechanisms of ferrostatin-1 on hematopoietic acute radiation syndrome of mice

Xiaohong Zhang[a,b,c,d], Mengxin Tian[a], Xin Li[a], Chunyan Zheng[a], Ailian Wang[e], Jundong Feng[a,c], Xiaodan Hu[a], Shuquan Chang[a,c], and Haiqian Zhang[a,b,d]

[a]Department of Nuclear Science and Technology, College of Materials Science and Technology, Nanjing University of Aeronautics and Astronautics, Nanjing City, P. R. China; [b]Jiangsu Key Laboratory for Biomaterials and Devices, Southeast University, Nanjing City, P. R. China; [c]Key Laboratory of Nuclear Technology Application and Radiation Protection in Astronautics (Nanjing University of Aeronautics and Astronautics), Ministry of Industry and Information Technology, Nanjing City, P.R. China; [d]Collaborative Innovation Centre of Radiation Medicine of Jiangsu Higher Education Institutions, Soochow University, Suzhou, P. R. China; [e]Department of Ophthalmology, First Affiliated Hospital, Bengbu Medical College, Bengbu, P. R. China

ABSTRACT

Purpose: Baicalein (an anti-ferroptosis drug) was recently reported to synergistically improve the survival rate of mice following a high dose of total body irradiation with anti-apoptosis and anti-necroptosis drugs. At the same time, our group has demonstrated that ferrostatin-1, a ferroptosis inhibitor, improves the survival rate of a mouse model of hematopoietic acute radiation syndrome to 60% for 150 days ($p < .001$). These phenomena suggest that ferroptosis inhibition can mitigate radiation damage. In this study, we continued to study the mechanisms by which ferrostatin-1 alleviated radiation-induced ferroptosis and subsequent hematopoietic acute radiation syndrome.

Materials and methods: Male ICR mice (8–10 weeks old) were exposed to doses of 0, 8, or 10 Gy irradiated from a ^{137}Cs source. Ferrostatin-1 was intraperitoneally injected into mice 72 h post-irradiation. Bone marrow mononuclear cells (BMMCs) and peripheral blood cells were counted. The changes in iron-related parameters, lipid metabolic enzymes, lipid peroxidation repair molecules (glutathione peroxidase 4, glutathione, and coenzyme Q10), and inflammatory factors (TNF-α, IL-6, and IL-1β) were evaluated using biochemical or antibody techniques.

Results: Ferrostatin-1 increased the number of red and white blood cells, lymphocytes, and monocytes in the peripheral blood after total body irradiation in mice by mitigating the ferroptosis of BMMCs. Total body irradiation induced ferroptosis in BMMCs by increasing the iron and lipid peroxidation levels and depleting the acyl-CoA synthetase long-chain family member 4 (ASCL4), lipoxygenase 15, glutathione peroxidase 4, and glutathione levels. Ferroptotic BMMCs did not release TNF-α, IL-6, or IL-1β at the early stage of radiation exposure. Ferrostatin-1 mitigated the lipid peroxidation of radiation-induced ferroptosis by attenuating increases in levels of hemosiderin and liable iron pool and decreases in levels of ASCL4 and glutathione peroxidase 4.

Conclusions: The onset of total body irradiation-induced ferroptosis in BMMCs involved changes in iron, lipid metabolic enzymes, and anti-lipid peroxidation molecules. Ferrostatin-1 could be a potential radiation mitigation agent by acting on these targets.

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KEYWORDS
Hematopoietic acute radiation syndrome; ferrostatin-1; iron; lipid metabolic enzyme; lipid peroxidation repair molecule

Introduction

Hematopoietic acute radiation syndrome (hARS) is an acute hematopoietic injury caused by total body irradiation or the irradiation of the most of body with a dose range of 1–10 Gy in a short period of time. Damage to bone marrow hematopoietic stem/progenitor cells is the pathogeny of hARS (Shao et al. 2014). This damage leads to a reduction in the number of peripheral hematocytes, which subsequently causes the clinical manifestations of infection, hemorrhage, and fever. Depending on the dose received, hARS can be classified as mild, moderate, severe, and extremely severe. Although much attention has been paid to developing strategies to ameliorate hARS, the mortality rate of severe and extremely severe hARS is very high.

Ferroptosis is a form of regulated cell death characterized by the iron-dependent accumulation of lipid reactive oxygen species (ROS), the inactivation or depletion of glutathione peroxidase 4 (GPX4), and the exhaustion of glutathione, which was first reported in cultured cancer cells (Dixon et al. 2012). It has since been associated with cell death in vivo following acute tissue injuries, such as cerebral injuries (Li et al. 2017), and kidney or heart ischemia/reperfusion (Friedmann Angeli et al. 2014; Fang et al. 2019). Therefore, the regulation of ferroptosis has proved to be a new therapeutic strategy in the treatment of cancers and

CONTACT Xiaohong Zhang zhangxiaohong@nuaa.edu.cn Department of Nuclear Science and Technology, Nanjing University of Aeronautics and Astronautics, No. 29 Yudao Street, Nanjing City 210016, P. R. China

ferroptosis-related diseases. Ferrostatin-1, a radical-trapping agent containing an arylalkylamine group (Skouta et al. 2014), which prevents lipid peroxidation to membrane lipids, had been identified as a ferroptosis inhibitor (Dixon et al. 2012), and is used to rescue ferroptosis-related diseases (Li et al. 2017).

The indirect effect of ionizing radiation arising from water radiolysis products (hydroxyl radicals, hydrogen radicals, and hydrated electrons) induces the most of ionizing radiation-induced damage in organisms (Miao et al. 2014). Therefore, both ionizing radiation-induced injuries and ferroptosis are ROS-related. Several research groups have reported that radiation induces ferroptosis in cancer cells (Lei et al. 2020; Ye et al. 2020). More importantly, Thermozier et al. (2020) found that baicalein, an anti-ferroptosis drug, synergistically improves the survival rate of hARS-mice with anti-apoptosis and anti-necroptosis drugs. These indicate that ionizing radiation is an exogenous inducer of ferroptosis (Stockwell and Jiang 2020), whereby targeting ferroptosis mitigates radiation damage. At the same time, our group demonstrated that the intraperitoneal injection of ferrostatin-1 is able to effectively increase the survival rate of hARS-mice to 60% for 150 days (Zhang et al. 2020). However, the mechanism by which ferrostatin-1 mitigates radiation-induced ferroptosis, and subsequent hARS, remains unknown.

The onset of ferroptosis involves imbalances in iron, lipids, amino acids, or the oxidative metabolism. Particularly, the induction of ferroptosis requires certain pre-conditions, including the existence of redox-active iron and phospholipids containing polyunsaturated fatty acyl groups and the failure of lipid peroxide repair network (Angeli et al. 2017), such as the inactivation of GPX4 and coenzyme Q 10 (CoQ10; Bersuker et al. 2019). In addition to these factors, after onset, ferroptotic cells are supposed to deliver damage-associated molecular patterns, which may mediate reactions related to inflammation (Proneth and Conrad 2019). In this study, we studied changes in bone marrow mononuclear cells (BMMCs) and peripheral blood cells after the administration of ferropstatin-1 to irradiated mice to elucidate the effect of ferrostatin-1 on hematopoietic syndrome; explored ferrostatin-1's effect on the factors involved in radiation-induced ferroptosis in BMMCs, that is, iron, lipid peroxidation and its corresponding enzymes, lipid peroxide repair network, and inflammatory factors, to identify the mechanisms by which ferrostatin-1 alleviates hematopoietic syndrome.

Materials and methods

Animal and radiation treatments

Male ICR mice (8–10 weeks old) were purchased from Nantong University Animal Center (Nantong, Jiangsu, China) and housed in cages for one week with a relative humidity of 55 ± 15%, a temperature of 23 ± 2 °C, and a 12 h/12 h light/dark cycle. This work was carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Mice were placed in a ventilated plexiglass cage and exposed to total body irradiation from a ^{137}Cs source (Hopewell Designs Inc., Alpharetta, GA, USA). The absorbed doses were 0, 8, and 10 Gy at a dose rate of 2 Gy/min. A PTW Farmer ionization chamber (30010; PTW-Freiburg, Freiburg, Germany) was used for dose calibration.

Reagent administration

Mice were randomly divided into four groups: (1) 0 Gy; (2) 0 Gy + ferrostatin-1; (3) radiation; (4) radiation + ferrostatin-1. Ferrostatin-1 (APExBIO Technology LLC, Houston, TX, USA) was dissolved in DMSO (0.01%, diluted with 0.9% saline) at 37 °C under ultrasonic conditions. Ferrostatin-1 (2 mg/kg) was intraperitoneally injected into mice 72 h post-irradiation. DMSO was injected into mice in the 0 Gy or radiation group. Each group contained six mice. Mice in the 0 Gy or 0 Gy + ferrostatin-1 group were sham-irradiated.

BMMCs counting

The femur was removed from the mice, and its bone marrow was rinsed with Dulbecco's modified Eagle's medium. BMMCs were extracted from the bone marrow cell suspension using lymphocyte separation medium (Tianjin Haoyang Bio., Tianjin, China). The BMMC suspension was dripped onto a slide and a biological binocular microscope (Olympus Optical Co., Tokyo, Japan) was used to observe the slide. BMMCs were counted on a blood cell counting plate.

Histomorphological evaluation

The removed femur was fixed in a 4% formalin solution for 24 h and decalcified with 10% ethylenediaminetetraacetic acid under microwave conditions. The treated bone samples were then dehydrated with ethanol and embedded in paraffin. Finally, the paraffin-embedded sample was sectioned and subsequently stained with hematoxylin and eosin. A biological binocular microscope (Olympus Optical Co.) was used to study the histomorphology of the bone marrow.

Blood cell counting

Blood samples were extracted from the orbital sinus of mice. A hematology analyzer (Nihon Kohden, Tokyo, Japan) was used to count the red and white blood cells, lymphocytes, monocytes, and platelets of the blood sample.

Iron content measurement

The supernatant of the rinse solution of the bone marrow was collected. The corresponding cell precipitate was cracked with NP-40 (Beyotime, Shanghai, China), and its supernatant was also collected. The two supernatants were combined, and a flame atomic absorption spectroscopy (Persee, Beijing, China) was used to measure the iron content in the mixed supernatants.

Prussian blue staining

The cell suspension extracted from the bone marrow was smeared onto a glass slide. The iron of the cell suspension was stained with a Prussian Blue Nucleus Fixation Kit (Jianchen, Bio., Nanjing, Jiangsu, China). Staining procedures were performed according to the manufacturer's instructions. A biological binocular microscope (Olympus Optical Co.) smearing with oil was used to study the iron distribution of bone marrow cells.

Enzyme-linked immunosorbent assay (ELISA)

After the cryolysis of BMMCs (three freeze-thaw cycles), the supernatant was collected and used to evaluate ferritin (Camilo Biological Co., Nanjing, Jiangsu, China), GPX4 (Camilo Biological Co.), and CoQ10 (Tongwei Biotechnology Co., Shanghai, China) levels with an ELISA kit. The supernatant of the rinse solution of the bone marrow and serum were collected to determine the levels of TNF-α (KeyGen Biotech Co., Nanjing, Jiangsu, China), IL-6 (KeyGen Biotech Co.), and IL-1β (KeyGen Biotech Co.). The ELISA procedures were followed according to the manufacturer's instructions.

Liable iron Pool (LIP) evaluation

Calcein acetoxymethyl ester (Calcein AM), a transmembrane and non-fluorescent molecule, has often been used to evaluate LIP. When Calcein AM enters cells, it is immediately cleaved by intracellular esterases, resulting in the formation of calcein, a fluorescent iron chelator. The binding of LIP to calcein quenches the fluorescence of calcein, which is used as the principle to evaluate the level of LIP (Kakhlon and Cabantchik 2002). The LIP evaluation procedures are as follows. Calcein AM (0.125 μM; Beyotime Co.) was added to the BMMC sediment. After incubation at 37 °C for 35 min, the cells were re-suspended in phosphate buffer solution, and their fluorescence (F1) was rapidly assessed using a fluorescence microplate reader (Infinite M Plex; Tecan Group Ltd., Mannedorf, Zurich, Switzerland) at a wavelength of 515 nm. Deferiprone (500 μM; APExBIO Technology LLC) was added to Calcein AM-treated BMMCs. After incubating for 1 h, the fluorescence of deferiprone-treated cells (F2) was measured. The change in fluorescence (F2-F1) was equivalent to the amount of LIP.

Malondialdehyde determination

The supernatant was collected after the ultrasonic pyrolysis of BMMCs, and the malondialdehyde level was evaluated using a Malondialdehyde Assay Kit (A003-1; Jiancheng Bio.). The corresponding procedures were performed according to the manufacturer's instructions.

Western blotting

The expression of two lipid metabolic enzymes of BMMCs, acyl-CoA synthetase long-chain family member 4 (ACSL4) and lipoxygenase 15, was detected by the western blotting. The primary antibodies for ACSL4 (sc-271800; Santa Cruz Biotech Inc., Dallas, Texas, USA) and lipoxygenase 15 (ab244205; Abcam Co., Cambridge, Cambs, UK) were used. The procedures were performed as previously reported (Wu et al. 2016).

Glutathione determination

The supernatant was collected after the cryolysis of BMMCs, and the glutathione level was detected using a Glutathione Assay Kit (KeyGen Biotech Co.). The procedures were performed according to the manufacturer's instructions.

Statistical analysis

All data analyzed are expressed as the mean ± standard deviation (SD). Statistical differences between groups were assessed using one-way analysis of variance, followed by a post-hoc Student-Newman-Keuls test. $p < .05$ was considered statistically significant.

Results

Ferrostatin-1 recovers hematopoietic function in mouse model of hARS

BMMCs are a cell population with a single round nucleus, which includes hematopoietic precursor cells. A change was observed in the number of BMMCs of 8 Gy-irradiated mice 24 h after exposure to ferrostatin-1. Ferrostatin-1 significantly increased the number of BMMCs in irradiated mice on the 4th day post-irradiation (Figure 1(A)). Hematoxylin and eosin staining showed that the number of BMMCs in 10 Gy-irradiated ferrostatin-1-exposed mice was very similar to those of control mice on the 120th day post-irradiation (Figure 1(B); 10 Gy-irradiated mice died within 20 days.). These data indicate that ferrostatin-1 can restore BMMCs in hARS-mice.

The red blood cells of the peripheral blood differentiate from the erythroid progenitor cells of bone marrow, lymphocytes differentiate from myeloid progenitor cells, monocytes differentiate from granulocyte-macrophage hematopoietic progenitor cells (GM-HPCs), and platelets differentiate from megakaryocyte progenitor cells. The major symptom of severe or extremely severe hARS is cytopenia, which is caused by the injury of these progenitor cells in the bone marrow. Next, the effect of ferrostatin-1 on the number of peripheral hematocytes in mice after total body irradiation was observed. Ferrostatin-1 was injected intraperitoneally into mice 72 h after being subjected to 8/10 Gy of gamma radiation. The numbers of red and white blood cells, lymphocytes, monocytes, and platelets were counted on days 15, 30, or 120 post-irradiation. As a result, ferrostatin-1 treatment was found to attenuate the decrease

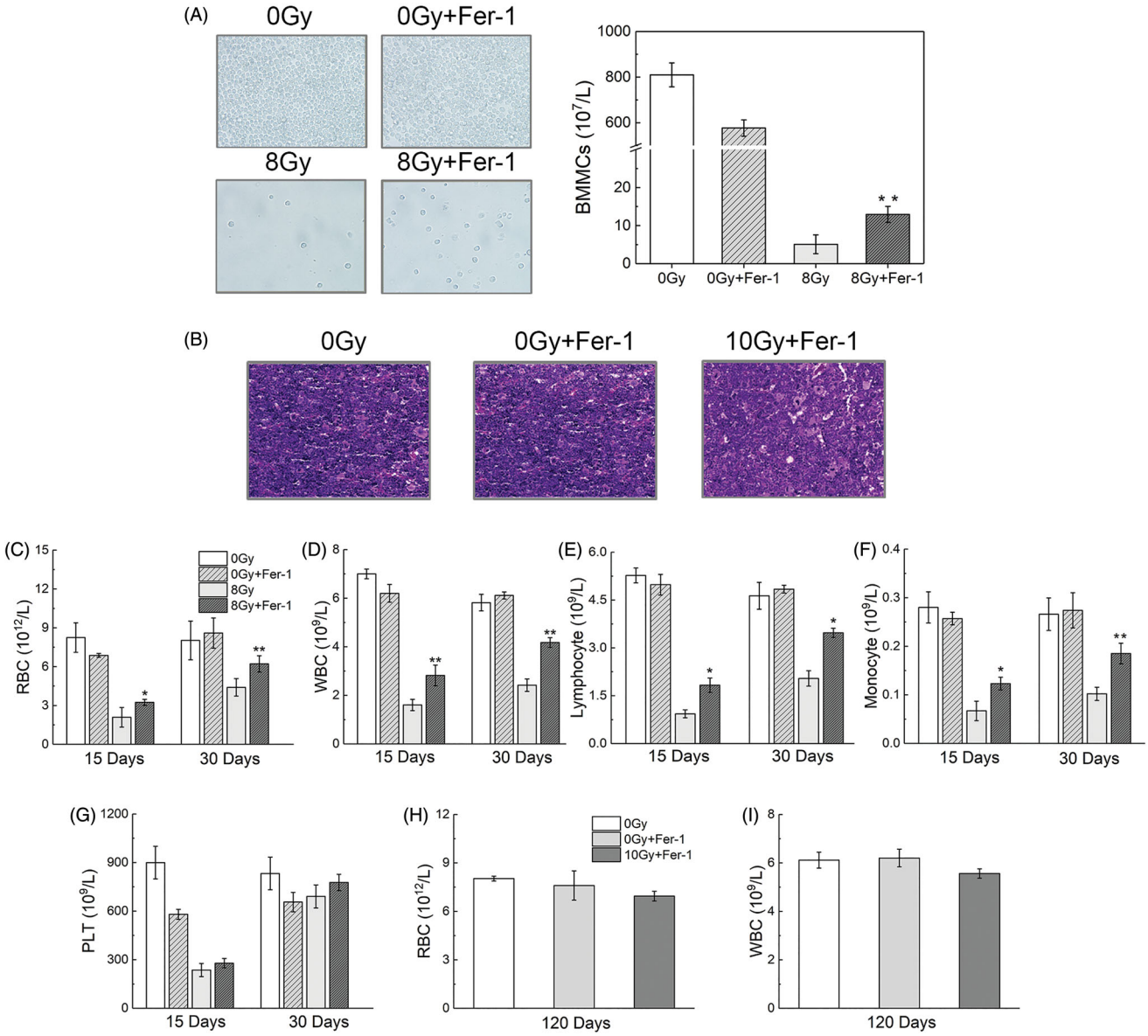

Figure 1. Ferrostatin-1 recovers the hematopoietic function of hARS-mice. Mice were injected with 2 mg/kg of ferrostatin-1 72 h after being subjected to gamma radiation. (A) Effect of ferrostatin-1 on the number of BMMCs (×40) in mice 4 days after 8-Gy of gamma radiation. (B) Effect of ferrostatin-1 on the histomorphology of femur bone marrow (× 20) in mice 120 days after 10-Gy of gamma radiation (10 Gy-irradiated mice died within 20 days). (C–G) Effect of ferrostatin-1 on counts of red blood cells, white blood cells, lymphocytes, monocytes, and platelets in mice 15 or 30 days after 8-Gy of gamma radiation. (H, I) Effect of ferrostatin-1 on counts of red blood cells and white blood cells in mice 120 days after 10-Gy of gamma radiation (10 Gy-irradiated mice died within 20 days). The data were presented as the mean ± SD ($n = 6$). * $p < .05$, and ** $p < .01$ vs 8 Gy group. RBC: red blood cell, WBC: white blood cell, PLT: platelet, and Fer-1: ferrostatin-1.

in red and white blood cell, lymphocyte, and monocyte counts on days 15 and 30 after 8 Gy of gamma radiation (Figure 1(C–F)). The red and white blood cell counts of 10 Gy-irradiated ferrostatin-1-exposed mice were very similar to those of control mice on the 120th day post-irradiation (Figure 1(H, I); 10 Gy-irradiated mice died within 20 days). Ferrostatin-1 treatment did not affect the number of platelets in irradiated-mice (Figure 1(G)). These data indicate that ferrostatin-1 can elevate the red and white blood cell, lymphocyte, and monocyte counts in the blood of hARS-mice. Together, the restoration of red and white blood cells, lymphocytes, and monocytes and BMMCs suggests that ferrostatin-1 is able to exert a protective effect on erythroid progenitor cells, myeloid progenitor cells, and GM-HPCs in the bone marrow of hARS-mice.

Ferrostatin-1 decreases iron level of bone marrow

Since hemosiderin-based iron participates in total body irradiation-induced ferroptosis, the effect of ferrostatin-1 on iron was assessed (Zhang et al. 2020). First, the total iron content of bone marrow 24 h after ferrostatin-1 was injected into 8 Gy-irradiated mice was measured. Gamma radiation was found to increase the total iron content. A decrease in the total iron content was observed in irradiated mice administered ferrostatin-1 compared to irradiated mice alone (Figure 2(A)). This result indicates that ferrostatin-1 decreased the total iron content in the bone marrow of hARS-mice. Then, the effect of ferrostatin-1 on hemosiderin in the bone marrow of 8 Gy-irradiated mice was observed. Similarly, ferrostatin-1 was found to

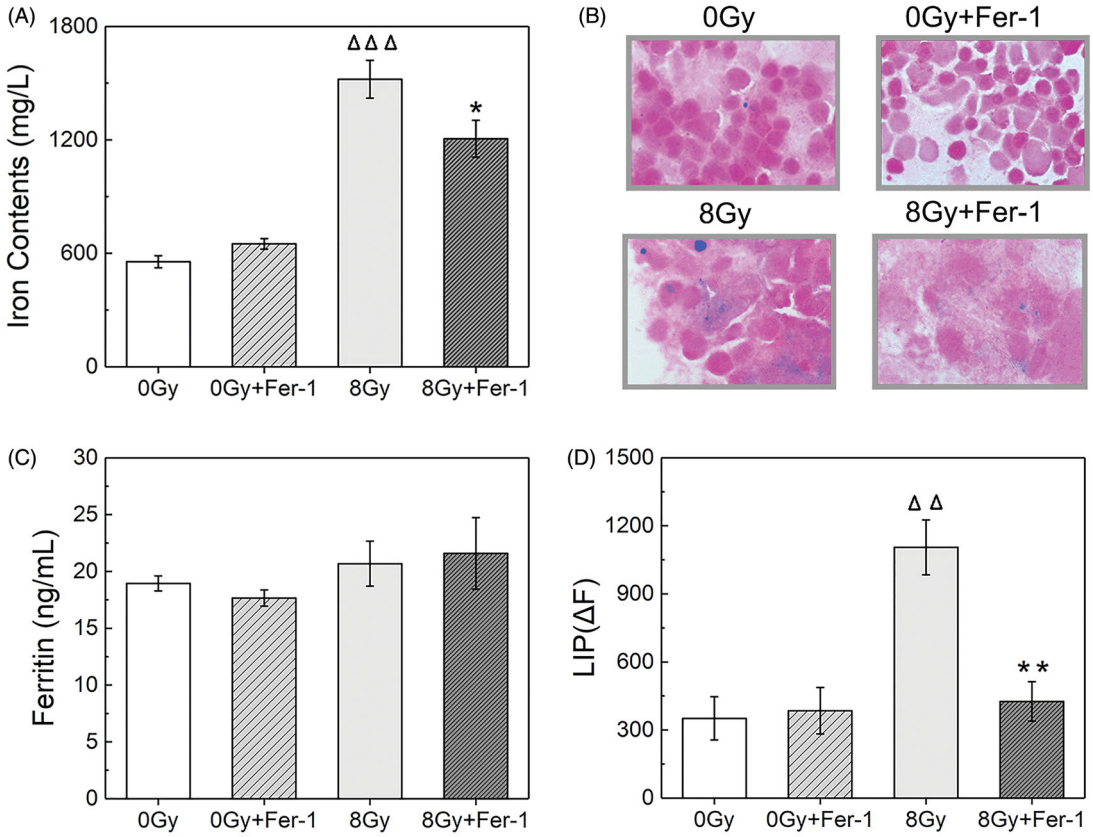

Figure 2. Effect of ferrostatin-1 on iron levels in the bone marrow of mice on the 4th day post-irradiation. Mice were injected with 2 mg/kg of ferrostatin-1 72 h after being subjected to gamma radiation. (A–D) Effect of ferrostatin-1 on the total iron content of bone marrow, hemosiderin (×100) of bone marrow cell suspension, ferritin of BMMCs, and cellular LIP of BMMCs. The data are presented as the mean ± SD ($n = 6$). $^{\Delta\Delta}p < .01$, and $^{\Delta\Delta\Delta}p < .001$ vs 0 Gy group; * $p < .05$, and ** $p < .01$ vs 8 Gy group. Fer-1: ferrostatin-1, and LIP: Liable iron pool.

attenuate the increased levels of hemosiderin caused by radiation exposure (Figure 2(B)). Hemosiderin is an iron-storage complex (Takami and Sakaida 2011). This result suggests that ferrostatin-1 is able to decrease the stored iron of irradiated bone marrow. When the iron is in excess, the pathway of hemosiderin-ferritin-liable iron pool (LIP) will act for the mobilization of stored iron (Picard et al. 1998; Saito et al. 2012). Within this pathway, ferritin is involved in the storage of iron, which mediates the transformation from hemosiderin to LIP. LIP contains redox-active iron, which is closely associated with the induction of ferroptosis. As such, changes in these two parameters and the effect of ferrostatin-1 on these two parameters were measured in the irradiated BMMCs. The ferritin levels did not change in irradiated BMMCs, and ferrostatin-1 did not affect the levels of ferritin (Figure 2(C)). However, gamma radiation was found to increase the level of cellular LIP, which was mitigated by ferrostatin-1 (Figure 2(D)). Together, these results suggest that the increase in the levels of hemosiderin and cellular LIP contributed to a radiation-induced elevation in the total iron content of the bone marrow. In addition, ferritin did not mediate the transformation of hemosiderin into cellular LIP; ferrostatin-1 decreased the high levels of total iron content by reducing the levels of hemosiderin and cellular LIP.

Ferrostatin-1 attenuates the depletion of lipid metabolic enzymes in BMMCs

Malondialdehyde is a byproduct generated during lipid peroxidation. The level of malondialdehyde in BMMCs was observed 24 h after ferrostatin-1 was injected into 8 Gy-irradiated mice. Gamma radiation was found to elevate the levels of malondialdehyde, and ferrostatin-1 mitigated the increase in malondialdehyde levels (Figure 3(A)). This indicates that ferrostatin-1 decreased the level of lipid peroxidation in irradiated BMMCs.

ACSL4 and lipoxygenase 15 are lipid metabolic enzymes. Ferroptosis has been previously associated with enzymatic lipid peroxidation. Changes in these two enzymes in total body radiation-induced ferroptosis and the effects of ferrostatin-1 on these two enzymes were evaluated. ACSL4 expression was found to be significantly decreased in irradiated BMMCs, and ferrostatin-1 mitigated the decrease in the level of ACSL4 (Figure 3(B)). This indicates that ACSL4 plays a role in total body radiation-induced ferroptosis, and ferrostatin-1 can target ACSL4. Although lipoxygenase 15 was also significantly decreased in irradiated BMMCs, its decrease was not attenuated by ferrostatin-1 (Figure 3(B)). This suggests that lipoxygenase 15 also participated in the lipid peroxidation of total body irradiation-induced ferroptosis, but ferrostatin-1 is unable to target lipoxygenase 15. Together,

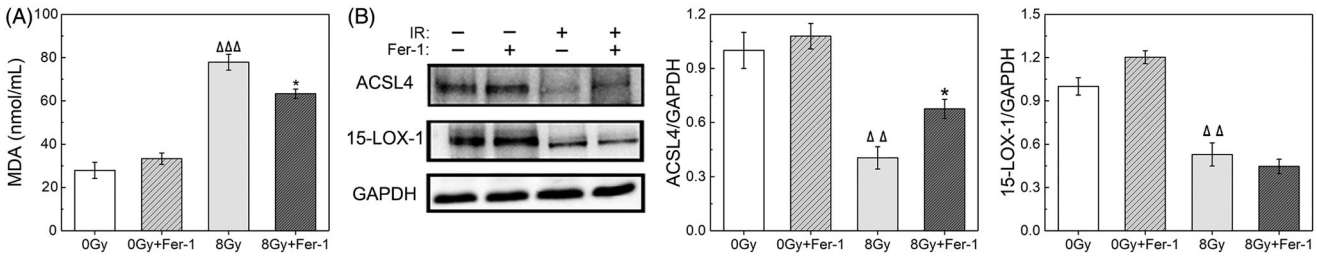

Figure 3. Effect of ferrostatin-1 on malondialdehyde levels and lipid metabolic enzyme expression of BMMCs on the 4th day post-irradiation. Mice were injected with 2 mg/kg of ferrostatin-1 72 h after being subjected to gamma radiation. (A, B) Effect of ferrostatin-1 on malondialdehyde level and ACSL4 and lipoxygenase 15 expression. The data are presented as the mean ± SD (n = 6). $^{\Delta\Delta}p < .01$, and $^{\Delta\Delta\Delta}p < .001$ vs 0 Gy group; $^{*}p < .05$ vs 8 Gy group. Fer-1: ferrostatin-1, ACSL4: acyl-CoA synthetase long-chain family member 4, and 15-LOX-1: lipoxygenase 15 of mice.

these indicate that ACSL4 and lipoxygenase 15 participated in the lipid peroxidation during total body radiation-induced ferroptosis, and ferrostatin-1 targets ACSL4 to mitigate lipid peroxidation.

Ferrostatin-1 increases the level of GPX4 in BMMCs

The dysfunction of the lipid peroxide repair network in the body is a key factor for the onset of ferroptosis. GPX4, glutathione, and CoQ 10 are involved in the lipid peroxide repair network of ferroptosis (Stockwell and Jiang 2020). Thus, changes in these three parameters in irradiated BMMCs and the effect of ferrostatin-1 on these parameters were evaluated. Gamma radiation decreased the levels of GPX4 and glutathione, and ferrostatin-1 attenuated the decrease in the level of GPX4 (Figure 4(A, B)). Gamma radiation or gamma radiation plus ferrostatin-1 did not affect the level of CoQ 10 (Figure 4(C)). These data suggest that the onset of radiation-induced ferroptosis involves the failure of the functions of GPX4 and glutathione, and ferrostatin-1 increases the level of GPX4 to mitigate ferroptosis.

Ferrostatin-1 does not affect the levels of inflammation factors

Ferroptosis is thought to be immunogenic and proinflammatory because ferroptotic cells release pro-inflammatory factors (Linkermann et al. 2014; Proneth and Conrad 2019). TNF-α, IL-6, and IL-1β have been reported to increase during ferroptosis in severe acute injury, and ferroptosis inhibitors have prevented these increases (Zhang et al. 2018; Ma et al. 2020). Changes in TNF-α, IL-6, and IL-1β in irradiated mice and the effects of ferrostatin-1 on these inflammatory factors should therefore be observed. TNF-α, IL-6, and IL-1β in the bone marrow or serum were detected 24 h after the administration of ferrostatin-1 to 8 Gy-irradiated mice. Gamma radiation or gamma radiation plus ferrostatin-1 did not change the TNF-α, IL-6, and IL-1β levels in the bone marrow (Figure 5(A–C)). These suggest that ferroptotic BMMCs induced by radiation did not release TNF-α, IL-6, and IL-1β, and correspondingly, ferrostatin-1 did not affect the basal expression of TNF-α, IL-6, and IL-1β. Gamma radiation did not affect the level of serum TNF-α. However, it significantly decreased the levels of serum IL-6 and IL-1β. At the same time, ferrostatin-1 did not attenuate the decrease in serum IL-6 and IL-1β (Figure 5(A–C)). Together, these data indicate that radiation-induced ferroptosis in BMMCs did not cause a proinflammatory effect, and ferrostatin-1 did not affect the expression of inflammatory factors.

Discussion

In our previous studies, ferrostatin-1 was found to effectively increase the survival rate and lifespan of hARS-mice. In the present study, we studied the mechanisms of ferrostatin-1 as a radiation mitigator further and found that ferrostatin-1 increased the counts of red and white blood cells, lymphocytes, and monocytes in the peripheral blood by mitigating radiation-induced ferroptosis in BMMCs. Total body irradiation induced ferroptosis in BMMCs by increasing the levels of iron and lipid peroxidation and depleting the levels of ASCL4, lipoxygenase 15, GPX4, and glutathione. Ferrostatin-1 attenuated the lipid peroxidation of ferroptosis by decreasing the level of iron and increasing the levels of ACSL4 and GPX4.

Ferrostatin-1 protected erythroid progenitor cells, myeloid progenitor cells, and GM-HPCs in irradiated bone marrow (Figure 1), indicating that radiation induces ferroptosis in these progenitor cells, which is hemorrhagic iron-related (Zhang et al. 2020). Studies in which iron overload has been found to impair hematopoietic progenitor cells in the bone marrow support our results (Chai et al. 2015; Hua et al. 2017).

Iron is a major induction factor of ferroptosis, which is also known as a non-canonical ferroptosis induction factor (Hassannia et al. 2019). Hemosiderin and LIP were found to increase in the radiation-related ferroptosis (Figure 2(B and D)). Hemosiderin is a complex of stored iron, which occurs in many hemorrhagic diseases and prevents iron-mediated oxidative damage. Therefore, it was unlikely that hemosiderin directly caused the iron-mediated lipid peroxidation during ferroptosis. LIP has been reported to serve as an initial trigger of ferroptosis due to its high redox activity (Doll and Conrad 2017). Thus, the iron stored in hemosiderin may potentially transform into LIP. Although ferritin did not mediate iron immobilization from hemosiderin to cellular LIP (Figure 2(C)), the existence of this pathway indicates that another pathway for the immobilization of stored iron may exist between hemosiderin and LIP.

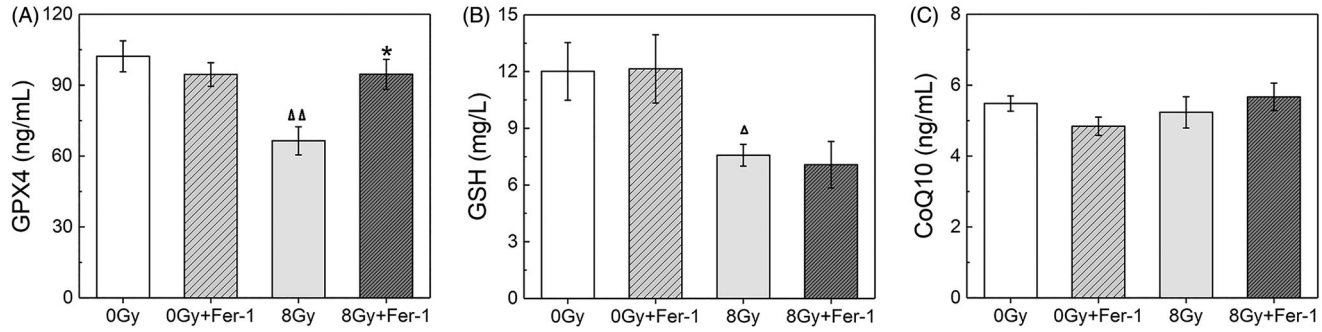

Figure 4. Effect of ferrostatin-1 on the lipid peroxidation repair network of BMMCs on the 4th day post-irradiation. Mice were injected with 2 mg/kg of ferrostatin-1 72 h after being subjected to gamma radiation. (A–C) Effect of ferrostatin-1 on GPX4, glutathione, and CoQ10 levels. The data are presented as the mean ± SD ($n = 6$). $^{\triangle}p < .05$, and $^{\triangle\triangle}p < .01$ vs 0 Gy group; $^{*}p < .05$ vs 8 Gy group. Fer-1: ferrostatin-1, GPX4: glutathione peroxidase 4, GSH: glutathione, and CoQ10: coenzyme10.

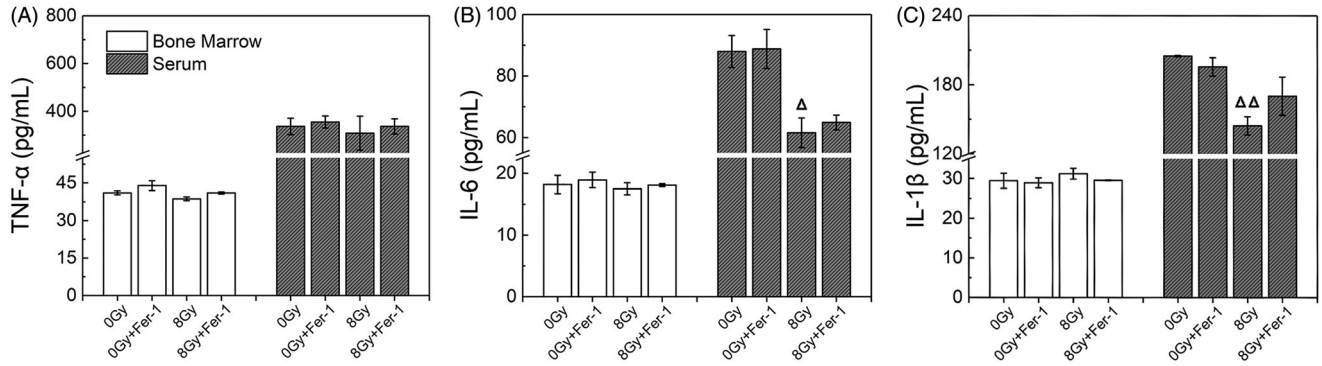

Figure 5. Effect of ferrostatin-1 on TNF-α, IL-6, and IL-1β in the supernatant of bone marrow cell suspension or serum on the 4th day post-irradiation. Mice were injected with 2 mg/kg of ferrostatin-1 72 h after being subjected to gamma radiation. (A-C) Effect of ferrostatin-1 on TNF-α, IL-6, and IL-1β levels. The data are presented as the mean ± SD (n = 6). $^{\triangle}p < .05$, and $^{\triangle\triangle}p < .01$ vs 0 Gy group. Fer-1: ferrostatin-1.

As for ferrostatin-1 decreasing hemosiderin (Figure 2(B)), this phenomenon may be related to the regulation of ferrostatin-1 at the level of hepcidin. Ferrostatin-1 decreases the level of hepcidin (Liu et al. 2020), which increases the expression of ferroportin 1, a transmembrane protein that transports iron from inside cells to the bloodstream (Knutson et al. 2005). High expression in ferroportin 1 promote the export of iron into the bloodstream, which decreases the level of hemosiderin in irradiated bone marrow, and subsequently decreasing the level of LIP in irradiated BMMCs (Figure 2(D)).

Lipid peroxidation is a typical pathological process of ferroptosis. There are two pathways for lipid peroxidation during ferroptosis: enzymatic lipid peroxidation and non-enzymatic lipid peroxidation which is initiated by ROS (Stoyanovsky et al. 2019). The depletion of ACSL4 participated in the process of lipid peroxidation in total body irradiation-induced ferroptosis, and ferrostatin-1 reversed the depletion of ACSL4 (Figure 3(B)), indicating that total body irradiation-induced ferroptosis contains the enzymatic lipid peroxidation, and that ferrostatin-1 mitigates lipid peroxidation via ACSL4. These results are consistent with those reported by Muller et al. (2017), who found that ACSL4 is depleted in a time-dependent manner during erastin-ferroptosis in NIH3T3, and that ferrostatin-1 prevents this type of ACSL4 depletion. However, high levels of ACSL4 expression have also been reported to increase cellular membranes with long polyunsaturated $\omega 6$ fatty acids, which promote the onset of ferroptosis (Doll et al. 2017). The differences in the expression of ACSL4 during the onset of ferroptosis cannot be explained, and will require further study.

Lipoxygenases are non-heme iron-containing enzymes that catalyze the peroxidation of free or esterified PUFAs at the bis-allylic position to generate various lipid hydroperoxides. Although ferrostatin-1 cannot target lipoxygenase 15 to mitigate total body irradiation-induced ferroptosis, the depletion of lipoxygenase 15 was found to be involved in the process of lipid peroxidation of this type of ferroptosis (Figure 3(B)). Shintoku et al. (2017) found that the level of lipoxygenase 15 is depleted in erasin- or RSL3-induced ferroptosis in HT1080, which is consistent with our results.

As for non-enzymatic lipid peroxidation, radiation-induced hydroxyl radicals initiate phospholipid peroxidation. The corresponding reactions are as follows:

$$OH^{\cdot} + PLH \rightarrow PL^{\cdot} + H_2O \quad (1)$$

$$PL^{\cdot} + O_2 \rightarrow PLOO^{\cdot} \quad (2)$$

$$PLOO^{\cdot} + PLH \rightarrow PLOOH + PL^{\cdot} \quad (3)$$

These reactions generate not only lipoxy-radicals but also lipid hydroperoxides. The high levels of lipid hydroperoxide, together with increased levels of iron, resulted in a cascade reaction of iron-related phospholipid peroxidation (Ursini and Maiorino 2020). The reactions are as follows:

$$Fe^{2+} + PLOOH \rightarrow Fe^{3+} + PLO^{\cdot} + OH^{-} \quad (4)$$

$$Fe^{3+} + PLOOH \rightarrow Fe^{2+} + PLOO^{\cdot} + H^{+} \quad (5)$$

Based on these theories, total body irradiation-induced ferroptosis involves the process of non-enzymatic lipid peroxidation. Taking into account these theories and results, total body irradiation-induced ferroptosis may include both non-enzymatic and enzymatic lipid peroxidation. Ferrostatin-1 decreased the level of lipid peroxidation (Figure 3(A)) by trapping phospholipid ROS with its arylalkylamine group (Skouta et al. 2014).

In addition to factors that induce lipid peroxidation, the dysfunction of the lipid peroxidation repair network is also a key for the generation of ferroptosis. The lipid peroxidation repair network for ferroptosis mainly consists of GPX4, glutathione, and CoQ10, which belong to three categories: antioxidant enzyme, water-soluble small-molecular antioxidant, and lipid-soluble antioxidant. GPX4 enzymatically suppresses the formation of phospholipid hydroperoxides of ferroptosis and protects membrane fluidity. Ferrostatin-1 mitigated lipid peroxidation in the irradiated-induced ferroptosis of BMMCs by increasing the level of GPX4 (Figures 3(A) and 4(A)). This is consistent with a previous study, which found that ferrostatin-1 inhibits erastin-induced ferroptosis in HFL-1 cells by enhancing GPX4 expression (Gong et al. 2019). Glutathione is regulated by a cystine-glutamate antiporter and is a water-soluble small-molecular antioxidant in the cytoplasm. Although ferrostatin-1 did not affect the level of glutathione, glutathione was found to be depleted during irradiated-induced ferroptosis (Figure 4(B)), which suggests that the cystine-glutamate antiporter of BMMCs may be inhibited. This result is supported by a previous study in which gamma radiation was found to decrease the level of glutathione in the liver and spleen of mice (Ran et al. 2014). CoQ 10, located on the mitochondrial membrane and reduced by ferroptosis suppressor protein 1, is a lipophilic antioxidant that suppresses lipid peroxidation and ferroptosis by scavenging $LOO^{\cdot}$ (Ursini and Maiorino 2020). Our work demonstrated that CoQ 10 did not participate in the mitigation of lipid peroxidation that occurred in total body irradiation-induced ferroptosis (Figure 4(C)). Based on these results and the locations of glutathione and CoQ 10, the intensity of lipid peroxidation in the mitochondrial membrane is likely to be lower than that in the cytoplasm of BMMCs following the onset of total body irradiation-induced ferroptosis.

No increase in inflammatory factors was observed in the bone marrow on the 4th day post-irradiation (Figure 5(A–C)), suggesting that total body irradiation-induced ferroptosis in the bone marrow does not cause the proinflammatory effect at the early stage of gamma radiation. Serum IL-6 and IL-1β decreased on the 4th day post-irradiation (Figure 5(B, C)), which could be attributed to the inhibition of inflammatory cells caused at the early stage of radiation exposure. Li et al. (2019) found that ferroptosis in radiation-induced lung fibrosis results in the upregulation of serum inflammatory factors on days 15, 30, and 60 post-irradiation. This difference in the excretion of serum inflammatory factors may be attributed to the later occurrence of infectious symptoms during hARS, which is due to the reduction in the number of white blood cells.

In summary, an increase in the level of iron and a decrease in the levels of ASCL4 and lipoxygenase 15, together with radiation-induced lipid hydroperoxide, resulted in a burst of both enzymatic and non-enzymatic lipid peroxidation, which subsequently induced ferroptosis in BMMCs. In addition, the imbalance in the lipid peroxidation repair network (i.e. decreased levels of GPX4 and glutathione) further exacerbated the lipid peroxidation. Ferrostatin-1 mitigated total body irradiation-induced ferroptosis in BMMCs by attenuating the increased levels of hemosiderin and LIP and the decreased levels of ASCL4 and GPX4, which subsequently increased the counts of red and white blood cells, lymphocytes, and monocytes in the peripheral blood of mice. This study provides an initial investigation into the mechanisms of ferrostatin-1 as a radiation mitigation agent in mice. Future works should focus on the effects of ferrostatin-1 on radiation mitigation in primates and humans.

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Notes on contributors

Xiaohong Zhang, PhD. She is a senior experimentalist of the Department of Nuclear Science and Technology, Nanjing University of Aeronautics and Astronautics. Her research interests are in the areas of radiation biology and biodosimetry.

Mengxin Tian, MD. She is a postgraduate of the Department of Nuclear Science and Technology, Nanjing University of Aeronautics and Astronautics. Her research interests are in the area of radiation biology.

Xin Li, MD. She is a postgraduate of the Department of Nuclear Science and Technology, Nanjing University of Aeronautics and Astronautics. Her research interests are in the area of radiation biology.

Chunyan Zheng, MD. She is a postgraduate of the Department of Nuclear Science and Technology, Nanjing University of Aeronautics and Astronautics. Her research interests are in the area of radiation biology.

Ailian Wang, MD. She is a chief physician of the Department of ophthalmology, the First Affiliated Hospital, Bengbu Medical College. Her research interests are in the area of ferroptosis.

Jundong Feng, PhD. She is an associate professor of the Department of Nuclear Science and Technology, Nanjing University of Aeronautics

and Astronautics. Her research interests are in the area of space radiation protection.

Xiaodan Hu, PhD. She is an associate professor of the Department of Nuclear Science and Technology, Nanjing University of Aeronautics and Astronautics. Her research interests are in the areas of radiation protection and degradation.

Shuquan Chang, PhD. He is the head of the Department of Nuclear Science and Technology, Nanjing University of Aeronautics and Astronautics. His research interests are in the area of radiation protection.

Haiqian, Zhang, PhD. He is a professor of the Department of Nuclear Science and Technology, Nanjing University of Aeronautics and Astronautics. His research interests are in the areas of radiation biology and biodosimetry.

ORCID

Xiaohong Zhang http://orcid.org/0000-0003-4717-2777

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