

Radioprotective effect of lactoferrin in mice exposed to sublethal X-ray irradiation

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Abstract. The radioprotective effect of lactoferrin (LF) was studied in mice subjected to sublethal X-ray irradiation. The mice were randomly divided into the Control (non-irradiated mice fed a standard diet without LF), IR (irradiated mice fed a standard diet) and IR+LF (irradiated mice fed LF) groups. The mice were fed daily for 7 days prior to irradiation and for 30 continuous days following irradiation. The survival ratio of the mice in the IR+LF group was significantly increased compared with the IR group between days 15 and 30 after irradiation. The body weight of the mice in the IR+LF group was increased compared with the IR group, and the difference was statistically significant. Blood was collected from the mice via the tail vein on days 2, 7, 14, 21 and 30 following irradiation. The laboratory indicators, including leukocyte, erythrocyte and platelet counts recovered more rapidly following irradiation in the IR+LF group compared with the IR group. Treatment of the irradiated mice with LF significantly reduced the DNA damage. In the hepatic tissue the level of superoxide dismutase in the IR+LF group was significantly increased, while malondialdehyde was significantly decreased compared with the IR group. These findings indicate that LF may prevent radiation damage and may have potential as a treatment for patients with cancer who receive radiotherapy.

Introduction

Radiotherapy is a common treatment method for a number of types of human cancer, with approximately half of all patients requiring radiotherapy for palliative or curative purposes (1). However, patients undergoing radiotherapy may develop adverse side-effects, including hematological toxicity, cytopenia, immune suppression and mucosal damage (2). Under ideal conditions, tumor tissue would receive a large dose of radiation, while normal healthy tissues would be protected from radiation injury. Therefore, the pathogenic processes induced by ionizing radiation and non-toxic radioprotective compounds that may protect normal tissues against radiation injury, are currently being extensively researched (3-5). Several compounds, including cysteine, aminothiol and cytokines, are known radioprotectors (6-8). Crescenti *et al* (9) reported that selenium, zinc and magnesium may also have radioprotective properties. Nishimura *et al* (10) reported that chitosan increased the hematocrit and survival rate in mice exposed to sublethal X-ray irradiation. Emami *et al* (11) reported that zinc exerted a protective effect against lethality in irradiated mice.

Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein, which is a component of exocrine secretions, including milk and saliva and is also present in neutrophil granules (12). LF has been reported to serve a role in host defense and has various biological properties, including antimicrobial effects and modulation of cell growth (13,14). In addition to serving a key role in immune homeostasis, LF also reduces oxidative stress and may control excessive inflammatory responses (13,15,16). Recently, Sriramoju *et al* (17) reported that LF exerts various beneficial effects on humans and animals, including inhibition of carcinogenesis and prevention of drug-induced toxicity. Irradiated mice on an LF diet exhibited a significantly higher survival rate compared with mice fed a standard diet (18). The prevention of chemotherapy-induced ovarian disorders in mice receiving oral LF has also been reported (19). In addition, the use of a gel containing LF in patients with oral cancer who were treated with radiotherapy, increased salivary secretion, inhibited xerostomia and improved oral bacterial flora (20).

However, studies on the radioprotective effects of LF are limited. The aim of the present study was to investigate *in vivo*

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whether LF may enhance resistance to high doses of ionizing radiation in mice and to elucidate the possible mechanisms of action. To determine this, the survival ratio and hematopoietic system toxicity in mice receiving whole-body, high doses (7.0 Gy) irradiation were assessed.

Materials and methods

Animals and irradiation. Male Balb/c mice (age, 6 weeks; weight, 20–23 g) were purchased from Unilever (Shanghai, China). All mice had free access to water and food; they were kept in a room maintained at 60±10% relative humidity and 20±2°C with a 12 h light/dark cycle. There were 5 mice per cage. A total of 60 mice were randomly assigned into 3 groups (n=20 per group) as follows: i) Control (non-irradiated mice fed a standard diet without LF); ii) IR (whole-body irradiated mice fed a standard diet without LF); and iii) IR+LF (whole-body irradiated mice fed a diet containing 0.1% bovine LF; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The mice in the IR and IR+LF groups were exposed to a sublethal radiation dose (7.0 Gy). The control mice were sham irradiated. The mice were irradiated using a 6-MV linear accelerator at a dose rate of 0.865 Gy/min (PRIMUS High Energy; Siemens AG, Munich, Germany). The mice were fed for 7 days prior to irradiation and for 30 continuous days following irradiation. The study protocol was approved by the Ethics Committee of Qianfoshan Hospital of Shandong Province (Jinan, China).

Peripheral blood cell counts. Blood was collected from the mice via the tail vein in EDTA tubes (BD Biosciences, Franklin Lakes, NJ, USA) on days 0, 1, 2, 3, 9, 14, 19 and 29 following irradiation. The blood was centrifuged at 1,000 × g for 20 min at 20±2°C and evaluated using an automated hematology analyzer (poch-100i; Sysmex Corporation, Kobe, Japan) to provide the complete blood cell counts. The measurements included leukocyte, erythrocyte and platelet (PLT) counts, as well as the hemoglobin. The normal reference values of hematological parameters were described previously (21).

Lymphocyte isolation and comet assay. A volume of 0.15 ml whole blood was layered onto the lymphocyte separation medium (cat. no. MRGMA0; R&D Systems, Inc., Minneapolis, MN, USA) and centrifuged for 2 min at 3,500 × g at 20±2°C. The lymphocytes were subsequently transferred to a 1.5 ml tube containing 1.2 ml 0.1 M PBS and centrifuged for 5 min at 2,000 × g at 20±2°C. The lymphocytes were washed twice with PBS. The cells were then suspended in PBS and the density was adjusted to 5–6×10⁴/ml. A comet assay was performed under neutral conditions as described by Banath *et al.* (22), with a slight modification. Specifically, special comet slides were used as opposed to conventional slides. All comet images were analyzed using CASP Lab software (version 1.2.3b2; CASPLab, Wroclaw, Poland) (23) and the percentage of DNA in the Olive Tail Moment (OTM) was recorded to characterize the lymphocytic DNA damage.

Biochemical analysis. The livers were removed, fixed in 4% paraformaldehyde solution at room temperature for 20 min and ground 30 days after radiation (5 mice per group). The obtained cells were washed with PBS and suspended in EDTA.

Superoxide dismutase (SOD) and malondialdehyde (MDA) activities in the liver were analyzed using SOD and MDA assay kits (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol.

Statistical analysis. Data are presented as the mean ± standard deviation (≥5 mice per group at each time point). Statistical analysis was performed using one-way analysis of variance with a post hoc Tukey's test (multiple comparison test) to determine the significance of differences among multiple groups. P<0.05 was considered to indicate a statistically significant difference. SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for the analyses.

Results

LF increases the survival rate of mice exposed to irradiation. In the present study, mice in the IR and IR+LF groups were exposed to 7 Gy radiation. The survival rate was monitored on days 1–30 following irradiation (Fig. 1). Kaplan-Meier analysis indicated that survival rates were significantly higher in the IR+LF group compared with the IR group between day 12 and 30 (P<0.05). On day 30 the survival rate of the IR+LF group was 50% and the survival rate of the IR group was 33%. The survival rate in the IR+LF group was significantly higher compared with that of the IR group (P<0.05). The differences between the IR+LF group and the control were also statistically significant (P<0.05). These results suggest that LF increased the survival rate of mice following exposure to radiation.

LF reduces the radiation-induced decrease in body weight. The body weights of the mice were measured at various time points following irradiation and the mean weight ± standard deviation was calculated among surviving mice (Fig. 2). The results revealed that the body weights significantly increased in the control group, remained mostly constant in the IR+LF group and decreased slightly in the IR group between day 8 and 10 after irradiation. Statistical analysis indicated that body weight was significantly higher in the IR+LF group compared with the IR group between days 20 and 30 (P<0.05). Furthermore, the body weights of the mice in the control group were significantly greater compared with the IR+LF group on days 20 and 25 (P<0.05). Furthermore, on day 30, no significant differences in body weight were identified between the control group and the IR+LF group.

LF enhances hematological repopulation following whole-body irradiation. Hematological parameters were recorded following irradiation, including changes in the leukocyte count (Fig. 3). The leukocyte count in the IR+LF group exhibited a progressive decline to 1.9×10⁹/l on day 3. In addition, the leukocyte count appears to stay steady between days 9 and 14 in the IR+LF group at ~2.6×10³/μl. On day 29 the leukocyte count of the IR+LF mice had stabilized to within the normal range (7.6×10⁹–10.9×10⁹/l) (21). The significant difference was identified between the control group and the IR+LF group except on day 29 (P<0.05). However, the leukocyte count of the IR mice remained low (0.35×10⁹/l) until day 14. Between day 9 and 29, the leukocyte counts in the IR group

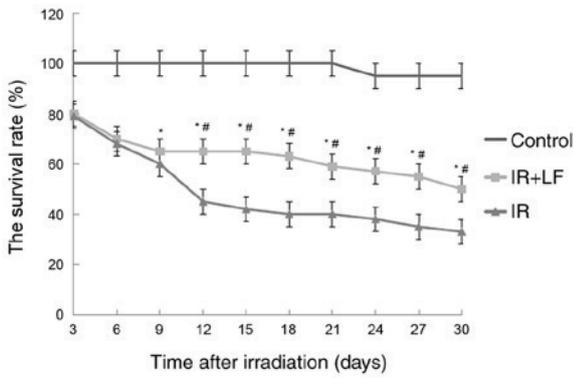


Figure 1. Survival rates of irradiated mice. Each vertical bar indicates the standard deviation. Data are presented as the mean \pm standard deviation (5 mice/group/time point). IR, irradiated mice fed a standard diet; IR+LF, irradiated mice fed lactoferrin. *P<0.05 vs. the control group; #P<0.05 vs. the IR group.

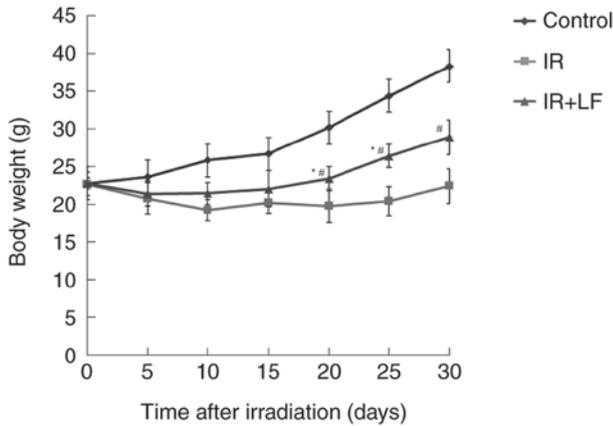


Figure 2. Body weights of irradiated mice. Each vertical bar indicates the standard deviation. Data are presented as the mean \pm standard deviation (5 mice/group/time point). IR, irradiated mice fed a standard diet; IR+LF, irradiated mice fed lactoferrin. *P<0.05 vs. the control group; #P<0.05 vs. the IR group.

were significantly lower compared with the IR+LF group (P<0.05).

In the IR+LF group the erythrocyte count decreased to $4.6 \times 10^{12}/l$ on day 9 and gradually recovered to a value of $6.47 \times 10^{12}/l$ on day 14 (Fig. 4). No significant difference was identified between the control group and the IR+LF group on day 29. In the IR group the erythrocyte count decreased to $2.17 \times 10^{12}/l$ on day 9. From day 9, the erythrocyte count in the IR+LF group was significantly greater compared with the IR group (P<0.05). The control group was significantly greater compared with the IR group between day 3 and 29 (P<0.05). These results indicate that LF improved erythrocyte repopulation in the mice.

Following a decrease post irradiation, the PLT count in the IR+LF group was restored to within a normal range on day 19 (Fig. 5) (21). However, in the IR group, the PLT count decreased to a minimum value at day 9 and slowly increased to a normal level (21) by day 29. The IR+LF group was significantly greater compared with the IR group between day 1 and 9, and 19 and 29 (P<0.05). The control group was significantly

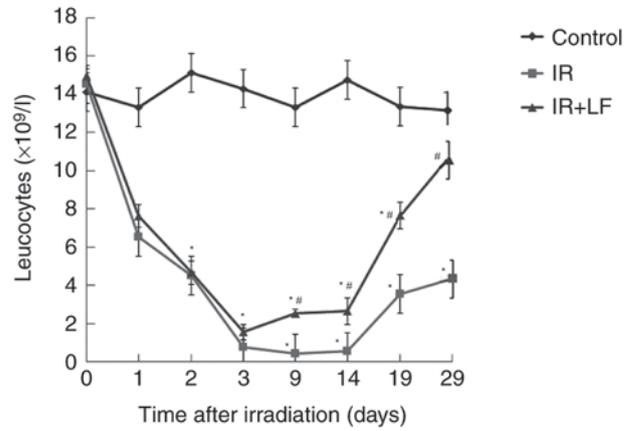


Figure 3. Leukocyte counts of mice following irradiation. Each vertical bar indicates the standard deviation. Data are presented as the mean \pm standard deviation (5 mice/group/time point). IR, irradiated mice fed a standard diet; IR+LF, irradiated mice fed lactoferrin. *P<0.05 vs. the control group; #P<0.05 vs. the IR group.

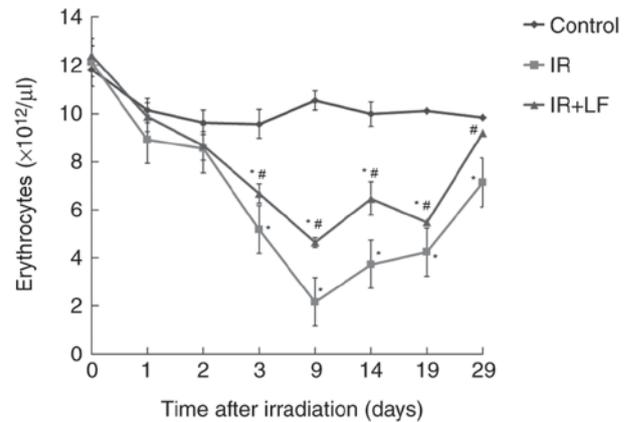


Figure 4. Erythrocyte counts of mice following irradiation. Each vertical bar indicates the standard deviation. Data are presented as the mean \pm standard deviation (5 mice/group/time point). IR, irradiated mice fed a standard diet; IR+LF, irradiated mice fed lactoferrin. *P<0.05 vs. the control group; #P<0.05 vs. the IR group.

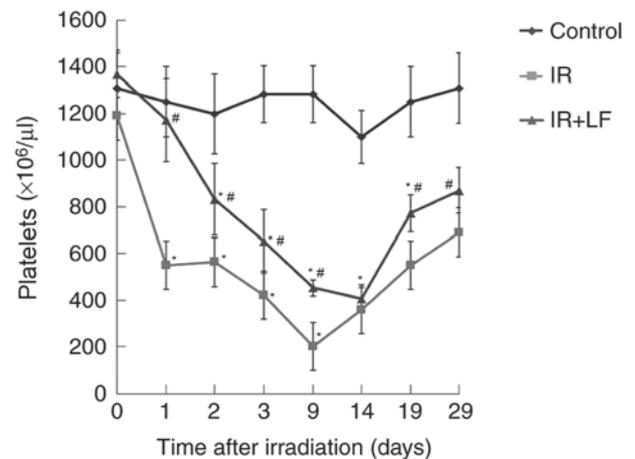


Figure 5. Platelet counts of mice following irradiation. Each vertical bar indicates the standard deviation. Data are presented as the mean \pm standard deviation (5 mice/group/time point). IR, irradiated mice fed a standard diet; IR+LF, irradiated mice fed lactoferrin. *P<0.05 vs. the control group; #P<0.05 vs. the IR group.

Table I. MDA level and SOD activity in hepatic tissue.

Group	SOD (U/ml)	MDA (pmol/l)
Control	41.25±0.41	4.31±0.02
IR	21.52±0.24 ^a	7.31±0.12 ^a
IR+LF	42.56±0.71 ^b	4.98±0.42 ^b

^aP<0.05 vs. the Control. ^bP<0.05 vs. the IR group. Data are presented as the mean ± standard deviation. n=5 per group. IR, irradiated mice fed a standard diet; IR+LF, irradiated mice fed lactoferrin; SOD, superoxide dismutase.

greater compared with the IR and IR+LF group (P<0.05). No significant difference was identified between the control group and the IR+LF group on day 29. These results indicate that LF improved PLT repopulation in the mice.

The results also demonstrated that IR induced a significant decrease in the level of hemoglobin between days 7 and 21 following irradiation. Post irradiation, the hemoglobin levels in the IR+LF and IR groups were significantly lower compared with the control group (P<0.05; Fig. 6). The hemoglobin level recovered faster and was consistently increased in the IR+LF group compared with the IR group. The hemoglobin levels in the IR+LF group were significantly higher compared with the IR group (P<0.05). These results indicate that LF significantly enhanced the recovery of hemoglobin during the experimental period compared with the IR group.

LF increases antioxidant capacity. The MDA level is associated with lipid peroxidation in the liver (24). The MDA level in hepatic tissue was significantly lower in the IR+LF group compared with the IR group, which suggests that the LF diet prevented hepatic lipid peroxidation (Table I). SOD activity indicates the generation of oxidative stress (25). The protective response to oxidative damage in the liver of IR mice decreased significantly following irradiation compared with the control group. However, the LF diet significantly prevented the change in SOD activity compared with the IR group.

LF decreases the OTM of lymphocytes following irradiation. Irradiation led to the breakage of DNA chains. The OTM percentage 24, 48 and 72 h post irradiation in the IR+LF group was significantly greater and lesser compared with the control and IR groups, respectively (P<0.05; Fig. 7). Following unwinding, DNA was affected by the electric field in the electrophoresis liquid, forming the distinctive comet tail formation (Fig. 8).

Discussion

A number of substances with radioprotective properties have been previously reported (26). Intraperitoneal injection of purified ginseng extract following 6.5 Gy X-ray irradiation significantly increased the 30 day survival rate in mice (27). In addition, Shigoka extract prepared from *Acatopanax senticosus* was also reported to increase the post-irradiation survival rate in mice (28). The aim of the present study was to demonstrate the protective effects exerted by LF against radiation-induced injury in mice. The results demonstrated

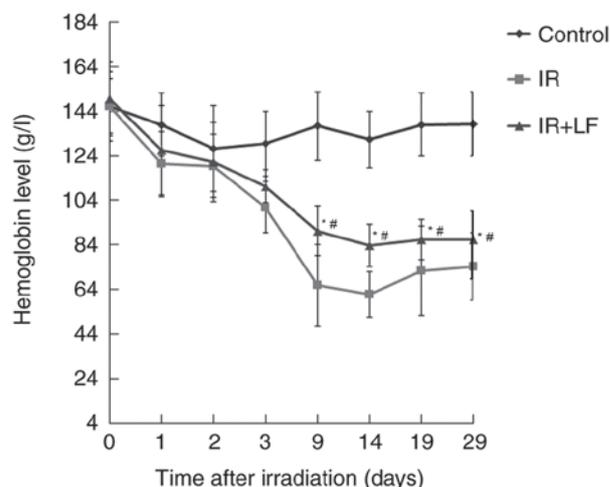


Figure 6. Hemoglobin levels of mice following irradiation. Each vertical bar indicates the standard deviation. Data are presented as the mean ± standard deviation (5 mice/group/time point). IR, irradiated mice fed a standard diet; IR+LF, irradiated mice fed lactoferrin. *P<0.05 vs. the control group; #P<0.05 vs. the IR group.

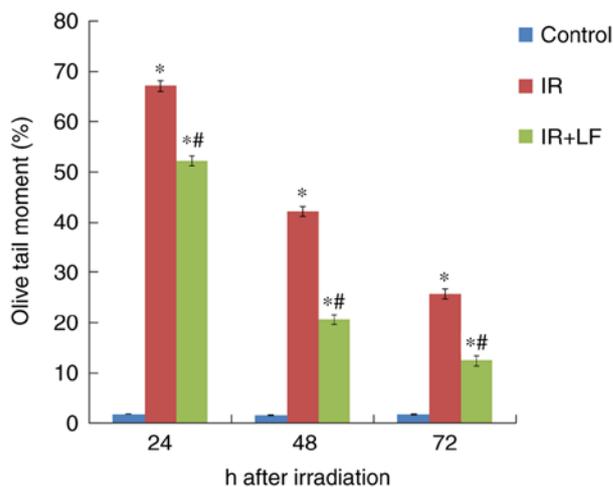


Figure 7. Olive Tail Moment of lymphocytes following irradiation. Data are presented as the mean ± standard deviation (5 mice/group/time point). IR, irradiated mice fed a standard diet; IR+LF, irradiated mice fed lactoferrin. *P<0.05 vs. the control group; #P<0.05 vs. the IR group.

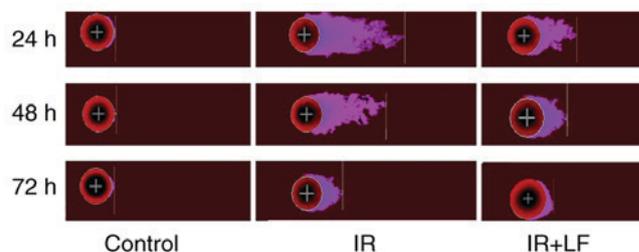


Figure 8. Comet images of lymphocytes following irradiation. Comet images were analyzed by CASPLab software.

that at day 30 following irradiation the survival rate of the mice was 17% higher in IR+LF group compared with the mice in the IR group, demonstrating that an LF diet significantly improves survival rates.

It has been previously established that the survival rate of mice following exposure to a sublethal dose of radiation depends, on the recovery of the hematopoietic system (29,30). To determine whether LF protects mice from IR-induced hematopoietic system injury, the mice were exposed to X-ray irradiation at a dose of 7.0 Gy.

It is known that the number of leukocytes is correlated with the radiation dose (31). The IR+LF group exhibited a rapid increase in the leukocyte count from day 14 onwards and on day 29 the count was restored to normal levels. However, in the IR group, the leukocyte count began to increase at day 14 in the IR group, but the count remained at a lower level. These results indicate that LF stimulated the recovery of leukocytes and exerted a radioprotective effect.

In the IR group the PLT count exhibited an initial decline following X-ray irradiation and on day 9 the count was at its lowest level, however it returned to normal by day 29. The IR+LF group exhibited a faster increase in PLTs compared with the IR group and they recovered to near normal levels at day 19. It has been previously reported that when infants were fed an LF-supplemented infant formula, their hemoglobin value was increased compared with the group fed a conventional infant formula (32); similar results were also observed in female marathon runners (33). In the present study, the red blood cell count and hemoglobin levels were increased in the IR+LF group compared with the IR group following irradiation, which indicates that LF exerted hematopoietic or radioprotective effects.

Radiation may increase the oxidative capacity and induce damage to cellular molecules; previous biochemical studies have been performed to define normal MDA and SOD levels in liver tissue (34-36). The results of the present study revealed that the MDA level in the hepatic tissue was significantly lower in the IR+LF group compared with the IR group, while SOD activity was significantly increased. These results reveal that LF exerted a protective effect on cellular molecules against radiation-induced oxidative damage.

The comet assay, which detects DNA damage, has been widely used in radiation biology (37-40). The comet assay is a rapid and sensitive microdosimetric technique, particularly useful in radiation accidents (41). In the IR+LF and IR groups, the comet assay was used to observe the degree of DNA damage by irradiation. The IR group exhibited a substantial increase in DNA damage, even at 30 days post irradiation, while the IR+LF mice exhibited significantly reduced DNA damage. The present study demonstrated that significant differences were identified between the IR group and IR+LF group following irradiation. Therefore, the comet assay demonstrated that LF effectively reduced radiation-induced DNA injury.

In conclusion, the results of the present study suggest that LF increases PLT and leukocyte counts and reduces DNA damage in mice following high-dose irradiation. In the future LF may have potential as a radioprotector to reduce the adverse effects of radiotherapy. However, the exact mechanism of action of LF has not yet been fully elucidated. Therefore, further studies are required to determine whether radiosensitizing or trapping is involved in this effect and to clarify the value of LF within the field of radiation protection.

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Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

Authors' contributions

LF fed the animals, collected blood from the mice and was a major contributor in the writing of the manuscript. DG performed the irradiation. DPD performed the histological examination. HYD performed the superoxide dismutase and malondialdehyde ELISAs. LQ analyzed the peripheral blood cells. JGL performed the lymphocyte isolation and comet assays. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Qianfoshan Hospital Affiliated to Shandong University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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