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Pre-treatment with rapamycin protects hematopoiesis against radiation injury

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► ***Original article***

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ABSTRACT

Background: Protection of hematopoietic system has become a primary goal in the development of novel medical countermeasures against ionization radiation and radiotherapy. This study was to explore the role of rapamycin in normal tissues against radiation. ***Materials and Methods***: Mice were pretreated with rapamycin by i.p. every other day for five times before 5 Gy or 8.5 Gy γ-ray whole body irradiation. Blood cell counts, HE staining of bone marrow and liver, bone marrow transplantation, CFU of spleen were used to measure the damage of hematopoiesis and extramedullary hemopoietic organs. Regular karyotype analysis and expression of γ-H2AX (by flow cytometry and western blot) were used to measure DNA damage. Rad 50 and DNA Lig 4 expression by western blot were to see the DNA repair ability. ***Results:*** The decrease of red blood cells and platelet induced by radiation were alleviated by pretreatment with rapamycin (d 7,15, $p<0.01$), and the long-term restoration of white blood cells, lymphocytes and bone marrow were enhanced in rapamycin pretreatment group (d 30,40,70, $p<0.05$). The transplantation experiment also indicates that the long-term reconstitution in lethally irradiated recipient mice was improved in rapamycin group ($p<0.05$). The hepatocellular injury by radiation was also reduced and the colony formation numbers of spleen after irradiation was improved in rapamycin group ($p<0.05$). Karyotype analysis indicates that rapamycin protected bone marrow cells from chromosome mutation. Furthermore, expression of DNA repair proteins Rad 50 and DNA Lig 4 was enhanced and DNA damage marker γ-H2AX was reduced in mice exposed to radiation by rapamycin pretreatment. ***Conclusion:*** Rapamycin pretreatment mitigates hematopoietic system from radiation injury in both bone marrow and extramedullary hematopoietic organs by improving genomic stability and increasing survival of hematopoietic stem and progenitor cells (HSPCs).

Keywords: *Rapamycin, hematopoiesis, ionization radiation, radiation injury and protection.*

INTRODUCTION

Exposure to ionizing radiation as the result of nuclear accidents or terrorist attacks is a significant threat and a major medical concern [1,2]. Radiation is also an effective and commonly employed therapy in the management of more than half of human malignancies [3]. Hematopoietic system injury, in particular hematopoietic stem cell (HSC) injury, is the primary cause of death after accidental or intentional exposure to a high dose of ionizing radiation [4,5]. Radiation causes hematopoietic cell senescence, hematopoiesis disorder, and leukemia by inducing ROS production, mitochondrial dysfunction and genomic injury of hematopoietic cells [6-9]. Therefore, protection of hematopoietic stem cells should be a primary goal in the development of novel medical countermeasures against ionization radiation.

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The mammalian target of rapamycin (mTOR) is an important regulator of HSC self-renewal and its overactivation contributes to HSC premature exhaustion in part via induction of HSC senescence [10]. Inhibition of mTOR with rapamycin has the potential to promote long-term hematopoiesis of *ex-vivo* expanded HSCs to facilitate the clinical application of HSC transplantation for various hematologic diseases [11]. mTOR complex 1 (mTORC1) is involved in genomic damage repair, mitochondrion regulation, and inhibition of ROS production by metabolism regulation [12-14]. It's also reported that leukemogenesis is associated with increased activities of mTORC1, and inhibition of mTORC1 by rapamycin might reduce the activity of leukemia cells, especially in PTEN depleted leukemia [15-17]. In addition, mTOR inhibitors were also applied to solid tumor therapy and radiosensitization study of cancer, such as bladder cancer, pancreatic cancer, colon cancer, prostate cancer, and non-small cell lung cancer [18-21]. mTOR inhibitors could enhance the radiosensitization during cancer therapy. Nevertheless, the effect of rapamycin on normal hematopoietic cells against radiation injury remains largely unclear. Currently, the combination of rapamycin with radiotherapy has become a hot topic in tumor therapy study. In this study, rapamycin was administrated to mice before whole body irradiation, and the hematopoietic system damage was examined to study possible radioprotective role of rapamycin.

MATERIALS AND METHODS

Mice in-vivo treatment

6 to 8 week old C57BL/6 male mice weighed 20-25g were obtained from Shanghai SLAC laboratory animal company (Shanghai, China) and divided into non-radiation and radiation group, each with control and rapamycin pre-treatment group. All the mice were fed and housed in the specific pathogen free (SPF) animal facilities of Soochow University. Mice in radiation group were exposed to 5 Gy of ^{60}Co γ ray with 2 Gy/min. Rapamycin group mice were injected with 4 mg/kg rapamycin (Merck Calbiochem, Billerica, MA, USA) (rapamycin was dissolved in absolute ethanol at 10 mg/ml for stock solution and diluted in 5% Tween-80 and 5% PEG-400) i.p. every other day for five times before radiation (described as previously [22]). All experiments with animals were complied with institutional protocols on animal welfare and approved by the Ethics Committee of Soochow University.

Routine blood analysis

Peripheral blood was obtained from tail vein of mice, 20 μl blood was diluted and counted with blood cell counter Sysmex KX21N (Sysmex, Kobe, Japan) for white blood cell (WBC), lymphocyte (LYM), red blood cell (RBC), hemoglobin (HGB) and platelet (PLT) analysis at different time point (0.5, 7, 15, 30, 40, 70 day post radiation), with at least five mice each time point with or without rapamycin treatment.

Liver and sternum HE staining

At different time points after radiation, livers (15th d) and sternum (5th d, 15th d, 30th d) of mice were obtained and fixed in 4% paraformaldehyde overnight for pathological observation with HE stain. Sternums were decalcified in 10% EDTA decalcifying solution (10% EDTA-Na_2 + 1.1% NaOH + 0.8% NaCl + 0.142% Na_2HPO_4) for one week, and then with a series steps of gradient dehydration, embedding, slicing up, dewaxing and rehydration, staining with hematoxylin and eosin, dehydration and mounting for photography with microscope.

CFU-S assay

At 15th day post 5 Gy radiation, spleens of mice were obtained and fixed in Tellyesniczky solution (90 ml 70% ethanol + 5 ml acetic acid + 5 ml 37% methanol) for overnight to form white spot (the white spots on spleen represent colony forming unit of spleen, CFU-S), the white spot of control and rapamycin treatment group was counted and photographed by stereo microscope, each group with at least five mice.

Bone marrow cell isolation and detection

To determine the bone marrow damage

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induced by irradiation and the potential mechanism, DNA double strand break marker γ-H2AX and DNA damage repair protein Rad50 and DNA Lig4 were measured by flow cytomery or western blot at 6 h post irradiation. Chromosome karyotype was analyzed at 30 day post irradiation. At different time points, bone marrow (BM) monocytes which includes most hematopoietic cells were isolated after mice anesthesiaed and killed. BM cells were flushed by a 25-gauge needle from the long bones (tibias and femurs) with HBSS without calcium or magnesium (Invitrogen, life technology, Grand Island, NY, USA). BM monocytes were isolated with Ficoll density gradient centrifugation (bone marrow suspension were added to Ficoll solution at 1:1 ratio and centrifuged at 400 g for 30 min, the middle white layer was extracted as BM monocytes).

For flow cytometry, 5×10^6 BM monocytes were fixed with 4% paraformaldehyde overnight at 4℃, permeabilized with 0.3% Triton X-100 for 30 min at 4℃, blocked with 0.5% BSA for 10 min at room temperature and then incubated with 1:100 Alexa Fluor®488 conjugated γ-H2AX antibody (CST, Danvers, MA, USA) for 30 min and suspended in 400 μl PBS and analyzed with flow cytometry for γ-H2AX level.

For western blotting, cellular proteins were extracted by lysing BM monocytes in extraction buffer (RIPA lysate from CST plus protease inhibitor from Roche, Basel, Swiss). The protein concentration was determined by BCA assay (Perice, Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein (30 μg) were fractionated by electrophoresis in SDS-polyacrylamide gel. The proteins were subsequently transferred to PVDF membranes. Antibodies against γ-H2AX (CST), Rad50 (Abcam, Cambridge, MA, USA), DNA Lig4 and GAPDH (CST) were applied to probe the membranes, respectively. The secondary antibodies (anti-rabbit or anti-mouse, CST) were conjugated to horseradish peroxidase. Signals were detected using the ECL system (Biological industries, Kibbutz Beit-Haemek, Israel).

For chromosome karyotype analysis, BM monocytes were treated with 0.1 μg/ml colchicine for 2 h, 0.075 M hypotonic KCl at 37°C for 20 min, fixed with methanol and acetic acid (3:1) for 20 min, flaked in slides and stained with Giemsa for microscopic observation.

Survival time and bone marrow transplantation

Mice were treated with or without rapamycin (4 mg/kg) by i.p. every other day for five times and then were given 8.5 Gy whole body irradiation, the median survival time of the mice were recorded (control group with 8 mice and rapamycin treatment group with 9 mice).

For transplantation experiment, bone marrow cells from control and rapamycin-treated mice (4 mg/kg by i.p. every other day for five times) at 4 h post 5 Gy irradiation were isolated from the long bones of tibias and femurs and then injected to lethally irradiated mice (10 Gy) by i.v. ($1*10^6$ cells per mouse), the median survival time of the lethally irradiated mice were recorded (control group with 5 mice and rapamycin treatment group with 6 mice).

Statistical analysis

The data are presented as Mean values ±SD from different mice of each group. Statistical analysis are performed using GraphPad Prism 5 Software. Error bars represent SD and p values calculated with a two-tailed Mann–Whitney test unless stated otherwise. (ns means no significance, *p<0.05, **p<0.01, ***p<0.001)

RESULTS

Rapamycin mitigates peripheral blood cell loss by irradiation

To test whether pretreatment of rapamycin reduces the damage on hematopoietic cells of mice exposed to ionizing radiation, mice were pretreated with rapamycin prior to 5 Gy whole-body γ-ray irradiation. All mice survived after 5 Gy irradiation. Peripheral blood cell counting was conducted sequentially at different time points (Day 0, 0.5, 7, 15, 30, 40, 70 post irradiation). Results showed that before radiation, white blood cells (WBC) and lymphocytes (LYM) numbers decreased but red

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blood cells (RBC) number and hemoglobin (HGB) level increased in rapamycin group compared with control (P<0.05), revealing an immunosuppressive role of rapamycin in the absence of irradiation (figure 1A-D). WBC and LYM began to decline sharply at 0.5th day post radiation, and began to restore at 15th day post radiation. WBC and LYM restored faster in rapamycin pretreatment group and almost reached to normal level at 70th day post radiation, with significant difference of control group (Day 30, 40, 70, P<0.05) (figure 1A and B). RBC and HGB level declined from the 7th day post radiation, possibly due to its tolerance, to the lowest at 15th day after radiation; pretreatment of rapamycin showed a protective role, manifested by its slowing down the decrease of RBC and HGB level, with significant difference at day 7, 15 from the control (P<0.05) (figure 1C and D). Platelet number was higher in rapamycin treatment group than the control in the majority of the time course (figure 1E). These results showed a protective role of rapamycin pretreatment post radiation, especially in the restoration of peripheral blood cells.

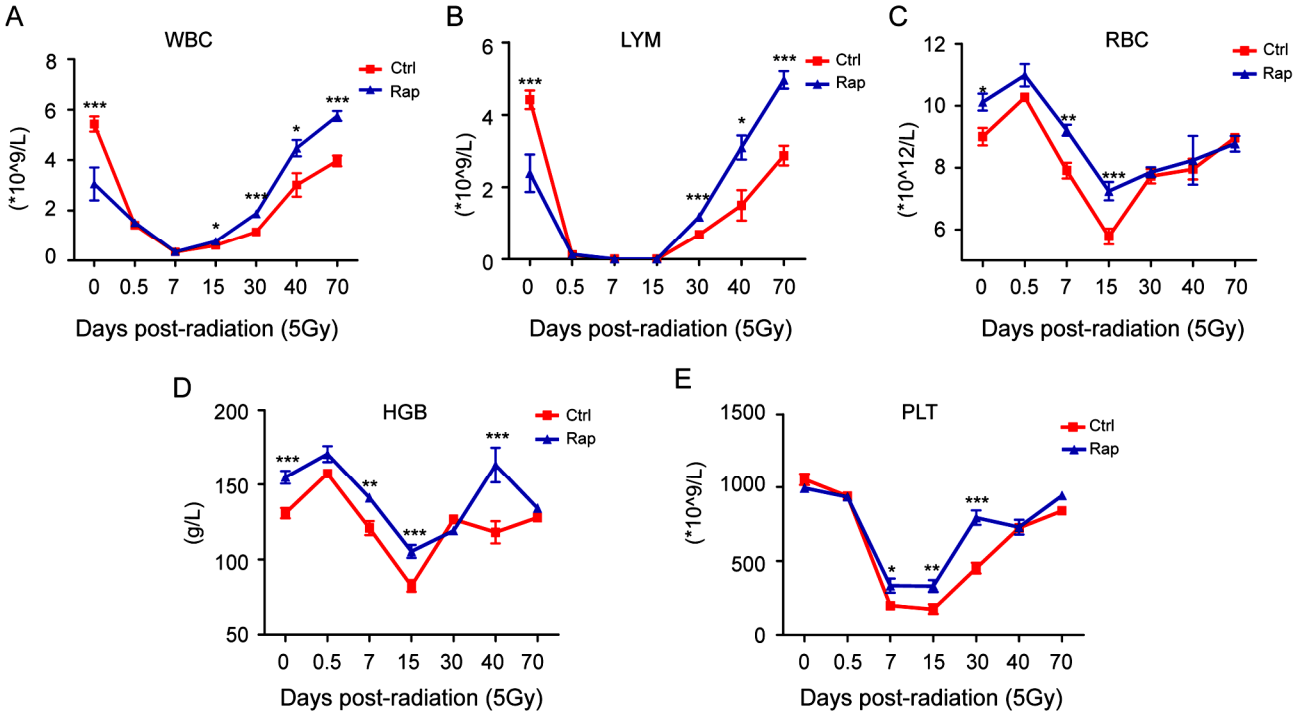

Figure 1. Rapamycin pretreatment mitigates peripheral blood cell loss by radiation.
Mice in control and rapamycin treatment group (n=5) received 5 Gy of γ ray radiation of whole body irradiation. Routine blood counting was conducted at day 0, 0.5, 7, 15, 30, 40, 70 after radiation) of WBC (A), LYM (B), RBC (C), HGB (D) and PLT (E). The results were shown as Means ± SD between rapamycin pretreatment groups with control group. *P＜0.05，**P＜0.01，***P＜0.001.

Rapamycin alleviates bone marrow damage by irradiation

Bone marrow is the main hematopoietic organ in mammalian adults. Bone marrow cell damage induced by radiation will jeopardize the peripheral blood cell number and long term restoration. To examine the bone marrow damage in irradiated mice with or w/o rapamycin pretreatment, bone marrow histological changes were measured by HE staining. Sternum from untreatment and radiation-treatment groups with or w/o rapamycin at day 5th, 15th and 30th post radiation were stained by HE to analyze the distribution and ratio of nucleated cells, mature red blood cells as well as the change of vessels. Results showed that before radiation, there was no difference on the histological change of bone marrow between rapamycin treatment and control. After radiation, nucleated cells

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decreased and red blood cells increased at 5th day, and the distribution of red blood cells became irregular due to the destruction of vessels. At 15th day post irradiation, nucleated cells were recovered, red blood cells were decreased, and vessels were reconstructed; however, the recovery of nucleated cells in control group was slower than the rapamycin-treatment group, and what is more, malignant erythropoiesis was found in the control group, especially in day 30 post radiation, but no malignant erythropoiesis was found in rapamycin pretreatment group and the nucleated cells recovered almost to normal (figure 2), which suggesting that rapamycin protected the bone marrow from radiation damage, and relieved the hematopoietic depression.

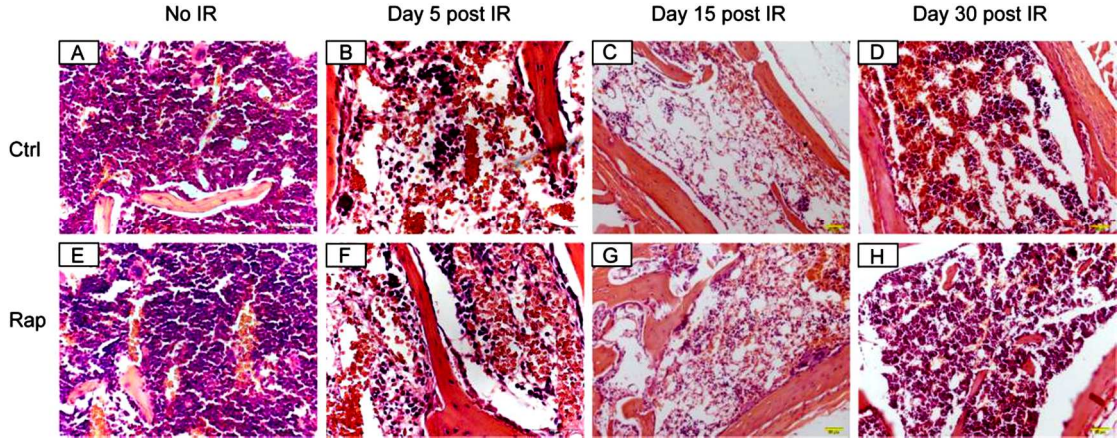

Figure 2. Rapamycin pretreatment alleviates bone marrow damage by radiation.
HE staining was performed to examine the effect of rapamycin on radioprotection. A, B, C and D showed the HE staining of the sternum in control group; E, F, G and H showed the HE staining of the sternum in rapamycin treatment group. A) and E): No IR (400×); B) and F): Day 5 post 5 Gy radiation (400×); C) and G): Day 15 post 5 Gy radiation (400×); D) and H): Day 30 post 5 Gy radiation (400×).

Rapamycin moderates extramedullary hematopoietic organ damage by irradiation

Liver and spleen are main extramedullary hematopoietic organs which serve as a compensatory hematopoiesis when bone marrow hematopoiesis fails or gets insufficient. To observe whether pretreatment of rapamycin mitigates the damage of liver and spleen caused by radiation, liver and spleen from each group were obtained at 15th day post 5 Gy radiation with or w/o rapamycin treatment. Livers were stained by HE to analyze the histological change. Spleens were immersed into tellyesniczky solution to count the white spot which indicates endogenous colony formation ability (CFU-S). The results showed that in the non-irradiation group, rapamycin showed no effect in the histological change compared with its control. In contrast, 15 days after radiation, severe hepatocyte hydropic degeneration was observed in radiation control group, but the histological degeneration was alleviated in the rapamycin treatment group (figure 3A). In CFU-S assay, results showed that at 15th days post 5 Gy radiation, the white spots of spleen (representing the colony formation ability of spleen) was less and smaller in the control group than rapamycin treatment group (figure 3B). These results indicate that rapamycin protects the extramedullary hematopoiesis capability in liver and spleen from radiation. The CFU-S assay determines the hematopoietic potential of survived hematopoietic stem/ progenitor cells (HSPCs) in spleens after irradiation [23, 24]. There is no colony formation in non-irradiated mice neither in control nor rapamycin group (data not shown), the colony numbers increased in rapamycin pretreatment group compared with control after irradiation, indicating that rapamycin decreased the damage of HSPCs and increased the survival of HSPCs.

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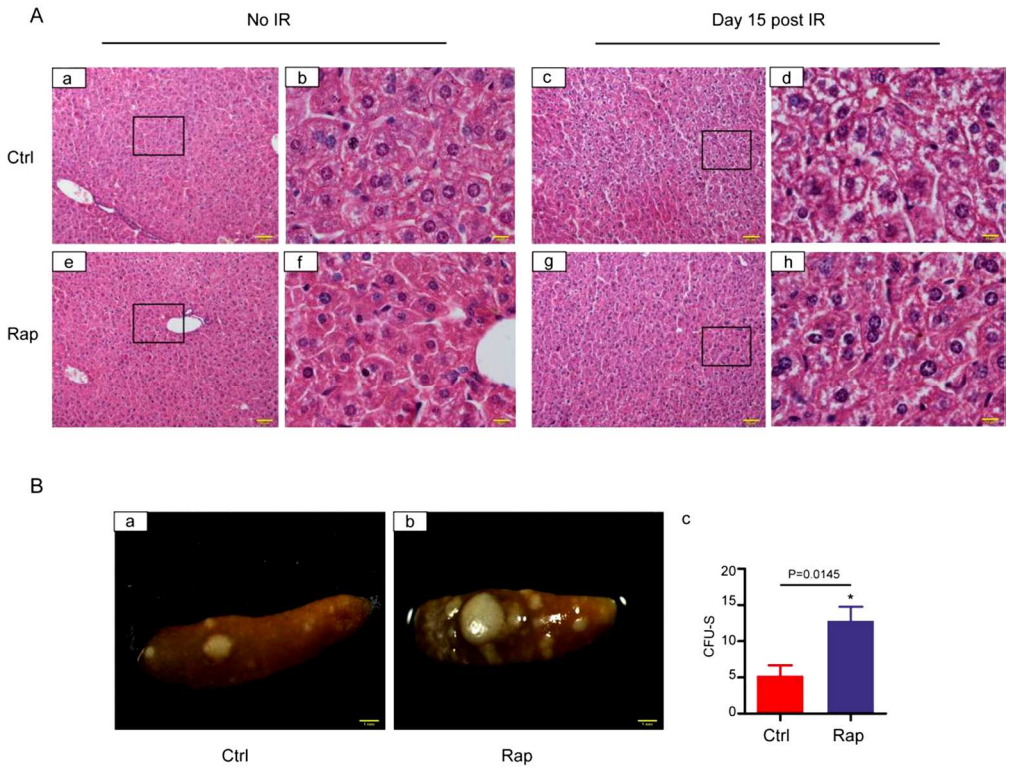

Figure 3. Rapamycin pretreatment moderates extramedullary hematopoietic organ damage by irradiation.
Histological change of livers at 15 day post 5 Gy radiation group with or without rapamycin pretreatment. a) and b): No radiation in control group; c) and d): Day 15 post 5 Gy radiation in control group; e) and f): No radiation in rapamycin treatment group; g) and h): Day 15 post 5 Gy radiation in rapamycin treatment group. a,c,e and g: ×100; b,d,f and h: ×400. (B) Colony formation unit of spleen (CFU-S) at day 15 post 5 Gy radiation in control and rapamycin group. a): CFU-S of mice in control group at day 15 post 5 Gy radiation. b): CFU-S of mice in rapamycin pretreatment group at day 15 post 5 Gy radiation. c): Statistical analysis of colonies in control and rapamycin groups. *P＜0.05.

Rapamycin protects bone marrow cell genome integrity against radiation injury

DNA damage marker γ-H2AX and DNA repair protein Rad50/DNA Lig4 was measured at 6 hours post radaiton by flow cytometry and western blot with or w/o rapamycin treatment. Bone marrow cell karyotype was analyzed at 30th day post radiation. The flow cytometry results showed that at 6 hours post 5 Gy radiation, the level of γ-H2AX which representes DNA damage increased in control group, but decreased in rapamycin pretreatment group (figure 4A and B), which is consistent with the increased expression of DNA repair protein Rad 50 and DNA Lig 4 level in western blot (figure 4C). Karyotype analysis showed that at 30th day post radiation, abnormal chromosomes were found in bone marrow cells of the irradiated mice, such as chromosome nick and break in radiation control group; however, no abnormality was found in rapamycin-treatment group (figure 4D). The above data indicates that rapamycin protects bone marrow cells from chromosome mutation and decreases DNA damage of BM cells induced by irradiation.

Rapamycin improves hematopoietic potential in irradiated mice

To test whether pretreatment of rapamycin could protect mice from high dose radiation, mice with or w/o rapamycin treatment (4 mg/kg to mice every other day for five times before radiation) were exposed to 8.5 Gy γ ray radiation, and were raised in specific pathogen free environment to record the survival time of the mice. The results showed that the survival time of mice in control group was 9 days in average after 8.5 Gy whole body irradiation, but the survival time of mice prolonged to 13 days in average in rapamycin treatment group, with

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significant difference (P<0.05) (figure 5A).

To detect whether rapamycin could directly protect the bone marrow hematopoietic stem cells, bone marrow cells from 5 Gy irradiated mice with or w/o rapamycin pretreatment were transplanted to 10 Gy lethally irradiated mice, and the survival time of the recipient mice were recorded. Results showed that the survival time of the recipient mice in control group was 7.5 days in average after 10 Gy whole body irradiation, but the survival time of the recipient mice prolonged to 12.5 days in average in rapamycin treatment group, with significant difference (P<0.05) (figure 5B). These data indicates that the long-term reconstitution of hematopoiesis in mice after radiation injury was improved by pretreatment of rapamycin.

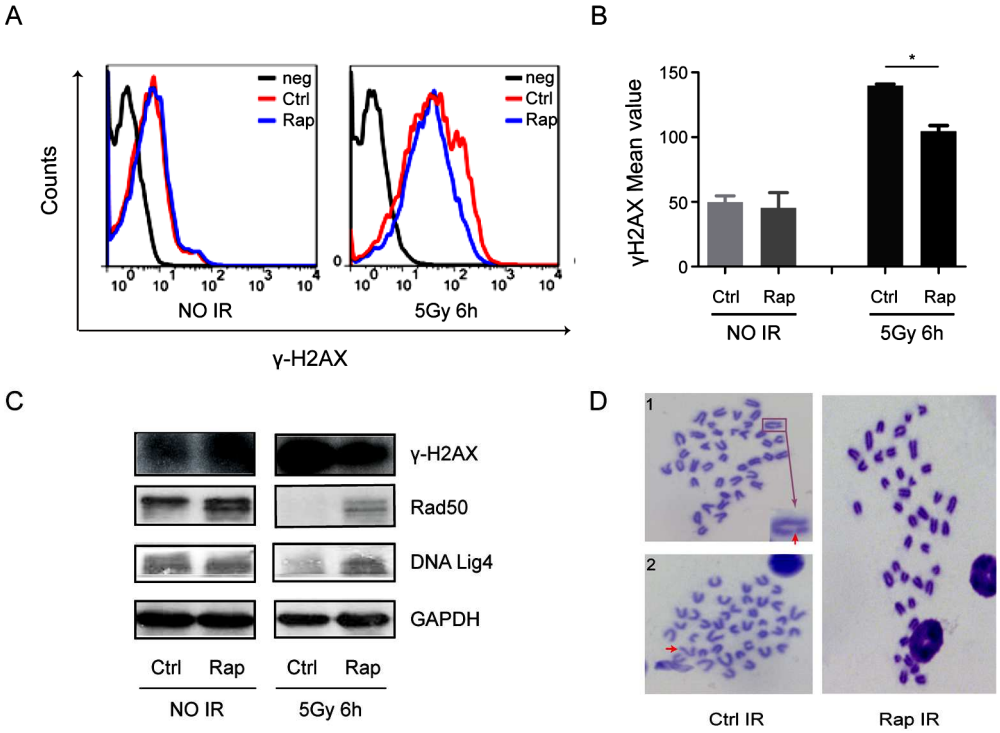

Figure 4. Rapamycin pretreatment protects bone marrow cell genome integrity against radiation injury

A: γ-H2AX expression at NO IR and 6 h after 5 Gy radiation measured by flow cytometry with fluorescent antibody to γ-H2AX. B: Statistical analysis of mean value of γ-H2AX by flow cytometry. C: γ-H2AX, Rad 50 and DNA Lig4 protein expression detected by western blotting antibodies indicated. D: Karyotype analysis of bone marrow cells at 30th day after 5 Gy radiation. The arrow showed chromosome nicks (1) and breaks (2) in the chromosomes of non-rapamycin-treated radiation control group BM cells.

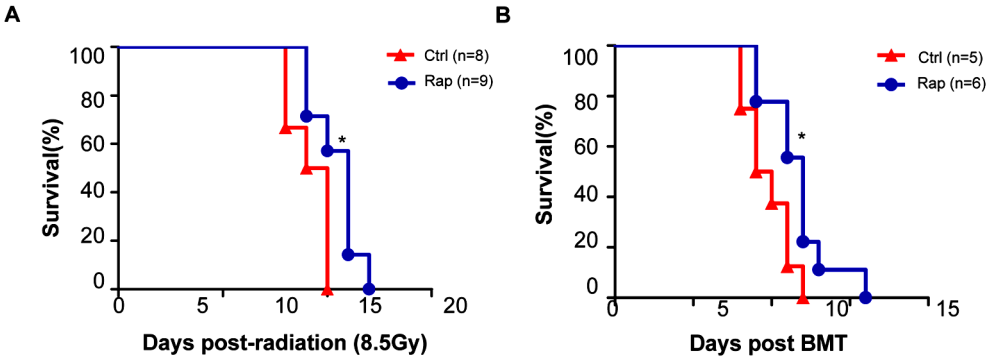

Figure 5. Rapamycin pretreatment improves hematopoietic potential in irradiated mice.

(A) Survival time of the mice receiving 8.5 Gy radiation in control and rapamycin treatment group. (B) Survival time of the lethally irradiated recipient mice post transplantation of bone marrow cells from 5 Gy irradiated control or rapamycin pretreatment donor groups. *P＜0.05.

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DISCUSSION

Rapamycin has been used for many years for immune-suppression in organ transplant to prevent rejection of the organ and recent findings suggest that rapamycin may also be useful in the treatment of leukemia while sparing normal hematopoietic progenitors [23, 24]. Our recent findings also indicate that autophagy mediated by rapamycin may play a protective role against DNA damage repair induced by radiation [25]. To investigate the role of rapamycin in normal tissues against radiation, rapamycin was pretreated to mice to see if it could protect the normal hematopoietic system in vivo of mice exposed to radiation.

The blood cell count results showed that before radiation, WBC and LYM numbers decreased, RBC and HGB level increased in rapamycin group compared with control, revealing an immunosuppressive role of rapamycin in the absence of irradiation. After radiation, different types of blood cells all decreased, but were alleviated in rapamycin pretreatment group, manifested in the lower decrease of RBC and platelet in short term (<30 days) and the long-term (>30 days) restoration of WBC and LYM, indicating the protective role of rapamycin in peripheral blood cells against radiation. The HE stain of bone marrow and liver also suggests the protective role of rapamycin in bone marrow and liver. Bone marrow is the main hematopoietic organ in vivo, and liver and spleen are the main extramedullary hematopoietic organs, indicating that rapamycin protects hematopoietic system against radiation. The CFU-S assay determines the hematopoietic potential of survived hematopoietic stem/progenitor cells (HSPCs) in spleens after irradiation [26, 27]. The colony numbers increased in rapamycin pretreatment group compared with control after irradiation, indicating that rapamycin decreased the damage of HSPCs and increased the survival of HSPCs. The bone marrow transplantation experiment also indicates that rapamycin protected bone marrow HSPCs, and improves the long-term restoration of hematopoietic system after irradiation. The experiment also showed that rapamycin pretreatment in 4 mg/kg dose could protect mice from 8.5 Gy high dose radiation damage.

Bone marrow cell damage level post irradiation is related with DNA damage repair capacity of bone marrow cells. Regulation of DNA damage repair may improve the therapeutic outcome of radiation by differentially targeting HR and NHEJ function in tumor and normal tissues. γ-H2AX, which represents DNA double-stranded breaks [28, 29], increased significantly in 6 hours after irradiation, but much lower in rapamycin pretreatment group. Rad 50 which represents HR pathway [30, 31] and DNA Lig 4 which represents NHEJ pathway [32, 33], increased in rapamycin pretreatment group of 6 hours after irradiation, indicating rapamycin protected mice by enhancing the DNA repair of bone marrow cells.

In summary, our study showed that rapamycin pretreatment protected mice from radiation damage. Pretreatment with rapamycin before radiation could relieve the hematopoietic injury induced by radiation, promote the restoration of peripheral blood cells and bone marrow, and also protect extramedullary hemopoietic organs of liver and spleen, increase the survival of HSPCs, possibly by mitigating injury on chromosome integrity.

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Conflicts of interest*: *Declared none.

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