

Original Article

Ginsenoside Rg1 enhances the resistance of hematopoietic stem/progenitor cells to radiation-induced aging in mice

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Aim: To investigate the effects of ginsenoside Rg1 on the radiation-induced aging of hematopoietic stem/progenitor cells (HSC/HPCs) in mice and the underlying mechanisms.

Methods: Male C57BL/6 mice were treated with ginsenoside Rg1 (20 mg·kg⁻¹·d⁻¹, ip) or normal saline (NS) for 7 d, followed by exposure to 6.5 Gy X-ray total body irradiation. A sham-irradiated group was treated with NS but without irradiation. Sca-1⁺ HSC/HPCs were isolated and purified from their bone marrow using MACS. DNA damage was detected on d 1. The changes of anti-oxidative activities, senescence-related markers senescence-associated β -galactosidase (SA- β -gal) and mixed colony-forming unit (CFU-mix), P16^{INK4a} and P21^{Cip1/Waf1} expression on d 7, and cell cycle were examined on d 1, d 3, and d 7.

Results: The irradiation caused dramatic reduction in the number of Sca-1⁺ HSC/HPCs on d 1 and the number barely recovered until d 7 compared to the sham-irradiated group. The irradiation significantly decreased SOD activity, increased MDA contents and caused DNA damage in Sca-1⁺ HSC/HPCs. Moreover, the irradiation significantly increased SA- β -gal staining, reduced CFU-mix forming, increased the expression of P16^{INK4a} and P21^{Cip1/Waf1} in the core positions of the cellular senescence signaling pathways and caused G₁ phase arrest of Sca-1⁺ HSC/HPCs. Administration of ginsenoside Rg1 caused small, but significant recovery in the number of Sca-1⁺ HSC/HPCs on d 3 and d 7. Furthermore, ginsenoside Rg1 significantly attenuated all the irradiation-induced changes in Sca-1⁺ HSC/HPCs, including oxidative stress reaction, DNA damage, senescence-related markers and cellular senescence signaling pathways and cell cycle, etc.

Conclusion: Administration of ginsenoside Rg1 enhances the resistance of HSC/HPCs to ionizing radiation-induced senescence in mice by inhibiting the oxidative stress reaction, reducing DNA damage, and regulating the cell cycle.

Keywords: ginsenoside Rg1; hematopoietic stem and progenitor cell (HSC/HPC); ionizing radiation; cellular senescence; oxidative stress; DNA damage; cell cycle

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Introduction

With the growing population, aging, extended lifespans, and anti-aging have become popular areas of research in life and social sciences. Studies have shown that tissue-specific stem cells replaces worn out and damaged cells to maintain the internal environment homeostasis of a normal body^[1, 2]. It has been proposed that the aging of an organism is actually due to the senescence of these stem cells. Studies have also shown that hematopoietic stem cell (HSC) senescence is closely relative to aging and senile diseases^[2–4]. Exploring the mechanism

of HSC senescence and anti-senescence provides the foundations for possible methods to delay aging.

Ginseng has been used to enhance stamina and the body's capacity to address fatigue and physical stress for thousands of years in Chinese medical science and is now commonly used around the world. Ginsenoside Rg1 is an active ingredient in ginseng, and this compound plays roles in anti-aging and anti-oxidation, as well as improves immunity and memory^[5, 6].

It has been reported that ionizing radiation causes acute tissue damage and long-term bone marrow injury, including DNA damage, which primarily contributes to the senescence, impaired self-renewal and reduced pluripotency of HSCs^[7, 8]. In the present study, we induced HSC senescence by ionizing

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radiation in an *in vivo* mouse model and investigated the anti-aging mechanism of ginsenoside Rg1 to provide foundations for possible ways to delay aging.

Materials and methods

Animals

Male C57BL/6 mice, 6–8 weeks old, were purchased from the Medical and Laboratory Animal Center of Chongqing and housed in a temperature- and light-controlled room with free access to water and food. All experiments were performed in accordance with the institutional and national guidelines and regulations and approved by the Chongqing Medical University Animal Care and Use Committee.

Ninety-nine mice were randomly divided into three groups: 1) the irradiated+Rg1 group, 2) the irradiated group and 3) the sham-irradiated control group. In the irradiated+Rg1 group and the irradiated group, mice were treated with ginsenoside Rg1 (20 mg·kg⁻¹·d⁻¹, intraperitoneally) or normal saline in the same volume for 7 d, followed by exposure to 6.5 Gy X-ray total body irradiation, which was delivered by a linear accelerator (Philips, SL75-14, UK) at a dose rate of 57.28 Gy/min; the irradiator was placed 75 cm from the target, and an irradiation field of 20 cm×20 cm was used. The interval time between the last injection and irradiation was 24 h. In the sham-irradiated control group, the mice were injected with NS and were not subjected to irradiation.

Reagents

Ginsenoside Rg1 (purity>95%) was purchased from Hongjiu Biotech Co, Ltd (Tonghua, China). IMDM medium, fetal bovine serum (FBS) and equine serum (ES) were purchased from Gibco (CA, USA). The Anti-Sca-1⁺ Micro Bead kit was obtained from Miltenyi Biotech Co (Bergisch Gladbach, Germany), and the SA-β-gal Staining kit was purchased from Cell Signaling (Boston, USA). The CFU-mix culture media were purchased from Stem Cell Co (CA, USA), whereas the SOD and MDA kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The comet assay kit was purchased from Research Bio-Lab Co, Ltd (Beijing, China). Anti-P16^{INK4a} antibody, anti-P21^{Cip1/Waf1} antibody and goat anti-rabbit antibody were obtained from Santa Cruz (CA, USA).

Isolation and purification of Sca-1⁺ HSCs from the mouse bone marrow

The mice were sacrificed by cervical dislocation, and the femurs were collected. A single-cell suspension of the bone marrow was obtained. HSCs positive for stem cell antigen 1 (Sca-1⁺) were isolated and purified by MACs as previously described^[9]. The numbers of Sca-1⁺ HSC/HPCs in each group were analyzed.

Detection of senescence-associated markers in the Sca-1⁺ HSC/HPCs

Senescence-associated β-galactosidase cytochemical staining

The Sca-1⁺ HSC/HPCs were collected on d 7 following TBI, and the senescence-associated β-galactosidase (SA-β-gal)

staining was carried out according to the manufacturer's instructions (Cell Signaling). Briefly, 1×10⁵ purified cells were washed twice with PBS, fixed in Fixative Solution for 10 min at room temperature, and stained with Staining Solution for 12 h at 37°C without CO₂. Approximately 1×10⁴ cells were separated on each slide, and 400 cells were totally analyzed for each group. The percentage of SA-β-gal-positive cells was calculated by counting the number of blue cells under the bright field illumination, and then dividing by the total number of cells.

Mixed colony-forming unit (CFU-Mix) of HSC/HPC culture

The Sca-1⁺ HSC/HPCs were collected on d 7 following TBI. The mixed colony-forming unit (CFU-mix) culture was performed as previously described^[9]. Briefly, 1×10⁴ Sca-1⁺ HSC/HPCs were mixed with 2-mercaptoethanol (1×10⁻⁴ mol/L), 3% L-glutamine, ES, rhEPO, IL-3 and rhGM-CSF in a final volume of 2 mL, and the cells were plated in 96-well plates and grown in a humidified atmosphere with 5% CO₂ at 37°C for 7 d. The number of CFU-Mix represents the pluripotency of the Sca-1⁺ HSC/HPCs.

The detection of SOD activity and MDA content

The Sca-1⁺ HSC/HPC cells (1×10⁶) from each group were collected on d 7 following TBI and lysed in an ice bath for 30 min. The supernatant was collected after centrifugation. SOD activity and MDA content were detected by chemical colorimetric analysis according to the manufacturer's instructions.

Single cell gel electrophoresis (comet assay)

The Sca-1⁺ HSC/HPCs in each group were collected on the d 1 following TBI, and 30 μL of the cell suspension was transferred on agarose-coated slides. The slides were placed in cold lysis buffer for 2 h and then incubated with proteinase K for 2 h. The DNA was denatured by electrophoresis and stained with ethidium bromide. The slides were examined under a fluorescent microscope. The comet parameters including the olive tail moment, and the tail lengths were measured and analyzed using the Comet A software to evaluate DNA damage.

RNA extraction and semi-quantitative RT-PCR

The Sca-1⁺ HSC/HPCs (1×10⁶) from each group were collected on d 7 following TBI. Total mRNA was extracted using the TRIzol Reagent (TaKaRa, Japan), according to the manufacturer's protocol. The OD₂₆₀/OD₂₈₀ ratio of the RNA was between 1.8 and 2.0. Changes in expression were verified by RT-PCR for p16^{INK4a} and p21^{Cip1/Waf1}, which play important roles in cell cycle regulation. The unique PCR primers are shown in Table 1. First-strand cDNA was performed using RT (TaKaRa, Japan). The DNA was amplified by an initial incubation at 94°C for 2 min, followed by 35 cycles of 94°C denaturation for 30 s, annealing at 58°C for 40 s, and 72°C extension for 40 s. GAPDH served as an internal standard. The PCR products were separated by electrophoresis in a 1% agarose gel. The optical intensities were quantified using Quantity One (Bio-Rad). The relative expression levels were calculated as

Table 1. Primers sequences.

Primer	Forward (5'-3')	Reverse (5'-3')	Size (bp)
p16 ^{INK4a}	TCCGCTGCAGACAGACTGGCCAG	CATCGCGCACATCCAGCCGAGC	295
p21 ^{Cip1/Waf1}	ATTCTGGTGATGTCGGACC	AAAGTCCACCGTTCTCGG	144
gapdh	GTGCTGAGTATGCTGGAGTCT	GAGTGGGAGTTGCTGTTGAAGT	602

the ratio of the optical intensity of the target gene to that of GAPDH.

Western blotting analysis

The Sca-1⁺ HSC/HPCs in each group were collected on d 7 following TBI. Total protein was extracted, and the concentration was measured using the BCA procedure. Samples containing 50 µg of protein were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with the P16^{INK4a} antibody or P21^{Cip1/Waf1} antibody at a 1:400 dilution. The secondary antibody was diluted 1:5000 in TBST. The membranes were visualized using the enhanced chemiluminescence detection system (Pierce). The level of β-actin was used as an internal control. The relative intensities were quantified using Quantity One (Bio-Rad).

FCM measurement of cell cycle

The Sca-1⁺ HSC/HPC cells from each group were collected on d 1, d 3, and d 7 following TBI and were fixed with 70% ice-cold ethanol overnight. The fixed cells were then incubated with 100 µL of bovine pancreatic ribonuclease (1 mg/mL) at 37 °C for 30 min, followed by incubation with PI (1 µg/mL) for another 30 min in the dark. The PI fluorescence was excited at a wavelength of 488 nm, and the emission was detected at 610 nm. The PI fluorescence of individual nucleus was measured by flow cytometry (FCM, FACS Vantage SE, BD), and ≥2×10⁴ cells were analyzed for each sample. The proportions of the cells in each cell cycle phase were determined using the PC-Lysys II software.

Statistical analysis

The data were analyzed by ANOVA using the SPSS version 13.0 software and represented as mean±SD. Differences were considered significant if *P*<0.05.

Results

The effect of ginsenoside Rg1 on the number of Sca-1⁺ HSC/HPCs

TBI was carried out on d 0. After the Sca-1⁺ HSC/HPCs were isolated and purified from mouse bone marrow cells by MACS, the purity of the cells was found to be 93.66%, and the survival of the separated cells was 99.4% according to the Trypan blue dye exclusion assay. In the irradiated group, the number of Sca-1⁺ HSC/HPCs was violently reduced on d 1, bottomed out on d 3, and had barely recovered by d 7, compared to the sham-irradiated control group. However, in the irradiated+Rg1 group, the number of Sca-1⁺ HSC/HPCs also

violently decreased 1 d after irradiation, but began to notably recover on d 3 and had remarkably recovered by d 7 (Figure 1). Moreover, there were no notable differences between the groups before TBI (d 0).

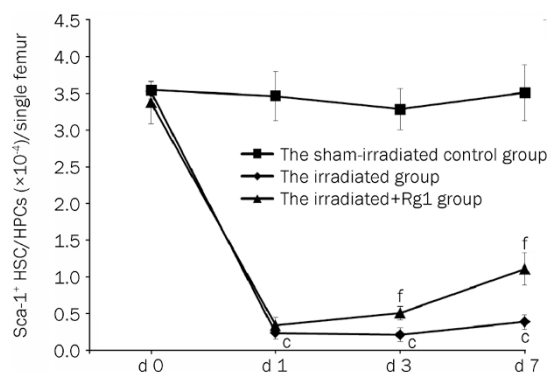


Figure 1. The effect of ginsenoside Rg1 on the number of Sca-1⁺ HSC/HPCs per femur of irradiated mouse (d 0 *n*=3, others *n*=10). Mice were excuted at the desired time. The femurs were collected and the Sca-1⁺ HSCs were isolated. The number of the HSC/HPCs in each group were analyzed. Data in d 0 represents cells collected before TBI. ^o*P*<0.01 vs the sham-irradiated control group. ^f*P*<0.01 vs the irradiated group.

The effect of ginsenoside Rg1 on the senescence of purified Sca-1⁺ HSC/HPCs from irradiated mice

SA-β-gal is one of the most widely used biomarkers for aging cells^[10]. Therefore, we performed SA-β-gal staining to investigate the effect of Rg1 on irradiation-induced Sca-1⁺ HSC/HPC senescence. As shown in Figure 2A, the aged cells are stained in blue in the cytoplasm. Moreover, the capacity to form CFU-Mix can evaluate the senescence of HSCs, and this ability decreased with the senescence of the HSCs. Therefore, we also performed CFU-mix culture and counted the numbers of mixed hematopoietic progenitor colonies (Figure 2B).

The Sca-1⁺ HSC/HPCs were collected on d 7 following TBI. Irradiation induced a remarkable increase in the percentage of cells stained positive for SA-β-gal (Figure 2A, Table 2) and a significant decrease in the number of CFU-mix formed by the Sca-1⁺ HSCs, compared to the sham-irradiated control group (Figure 2B, Table 2). However, in the irradiated+Rg1 group, the proportion of SA-β-gal positive HSC/HPCs was significantly reduced (Figure 2B, Table 2) and the number of CFU-mix was remarkably increased, as well as the number of colony forming cells, compared to the irradiated group (Figure

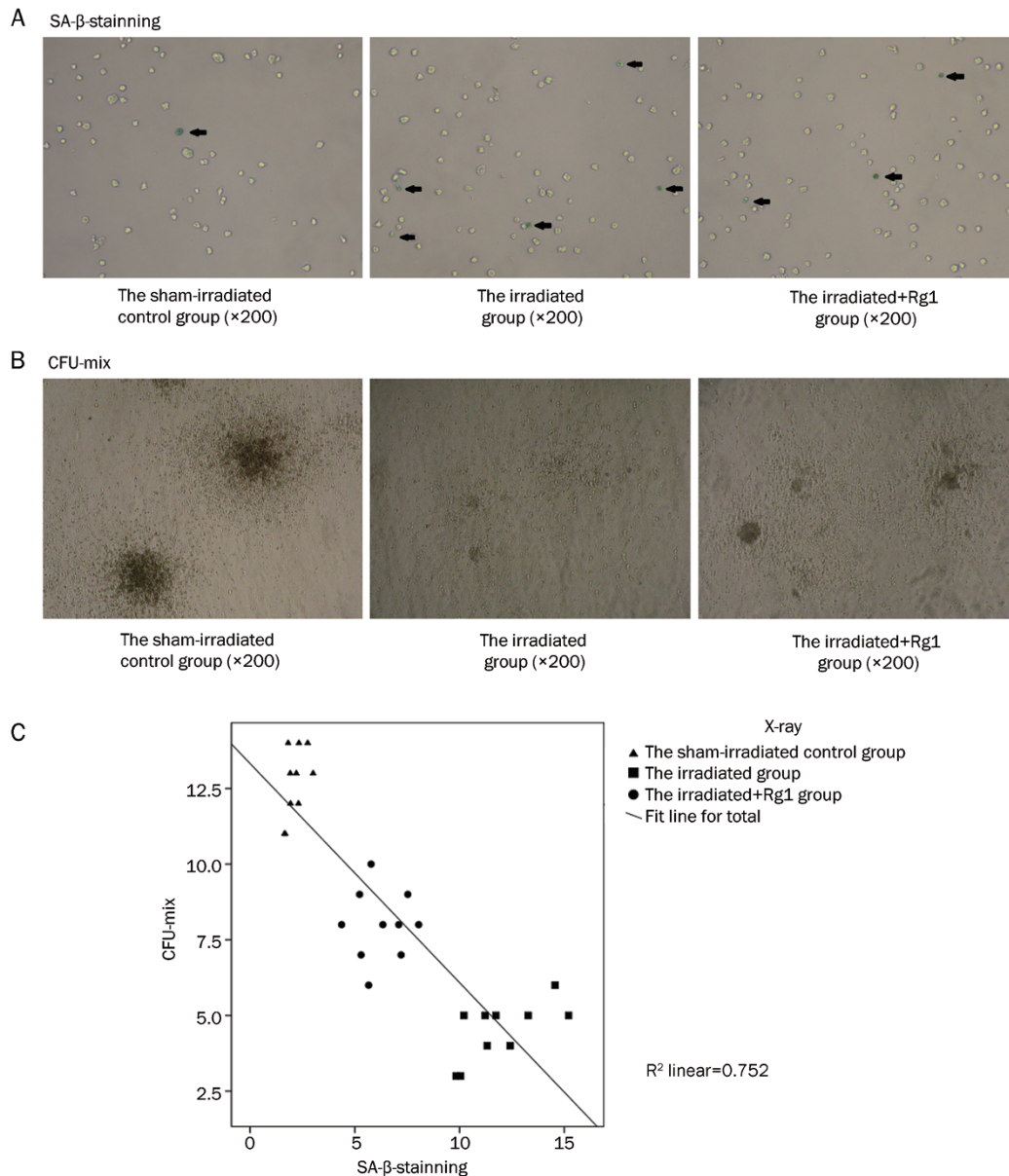


Figure 2. The effect of ginsenoside Rg1 on the Sca-1⁺ HSC/HPCs senescence of irradiated mouse. The Sca-1⁺ HSC/HPCs were collected on d 7 following TBI. (A) The senescence-associated β -galactosidase (SA- β -gal) staining was carried out. The aged cells are stained in blue in the cytoplasm (arrow). (B) The capacity of Sca-1⁺ HSC/HPCs to form hematopoietic progenitor colonies were evaluated by CFU-mix culture. (C) The correlation analysis between the percentage of SA- β -gal positive cells and the number of CFU-mix.

Table 2. The effect of ginsenoside Rg1 on SA- β -gal staining and CFU-mix forming of Sca-1⁺ HSC/HPCs in the irradiated mice (mean \pm SD, $n=10$). ^c $P<0.01$ vs the sham-irradiated control group. ^f $P<0.01$ vs the irradiated group.

Group	The percentage of SA- β -gal positive cells (%)	The number of CFU-Mix (per 10 ⁴ Sca-1 ⁺ HSC/HPCs)
The sham-irradiated control group	2.16 \pm 0.45	12.70 \pm 1.16
The irradiated group	11.99 \pm 1.87 ^c	4.50 \pm 0.97 ^c
The irradiated+Rg1 group	6.26 \pm 1.18 ^f	8.00 \pm 1.15 ^f

2B, Table 2). Furthermore, we performed correlational analyses and found a negative correlation existing between the positive rate of SA- β -gal and the number of CFU-mix ($R=0.867$) (Figure 2C).

The effect of ginsenoside Rg1 on the SOD activity and MDA content of the Sca-1⁺ HSC/HPCs from irradiated mice

Superoxide dismutase (SOD) is responsible for the degeneration of reactive oxygen species (ROS), which can cause cells to senesce. Malondialdehyde (MDA), a product of ROS, can be used as an oxidative stress biomarker. By measuring the SOD activity and MDA content of cells, we determined the anti-

oxidant ability and oxidative damage of the HSC/HPCs.

The Sca-1⁺ HSC/HPC cells (1×10⁶) from each group were collected on d 7 following TBI. Compared to the sham-irradiated control group, SOD activity decreased and MDA content increased in the Sca-1⁺ HSC/HPCs in the irradiated group. Meanwhile, Rg1 partially rescued the reduction of SOD activity, leading to a decrease in MDA content in the irradiated+Rg1 group (Table 3).

Table 3. The effect of ginsenoside Rg1 on the activity of SOD and the content of MDA of Sca-1⁺ HSC/HPCs in the irradiated mice (mean±SD, n=10). ^aP<0.01 vs the sham-irradiated control group. ^fP<0.01 vs the irradiated group.

Group	The activity of SOD (U/mL)	MDA (nmol/mg prot)
The sham-irradiated control group	27.19±3.50	2.50±0.48
The irradiated group	19.24±2.52 ^a	6.34±1.07 ^c
The irradiated+Rg1 group	24.48±3.02 ^f	4.21±0.44 ^f

The effect of ginsenoside Rg1 on the DNA damage of Sca-1⁺ HSC/HPCs in irradiated mice

The comet assay was used to determine the DNA damage present in the cells. This damage is mostly expressed by the length of the comet tail and the Olive moment. The Sca-1⁺ HSC/HPCs in each group were collected on the d 1 following TBI. Spherical and intact nuclei from Sca-1⁺ HSC/HPC cells were observed in the sham-irradiated control group, while the nucleus was observed as a bright orange head with a long comet tail in the irradiated group. In the irradiated+Rg1 group, the comet tails were smaller than those of the irradiated group (Figure 3). A computerized system was used to further analyze these samples. As expected, the comet tail length and the Olive moment of the irradiated group were both significantly larger than those of the sham-irradiated control group. Meanwhile, in the irradiated+Rg1 group, the injection of ginsenoside Rg1 resulted in a remarkably smaller comet tail length and the Olive moment than the irradiated group (Table 4).

Table 4. The effect of ginsenoside Rg1 on the DNA damage of Sca-1⁺ HSC/HPCs in the irradiated mice (mean±SD, n=10). ^cP<0.01 vs the sham-irradiated control group. ^fP<0.01 vs the irradiated group.

Group	Tail length (μm)	Olive moment
The sham-irradiated control group	4.42±2.97	1.24±1.10
The irradiated group	90.54±21.67 ^c	53.09±19.11 ^c
The irradiated+Rg1 group	47.21±8.01 ^f	22.15±7.50 ^f

The effect of ginsenoside Rg1 on the expression of P16^{INK4a} and P21^{Cip1/Waf1}

The P16^{INK4a}-retinoblastoma (Rb) pathway and the P19^{Arf}-Mdm2-P53-P21^{Cip1/Waf1} pathway are two main signal transduction pathways involved in cell aging processes. Therefore, we performed RT-PCR and Western blot analyses to explore the mRNA and protein expressions, respectively, of P16^{INK4a} and P21^{Cip1/Waf1}, which are located in the core positions of the two senescence-associated pathways. The expression of GAPDH and β-actin were used as the internal controls for the mRNA and western blot analyses, respectively. The mRNA of Sca-1⁺ HSC/HPCs (1×10⁶) from each group were collected on day 7 following TBI. As shown in Figure 4 and Table 5, both the mRNA and protein expression of P16^{INK4a} and P21^{Cip1/Waf1} were significantly higher in the irradiated group than in the sham-irradiated control group. In the irradiated+Rg1 group, both the mRNA and protein expression of P16^{INK4a} and P21^{Cip1/Waf1} were significantly lower than that of the irradiated group.

The effect of ginsenoside Rg1 on the cell cycle distribution of Sca-1⁺ HSC/HPCs in irradiated mice

Because P16^{INK4a} and P21^{Cip1/Waf1} play roles in cell cycle regulation and because cells are arrested at the G₁ stage when HSCs age^[11], we examined the cell cycle profile of these cells using FACS. The Sca-1⁺ HSC/HPC cells from each group were collected on d 1, d 3, and d 7 following TBI respectively. In both the irradiated group and the irradiated+Rg1 group, the Sca-1⁺ HSC/HPCs displayed a G₁ phase arrest, and the percentages of cells in S and M phases was significantly decreased compared to the sham-irradiated control group on d 1. However, the G₁ arrest was barely overcome in the irradiated group, while the number of irradiated+Rg1 cells arrested at in G₁

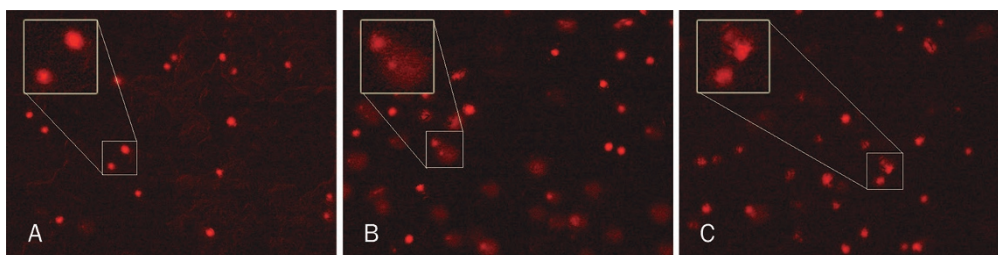


Figure 3. The effect of ginsenoside Rg1 on the DNA damage of Sca-1⁺ HSC/HPCs in the irradiated mice. The Sca-1⁺ HSC/HPCs in each group were collected on the d 1 following TBI. The comet assay was used to determine the DNA damage present in the cells. Enlarged images shown in the left top corner are from the framed region. (A) The sham-irradiated control group (×100). (B) The irradiated group (×100). (C) The irradiated+Rg1 group (×100).

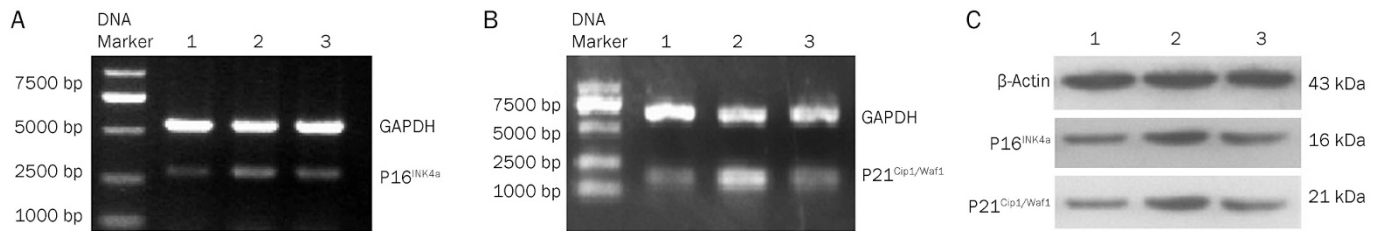


Figure 4. The effect of ginsenoside Rg1 on the mRNA and protein expression of $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ in the irradiated mice. The mRNA of Sca-1⁺ HSC/HPCs (1×10^6) from each group were collected on d 7 following TBI. Gapdh was used as the internal control. The experiments were performed three times with similar results. (A) The sham+irradiated control group. (B) The irradiated group. (C) The irradiated+Rg1 group.

Table 5. The effect of ginsenoside Rg1 on the mRNA and protein expression of $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ in the irradiated mice (mean \pm SD, $n=10$). ^c $P < 0.01$ vs the sham-irradiated control group. ^f $P < 0.01$ vs the irradiated group.

Group	mRNA expression		Protein expression	
	$P16^{INK4a}/GAPDH$	$P21^{Cip1/Waf1}/GAPDH$	$P16^{INK4a}/\beta\text{-actin}$	$P21^{Cip1/Waf1}/\beta\text{-actin}$
The sham-irradiated control group	0.489 \pm 0.032	0.457 \pm 0.032	0.224 \pm 0.013	0.309 \pm 0.014
The irradiated group	0.802 \pm 0.041 ^c	0.789 \pm 0.045 ^c	0.831 \pm 0.037 ^c	0.823 \pm 0.033 ^c
The irradiated+Rg1 group	0.673 \pm 0.033 ^f	0.621 \pm 0.029 ^f	0.613 \pm 0.024 ^f	0.578 \pm 0.027 ^f

phase was significantly reduced on d 3 and d 7 (Figure 5).

Discussion

The effect of ginsenoside Rg1 on the senescence of Sca-1⁺ HSC/HPCs

HSC/HPCs are a type of stem cells that has high self-renewal capacity and multi-lineage differentiation potential. They continuously proliferate and differentiate via unequal division to maintain the homeostasis of the peripheral blood cells. It has been reported that ionizing radiation causes HSCs damage, induces cells senescence and apoptosis, and results in the loss of the hematological-reconstitution function^[7,8].

In this study, the number of Sca-1⁺ HSC/HPCs was violently reduced following exposure to 6.5 Gy X-ray TBI, and these levels barely recovered; however, in the irradiated+Rg1

group, a notable increase in the number of cells was observed on d 3 (Figure 1). This result suggests that ionizing irradiation is fatal to HSC/HPCs, but ginsenoside Rg1 can help restore the HSC/HPCs.

Furthermore, multiple aging indexes were used to evaluate the effect of Rg1 on the senescence of the surviving Sca-1⁺ HSC/HPCs. SA- β -gal, which reflects the function of the lysosomes, accumulates in aging cells as the lysosomes begin to malfunction. The capacity to form CFU-mix colonies decreases with the regression of self-renewal and multi-differentiation potential in aging HSCs and is another widely used biomarker for HSC/HPC senescence^[12].

In the present study, we found that the proportion of SA- β -gal positive HSC/HPCs was significantly increased (Figure 2A, Table 2) and the number of CFU-mix and colony form-

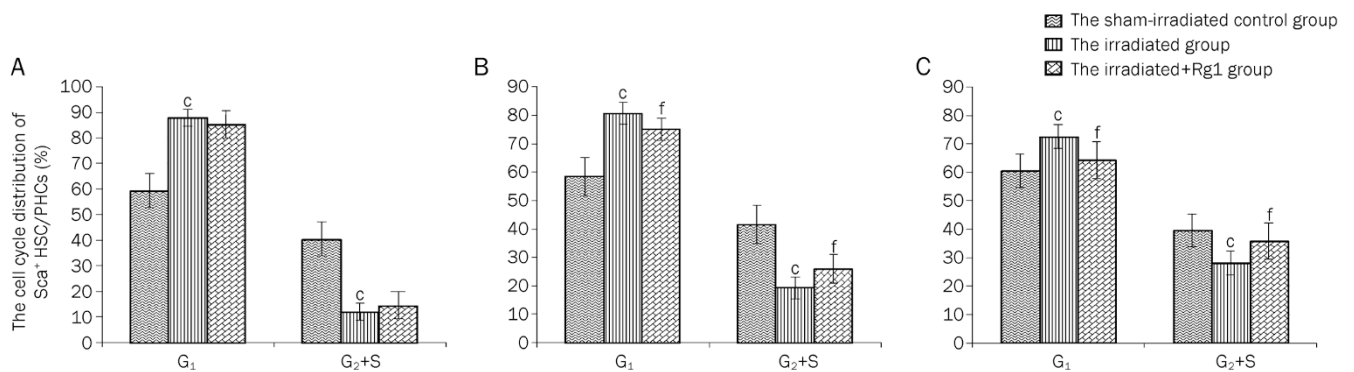


Figure 5. The effect of ginsenoside Rg1 on the cell cycle distribution of Sca-1⁺ HSC/HPCs in the irradiated mice ($n=10$). The Sca-1⁺ HSC/HPC cells from each group were collected on d 1 (A), d 3 (B), and d 7 (C) following TBI. Cell cycle was measured by flow cytometry, and $\geq 2 \times 10^4$ cells were analyzed for each sample. ^c $P < 0.01$ vs the sham-irradiated control group. ^f $P < 0.05$ vs the irradiated group.

ing cells were remarkably reduced in the irradiated group. However, daily injection of ginsenoside Rg1 partially reversed these irradiation-induced, senescence-associated index changes. Further analysis showed a negative correlation existing between the positive rate of SA- β -gal accumulation and the number of CFU-mix cells. These results are consistent with previous studies that showed HSCs senescence is accompanied by a regression of self-renewal and multi-differentiation potential^[12]. These results suggested that ginsenoside Rg1 was able to counteract the persistent senescence of the HSC/HPCs induced by irradiation.

The effect of ginsenoside Rg1 on the oxidative stress and DNA damage of Sca-1⁺ HSC/HPCs in irradiated mice

Irradiation induces an abnormal generation of reactive oxygen species (ROS), which causes intercellular oxidative damage and results in cell senescence. It has been reported that aberrant increases in ROS can disrupt HSC quiescence by stimulating entry into the cell cycle, which comprises the ability of the HSCs to self-renew and leads to premature exhaustion^[13-15]. Superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Thus, it is an important anti-oxidant defense enzyme in nearly all cells. ROS degrades polyunsaturated lipids, thereby forming malondialdehyde (MDA). The production of MDA is used as a biomarker to measure the level of oxidative stress in an organism. MDA levels increase continuously with cell age, so this biomarker also reflects cellular senescence. By measuring the SOD activity and MDA content of the cells, we determined the anti-oxidant ability and oxidative damage of the HSC/HPCs.

We found that SOD activity decreased and MDA content increased in the Sca-1⁺ HSC/HPCs following TBI. These results demonstrated that irradiation reduced SOD activity and resulted in oxidative damages in the Sca-1⁺ HSC/HPCs. Several studies have shown that ginsenoside Rg1 treatment increases SOD activity in neurons, livers and lungs^[16-18]. Similarly, in the present study, ginsenoside Rg1 partially rescued the reduction of SOD activity and increase in MDA content. This suggests that ginsenoside Rg1 can improve the activity of SOD, which is important in the breakdown of ROS, to prevent HSC/HPCs from irradiation-induced oxidative stress. However, the mechanism by which ginsenoside Rg1 increases SOD activity remains unclear.

Both irradiation and oxidative stress induce DNA damage, which is a common mediator of cellular senescence^[19]; therefore, we examined the levels of DNA damage in these cells. The comet assay, a sensitive technique to analyze DNA damage, has been used in radiation studies of all cell types^[20]. The comet tail length and the Olive moment are the two main parameters to evaluate DNA damage. At low doses of radiation, a linear relationship occurs between the tail length and the amount of DNA damage; however, at high doses of radiation, the level of DNA damage is related to the Olive moment^[21].

Using comet assay analysis, we found that irradiation

significantly increased the comet tail length and the Olive moment, while the injection of ginsenoside Rg1 reduced these parameters. These results suggest that ginsenoside Rg1 is able to reduce the levels of DNA damage induced by irradiation. Studies have shown that radiation-induced damage and oxidative stress are closely related. Cytoplasmic irradiation can result in damage to nuclear DNA, and experiments with free radical scavengers have shown that this DNA damage is dependent on ROS generation^[22, 23]. We propose that ginsenoside Rg1 might reduce DNA damage by increasing SOD activity, leading to reduced oxidative stress.

The effect of ginsenoside Rg1 on the expression of P16^{INK4a} and P21^{Cip1/Waf1} and cell cycle progression

Cell senescence is mediated through several signal transduction pathways, including the p16^{INK4}-Rb pathway and the P19^{Arf}-Mdm2-p53-p21^{Cip1/Waf1} pathway. P16^{INK4} and P21^{Cip1/Waf1}, which are inhibitors of cyclin-dependent kinases (CDK), are in key positions to regulate cellular senescence due to their regulation of the cell cycle^[24, 25].

P16 prevents cells from escaping G₁ phase by binding CDK4 to inhibit the assembly of the cyclin D-CDK complex, and expression of P16 is significantly higher in aged cells^[26]. It has been reported that p16^{INK4a} gene delivery to young cells induced a senescent phenotype, while p16^{INK4a} knockout HSCs and neural stem cells exhibited delaying aging and enhanced function^[27].

P21 inhibits the phosphorylation of the cyclin D-CDK4 and cyclin E-CDK2 complexes, resulting in the dephosphorylation of the Rb protein and the inhibition of E2F release and DNA synthesis^[28, 29]. P21 also binds to PCNA, a cofactor of DNA polymerase, to form the P21-PCNA complex. This complex prevents the polymerase from extending, thereby causing the polymerase to deviate from the templates and results in DNA replication inhibition^[30]. Above all, P21 prevents cells from entering S phase, during which DNA synthesis occurs. As a result of the overexpression of P21, cells are arrested in G₁ phase, and cell senescence is induced.

A previous study showed that ginsenoside Rg1 pretreatment significantly reduced the t-BHP-induced increase in P16 and P21 expression in human WI-38 diploid fibroblast cells^[31]. Similarly, we observed an obvious increase in P16^{INK4a} and P21^{Cip1/Waf1} expression in the Sca-1⁺ HSC/HPCs after irradiation; however, daily injection of ginsenoside Rg1 remarkably attenuated these changes.

Both P16^{INK4} and P21^{Cip1/Waf1} are important in the regulation of the cell cycle^[24, 25], which is closely related to cell senescence. The cell cycle is an ordered set of events consisting of four phases: G₁, S, G₂ and M. One of the hallmarks of aging cells is that they sustain metabolism but gradually lose mitosis reactivity and DNA synthesis activity, resulting in their arrest in G₁ phase without entering S phase^[11]. Our cell cycle analysis showed that ginsenoside Rg1 significantly reversed the G₁ arrest induced by irradiation. This suggests that ginsenoside Rg1 can regulate the expression of P16^{INK4a} and P21^{Cip1/Waf1}, thereby propelling G₁ phase cells to reenter S phase and delay

HSC/HPCs senescence.

Conclusion

Our study showed that ginsenoside Rg1 could improve the resistance of hematopoietic stem/progenitor cells to radiation-induced senescence using an *in vivo* mice model. We propose that ginsenoside Rg1 exerts anti-aging effects through its anti-oxidant properties, which can relieve the DNA damage induced by chronic oxidative stress and reduce the expression of P16^{INK4a} and P21^{Cip1/Waf1} to propel the Sca1⁺ cells in G₁ phase into S phase. Ginsenoside Rg1 cannot prevent cellular damage induced by irradiance but instead exerted an anti-oxidative effect by rescuing SOD activity to delay senescence. The present study may provide a theoretical and experimental foundation for the application of ginsenoside Rg1 in delaying cellular senescence.

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Author contribution

Cui CHEN, Jian-wei WANG, and Ya-ping WANG designed the research; Yue ZHOU, Ke SHUN, Shan GENG, and Jun LIU performed the research; Jie CHEN and Tin-yu LI analyzed the data; Cui CHEN, Xin-Yi MU, and Ya-Ping WANG wrote the paper.

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