# Salidroside, a scavenger of ROS, enhances the radioprotective effect of Ex-RAD® via a p53-dependent apoptotic pathway

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Received March 14, 2017; Accepted August 9, 2017

DOI: 10.3892/or.2017.5940

**Abstract.** Salidroside (Sal), the predominant component of a Chinese medicinal herb, *Rhodiola rosea* L., has become an attractive bioagent due to its significant anti-radiation, antioxidant and immune adjustment effects. We explored the radioprotective effect of Sal to ascertain whether it could enhance the anti-radiation effect of ON 01210.Na (Ex-RAD®) *in vivo* and *in vitro*, and elucidate its underlying mechanism. Our data demonstrated that Sal inhibited radiation-induced apoptosis, scavenged reactive oxygen species (ROS), and decreased the DNA damage of human umbilical vein endothelial cells (HUVECs). Sal downregulated the expression of Bax and p53 and increased the ratio of Bcl-2/Bax, which indicated that Sal inhibited the radiation-induced apoptosis through p53-dependent pathways. The radioprotection of the Sal pretreatment was also evidenced by an increasing survival rate of the mice, maintaining antioxidant enzyme levels in the liver, and accelerating hematopoietic recovery. The results suggest that Sal exhibits an excellent radioprotective effect with powerful antioxidant activity *in vitro* and *in vivo*. Sal enhanced the radioprotective effect of Ex-RAD by improving the antioxidant effect, the scavenging of ROS, by accelerating hematopoietic recovery and DNA repair as well as by regulating apoptotic and repair signaling pathways. Combined modality treatments were more effective than single-agent treatments, demonstrating the value of multiple-agent radioprotectants.

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*Key words:* salidroside, Ex-RAD, radioprotection, ROS, apoptosis, DNA repair

## Introduction

Ionizing radiation has provided numerous advantages since the discovery of X-rays in the 1890s with industrial, military, and medical uses, but is associated with problems as well. High-dose ionizing radiation has destroyed countless lives and caused untold suffering among the victims of huge nuclear accidents and terrorist activity (1,2). For years, scientists have grappled with finding novel radioprotective agents. Recently, an effective radioprotectant called amifostine (WR-2721), has been clinically approved by the US FDA for patients undergoing radiotherapy. However, in addition to its expensive price tag, this drug has serious side-effects, which has limited its effectiveness (3,4).

In view of this grim situation, radiation protection research has received intense focus. ON 01210.Na (Ex-RAD®; Fig. 1A), 4-carboxystyryl-4-chlorobenzylsulfone, was developed for modifying cell cycle distribution patterns in cancer cells subjected to radiation therapy. It has also been developed as a novel radioprotectant by Onconova Therapeutics Inc. (Newtown, PA, USA). Pre-clinical pharmacokinetic studies have shown that Ex-RAD is rapidly cleared from plasma and has no observable toxicity. Radiation protection was observed with dose escalation to 9.8 Gy. More than 78% of animals treated with Ex-RAD survived a dose of 8 Gy as compared to <20% in an untreated group (5). Ex-RAD received FDA approval as an investigational new drug in December 2008 due to its significant survival advantage and low toxicity. A previous study showed that Ex-RAD alleviated radiation-induced DNA damage and may be an inhibitor of p53-dependent apoptosis as demonstrated in *in vitro* models (6). A similar mechanism also occurred *in vivo*. Ex-RAD-treated mice exhibited lower phospho-ATM and p53, along with a higher Bcl-2/Bax ratio in the spleen. In addition, Ex-RAD treatment significantly mitigated the hematopoietic and gastrointestinal toxicity before and after exposure to ionizing radiation by accelerating recovery of peripheral blood cells, protecting the granulocyte macrophage colony forming units (GM-CFU), and enhancing intestinal crypt survival in mice (7,8).

Compared to the majority of synthetic radioprotectors, natural radioprotective agents are less toxic and have less undesirable side-effects. *Rhodiola rosea* L. is a traditional Chinese herb, which mainly grows at an altitude of 1,600-4,000 meters in

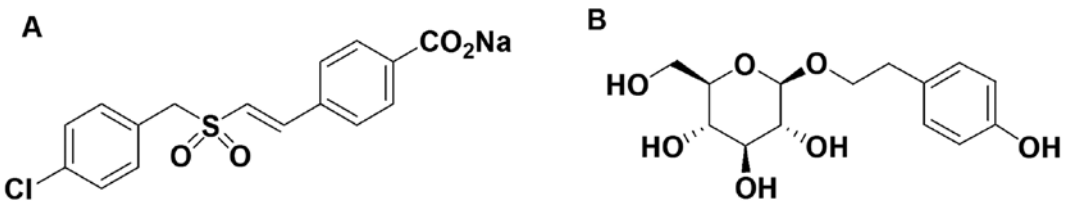


Figure 1. Chemical structure of (A) Ex-RAD and (B) salidroside.

the alpine zone characterized by hypoxia and strong ultraviolet radiation. Salidroside (Sal; Fig. 1B) is the major water-soluble pharmacological ingredient of *Rhodiola rosea* L., which is known for its multiple pharmacological properties and in particular its antioxidative effects. Sal was found to inhibit the UVB-induced apoptosis of HaCaT cells via modulating the expression of NF-κB, BCL-2 and CDK6 (9). It also increased SOD activity and decreased the malondialdehyde (MDA) level in myocardial tissues to protect against myocardial ischemia injury (10). Moreover, Sal was found to scavenge free radicals, reverse DNA damage and alter expression of cytokines and antioxidative enzymes induced by reactive oxygen species (ROS) (11,12). Additionally, Sal was reported to protect endothelial progenitor cells from γ-radiation-induced apoptosis and promote hematopoietic recovery in mice (13).

Ionizing radiation directly causes DNA strand breakage, or acts on water molecules, generating free radicals to damage DNA indirectly. Potential radioprotectors may help to alleviate radiation damage through scavenging free radicals, enhancing DNA repair and inhibiting death signaling pathways (14). In the early 1980s, scholars realized that not all radioprotectants mitigate damage through similar mechanisms; the use of multiple agents may in some instances provide significantly better protection than single agents (15). Currently, attention has been paid to identify highly efficient and low toxic traditional Chinese medicine for the combined treatment of radiation damage. The structure of Sal contains phenolic hydroxyl groups and unsaturated bonds. It is a natural powerful antioxidant, which has the function of antioxidation and scavenging action. In addition, Ex-RAD functions as a p53 inhibitor to block the apoptotic pathway, protecting mice from whole body lethal irradiation. In the present study, based on these clues, we investigated the hypothesis that Sal may enhance the anti-radiation effect of Ex-RAD *in vivo* by accelerating hematopoietic recovery, scavenging ROS and increasing antioxidant capacity. Further exploration *in vitro* also revealed that Sal influenced DNA damage and enhanced the anti-radiation effect of Ex-RAD via a p53-dependent apoptotic pathway.

## Materials and methods

*Chemicals and reagents*. Ex-RAD was synthesized in our laboratory with purity >99%. Sal was purchased from Xi'an Plants of Grass Technology Co. Ltd. (Shaanxi, China) with a purity >99%. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Solarbio Science & Technology Co. (Beijing, China). Chemicals for electrophoresis and immunoblots were purchased from Abbkine, Inc. (Redlands, CA, USA). The SOD/CAT/MDA/GSH assay kits were purchased from the Nanjing Jiancheng Institute of Biotechnology (Xi'an, China). The cell apoptosis detection kit with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) and intracellular ROS detection kits were purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). Antibodies for p53, Bax, Bcl-2 and γ-H2AX were purchased from Abcam (Cambridge, UK).

*Radioprotection activity in vivo*. Six- to eight-week-old male BALB/c mice were provided by the Animal Center of the Fourth Military Medical University (Xi'an, China) and acclimated in the laboratory for 1 week prior to the experiments, and housed (10/cage) in an air conditioned facility under standard maintenance conditions (12:12 h light-dark cycle; 21-23˚C; 55-65% relative humidity). All animals were provided with sufficient food and clean water. The experimental protocol involving animals was reviewed and approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University. All the studies carried out on animals strictly complied with the Guidelines for the Care and Use of Laboratory Animals. For irradiation, mice were placed in a rectangular, well-ventilated plastic mouse holder, exposed to general irradiation from a $^{60}$Co γ-ray irradiator (cobalt tank, irradiation rate 200 cGy/min; Fourth Military Medical University).

Ex-RAD was suspended in vehicle as reported in a previous study (6). Ex-RAD (500 mg/kg) was subcutaneously (s.c.) administered using a 1-ml sterile syringe with a 25G needle at 24 h and 15 min (two doses) before irradiation (16). Sal (100 mg/kg) was dissolved in normal saline (NS) and was chronically administered to animals for 7 days before general irradiation.

To determine whether Sal enhances the anti-radiation effect of Ex-RAD *in vivo*, a survival assay of mice was investigated. In the present study, 75 mice were randomly divided into five groups (n=15/group) as follows: CON (control), VEH (irradiation + vehicle), SAL (irradiation + Sal 100 mg/kg), EX (irradiation + Ex-RAD 500 mg/kg) and SAL + EX (irradiation + Sal 100 mg/kg + Ex-RAD 500 mg/kg). Mice in the irradiation groups were exposed to 8 Gy of whole body γ-radiation. Survival was monitored for 30 days post-radiation, and the data are expressed as the survival rate.

A peripheral blood study was investigated to evaluate the mitigation of hematopoietic toxicity of Sal on whole body radiation mice. Animals (n=10) were pretreated with Ex-RAD and Sal prior to sub-lethal total body irradiation of 5 Gy and the method of administration and dosage were mentioned above. Mice were humanely anesthetized with pentobarbital sodium (50 mg/kg i.p.). Blood (0.6-1.0 ml) was collected from the posterior vena cava using a 23G needle on day 7. Blood was quickly collected in EDTA tubes directly from the heart. Total white blood cells (WBC), red blood cells (RBC),

hemoglobin (HGB) and platelets (PLT) were counted using automated hematologic analyzer, Cell-Dyn 3000 (Unipath Corp., Mountain View, CA, USA).

After 7 days following radiation, liver tissue samples were homogenized with ice cold 150 mM NaCl and centrifuged at 15,000 rpm at 4˚C for 15 min. The supernatants were used for the determination of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and MDA. The assay procedure was performed according to the kit's instructions.

*Cell cultures and treatment*. Human umbilical vein endothelial cells (HUVECs) were purchased from the Chinese Academy of Sciences and incubated at 37˚C in 5% $CO_2$ in high-glucose DMEM supplemented with 10% FBS serum and antibiotics. HUVECs were divided into six groups: CON, VEH (irradiation + vehicle), SAL (irradiation + Sal 40 $\mu$mol/l, EX (irradiation + Ex-RAD 20 $\mu$mol/l) and SAL + EX (irradiation + Sal 40 $\mu$mol/l + Ex-RAD 20 $\mu$mol/l). Ex-RAD and Sal were dissolved in fresh DMEM, and their combination was dissolved in Ex-RAD medium. Cells were serum-starved for 4 h and then treated with the indicated concentrations of drugs 2 h before radiation exposure.

*Colony survival assay*. HUVCEs were seeded into 12-well plates at a density of $1x10^3$ cells/well. Cells were treated with the drugs as mentioned above for 2 h before γ-radiation (5 Gy). After 14 days post-radiation, cell colonies were stained with crystal violet and counted. The experiment was performed at least three times.

*Annexin V/PI staining*. The radiation-induced apoptosis of HUVCEs was assessed using a flow cytometry method that measures Annexin V-FITC/PI staining. Briefly, cells were pretreated with the drugs for 2 h before 5 Gy γ-radiation. After being cultured for another 48 h, the cells were collected, washed twice with cold phosphate-buffered saline (PBS), and then stained with Annexin V and PI for 15 min in the dark. Finally, the cells were analyzed by a flow cytometer. In regards to the analysis, Annexin V-positive and PI-negative cells represent early apoptotic populations, Annexin V-positive and PI-positive cells represent late apoptotic or dead proportions.

*Measurement of radiation-generated intracellular ROS*. 2',7'-Dichlorofluorescein diacetate (DCFH-DA) is a highly sensitive probe which is used for detection of intracellular ROS. This non-fluorescent dye diffuses readily into cells and yields DCFH which cannot pass out of cells. In the presence of peroxidase, DCFH can generate the fluorescent compound dichlorofluorescein (DCF), which can be analyzed by flow cytometry using a laser excitation and emission wavelength of 492-495 and 517-527 nm, respectively. Cells were treated with different concentrations of the drugs at 37˚C for 2 h before 5 Gy γ-radiation. After 48 h, the cells were washed with cold PBS and 10 $\mu$M DCFH-DA was added for 30 min to measure the intracellular ROS by flow cytometry.

*Single-cell alkaline gel electrophoresis (COMET assay)*. The single cell gel electrophoresis or Comet assay is a sensitive, reliable, and rapid method for DNA double- and single-strand breaks, alkali-labile sites and delayed repair site detection, in eukariotic individual cells (17). Cells were treated with drugs at 37˚C for 2 h before 8 Gy γ-radiation, and then harvested and suspended in cold PBS for COMET assay after 2 h of radiation. Specific procedures are outlined in a previous study (18).

*γ-H2AX immunofluorescence*. The number of γ-H2AX foci was used as a biomarker of radiation-induced DNA double-strand breaks (DSBs) (19). HUVECs were seeded in a glass bottom cell dish (NEST Instruments), and allowed to attach overnight. Cells were treated with the different drugs for 2 h before 8 Gy γ-radiation on ice, and 1 h after radiation the cells were washed with cold PBS, and fixed in 4% paraformaldehyde for 15 min. We used 5% serum to block the non-specific protein interactions and then the cells were incubated with the primary antibody (phosphorylated H2AX) at 4˚C, overnight. Cell dishes were washed in PBS before addition of the secondary antibody [Cy3-goat anti-rabbit IgG (H + L)] for 1 h at 37˚C, and then incubated with DAPI (5 $\mu$g/ml) for 10 min. We used a laser scanning confocal microscope to acquire the digital images. The γ-H2AX foci counts were calculated using ImageJ foci counter.

*Western blotting*. Cultured cells were exposed to 5 Gy γ-radiation and cultured for 24 h. Then, the cells were collected and lysed in RIPA lysis buffer on ice for 1 h. Cell lysates were centrifuged at 12,000 rpm for 5 min at 4˚C, and cell supernatants were collected. The protein concentration was confirmed using a BCA protein assay kit (Beyotime Institute of Biotechnology). Proteins (20 $\mu$g) from each group were separated on 10% SDS-polyacrylamide gels and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham, Braunschweig, Germany). The membranes were blocked in 5% skimmed milk dissolved in Tris-buffered saline and Tween-20 (TBST) (20 mM Tris, pH 7.5) for 2 h and then incubated with primary antibodies (p53, Bax, Bcl-2, γ-H2AX) at 4˚C overnight. After three washings in TBST, the membranes were incubated with the secondary antibody conjugated with anti-rabbit IgG peroxidase for 2 h at room temperature. Bands were monitored with chemiluminescence using an ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

*Statistical analysis*. Values are expressed as mean ± standard error (SE) or as a percentage. Analysis of variance (ANOVA) was used to determine whether there was a significant difference among the groups. If the difference was significant, a pairwise comparison by Tukey-Kramer was used to identify which group was different from the other. Log-rank (Mantel-Cox) test was used to compare survival rates among groups. Values of $P<0.05$ were considered as statistically significant.

## Results

*Radioprotection of Sal in vivo*. The mice in the irradiation group exhibited a reduction in food and water intake, epilation, weight loss, emaciation, diarrhea and hemafecia within 3 to 5 days after exposure to 6 Gy radiation (18). The results of the survival studies are expressed as survival rate (Fig. 2A). In the VEH group, no mouse survived to day 30. Although only 4 mice in the SAL group survived to day 30, the mean

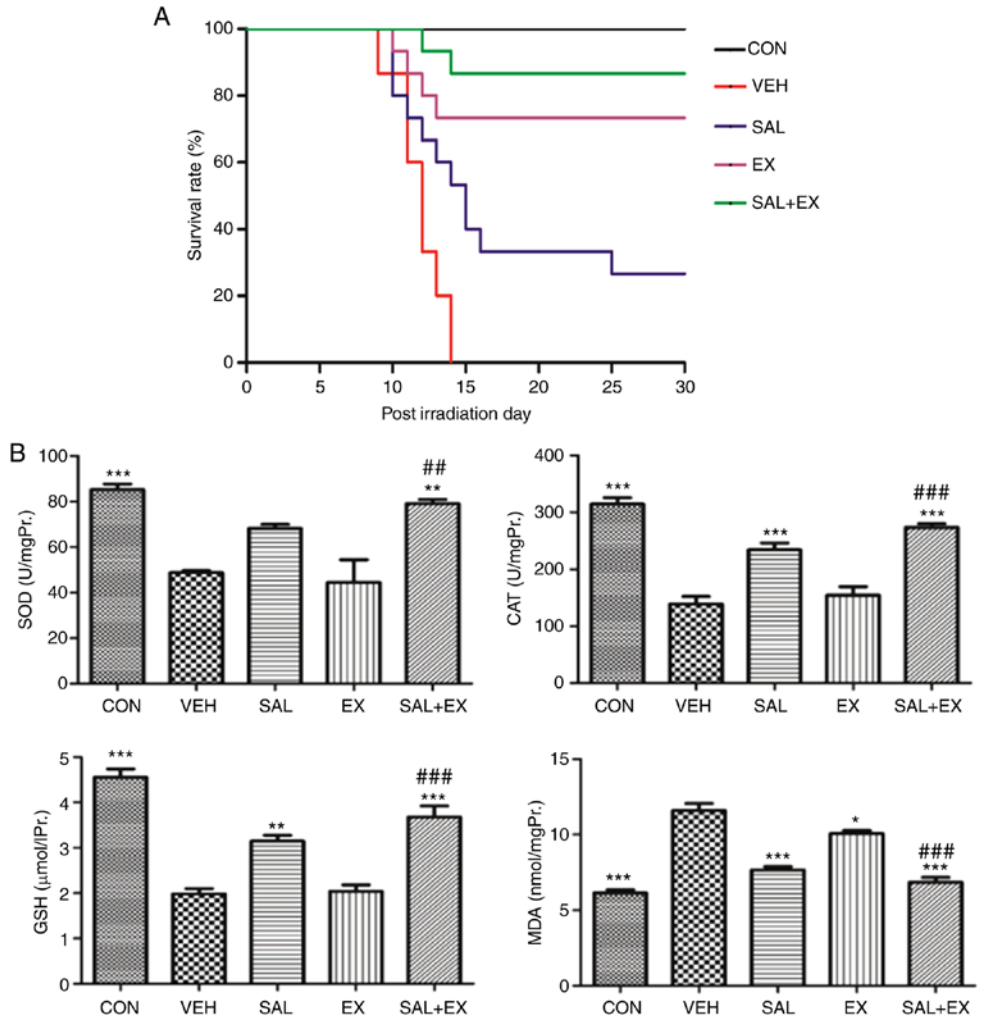


Figure 2. (A) Effect of drugs on the survival time of mice exposed to whole body irradiation at a dose of 8 Gy. (B) Effects of drugs on liver superoxide dismutase (SOD), glutathione peroxidase (GSH), catalase (CAT) and malondialdehyde (MDA) levels in mice after irradiation. Data are expressed as mean ± SE; $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 vs. VEH; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. EX. Groups: CON (control), VEH (irradiation + vehicle), SAL (irradiation + Sal 100 mg/kg), EX (irradiation + Ex-RAD 500 mg/kg) and SAL + EX (irradiation + Sal 100 mg/kg + Ex-RAD 500 mg/kg).

survival time was significantly higher from 12 to 15 days when compared to the VEH group (P=0.0055; log-rank test). The EX group (500 mg/kg) administered s.c. at 24 h and 15 min before irradiation showed a significant increase in survival, with 11 of 15 mice still alive on day 30 (P<0.0001). The SAL + EX pretreatment group showed an increased survival rate (from 73 to 86%; P<0.05, significant difference) compared to the EX group. Thirteen of 15 mice remained alive on day 30. These results collectively indicated that Sal administration significantly enhanced the radioprotection activity of Ex-RAD.

To evaluate the antioxidant activity of Sal on irradiated mice, we measured SOD, GSH, CAT activity and MDA levels in liver homogenates. The average test results are presented in Fig. 2B. γ-radiation significantly reduced the level of antioxidant enzymes involving SOD and CAT (P<0.001). However, Sal (100 mg/kg) significantly (P<0.001) increased the antioxidant enzyme level. Compared with the VEH group, SOD and CAT levels in the EX group had no difference. The Sal combination group had obviously increased SOD and CAT activity compared with the EX group (P<0.01; P<0.001, respectively). As to the content of GSH, the same change was noted in the combination group. Sal increased the GSH level in the Ex-RAD-treated group (P<0.001). Conversely, the MDA levels in the drug treatment groups were significantly decreased compared with the VEH group (P<0.05), and the SAL + EX pretreatment group was the most obvious group. These results indicated that Sal protects mice against oxidative damage, and Sal enhances the radioprotective activity of Ex-RAD by improving the antioxidant capability.

The peripheral blood data are shown in Table I. Total WBC and PLT counts began to decline shortly after radiation. However, recovery was accelerated in the Sal and Ex-RAD treated mice after 7 days total body irradiation. WBC and PLT counts were significantly increased in the drug-treated mice compared with the VEH group (P<0.05). In addition, the combination group was the fastest recovery group compared with the Ex-RAD and Sal single use groups (P<0.05). There was no statistical significance between these groups in regards to HGB and RBC counts.

*Clonogenic protection and anti-apoptotic effects of Sal in HUVECs*. HUVECs are sensitive to γ-radiation (6,20). We

Table I. Effects of the drugs on red blood cells, white blood cells, hemoglobin and platelets in the peripheral blood of mice after 7 days following whole body irradiation at a dose of 5 Gy.

| Groups | WBC (x10E9/l) | RBC (x10E12/l) | HGB (g/l) | PLT (x10E9/l) |
|---|---|---|---|---|
| CON | 8.20±0.62[b] | 10.95±0.67 | 160.75±3.59 | 522±25.13[b] |
| VEH | 1.79±0.29 | 10.22±0.23 | 150.75±4.11 | 84.25±5.12 |
| SAL | 3.42±0.63[a] | 10.68±0.15 | 147±5.94 | 191.25±7.18[b] |
| EX | 4.17±0.27[b] | 11.04±0.62 | 153±8.64 | 332.25±29.85[b] |
| SAL + EX | 6.28±0.87[b,c] | 10.24±0.32 | 157.5±3.70 | 483.5±43.71[b,d] |

Values are expressed as mean ± SE of 10 mice. [a]Significant difference (P<0.05) vs. VEH. [b]Significant difference (P<0.001) vs. VEH. [c]Significant difference (P<0.05) vs. EX. [d]Significant difference (P<0.001) vs. EX. WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; PLT, platelets. Groups: CON (control), VEH (irradiation + vehicle), SAL (irradiation + Sal 100 mg/kg), EX (irradiation + Ex-RAD 500 mg/kg) and SAL + EX (irradiation + Sal 100 mg/kg + Ex-RAD 500 mg/kg).

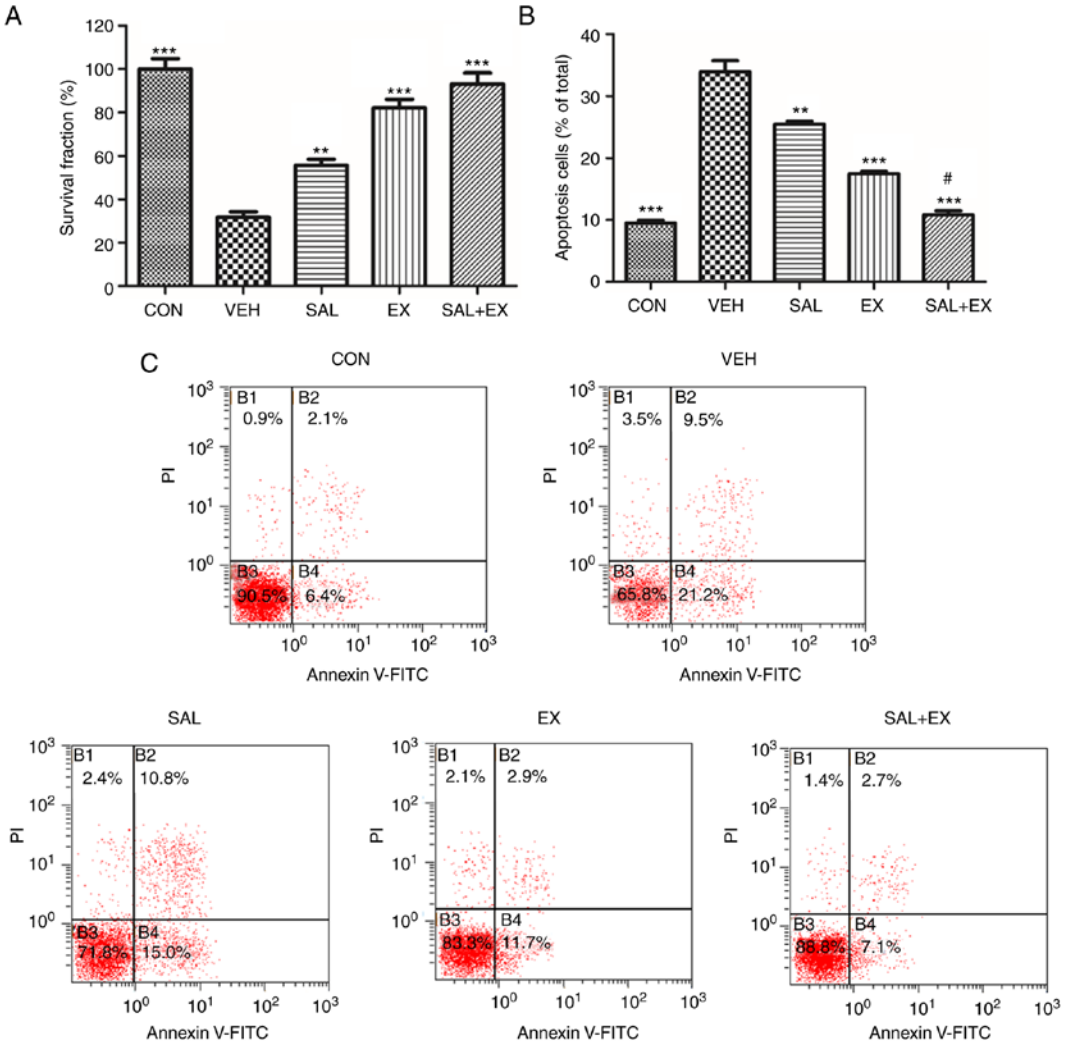


Figure 3. (A) Clonogenic survival assay. Values are expressed as percentages. The anti-apoptotic effect of the drugs was analyzed using Annexin V/propidium iodide staining in a flow cytometry assay. (B) Bar graph of apoptotic cells expressed as a percentage of total cells for each group from three experiments. (C) Specific apoptosis data in HUVECs after 5 Gy γ-radiation. B1, B2, B3 and B4 represent dead, late apoptosis, vital and early apoptotic cells, respectively. Data are presented as means ± SE (n=3); **P<0.01, ***P<0.001 vs. VEH; #P<0.05 vs. EX. Groups: CON (control), VEH (irradiation + vehicle), SAL (irradiation + Sal 100 mg/kg), EX (irradiation + Ex-RAD 500 mg/kg) and SAL + EX (irradiation + Sal 100 mg/kg + Ex-RAD 500 mg/kg).

used a clonogenic survival assay to examine the viability of irradiated HUVECs. Pretreatment of Sal and Ex-RAD before radiation provided excellent protection compared with the VEH group (Fig. 3A; P<0.01). However, there was no significant difference between the EX and SAL + EX group.

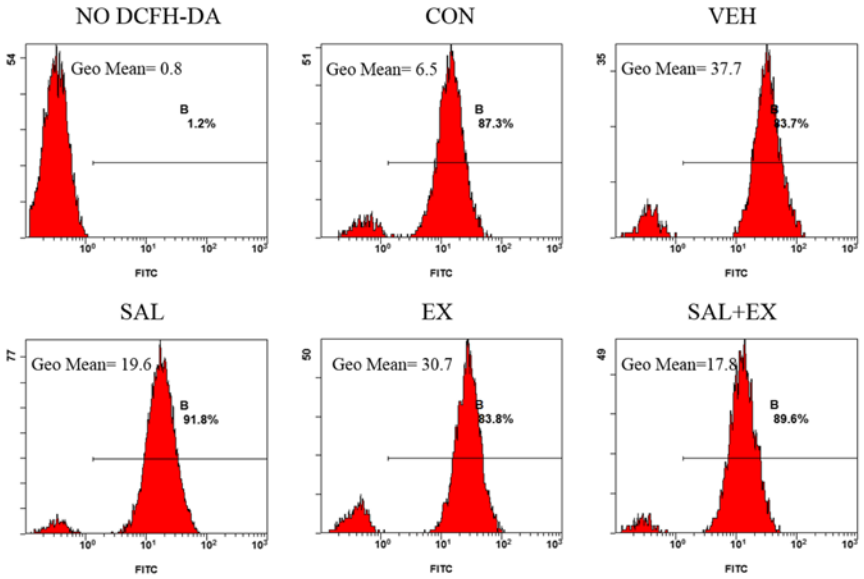


Figure 4. Identification of reactive oxygen species (ROS) after 5 Gy γ-radiation using oxidized DCFH-DA and flow cytometry. Two experiments with similar results were performed. Geometric mean (Geo Mean) was used to calculate the total intensity of fluorescence. Groups: CON (control), VEH (irradiation + vehicle), SAL (irradiation + Sal 100 mg/kg), EX (irradiation + Ex-RAD 500 mg/kg) and SAL + EX (irradiation + Sal 100 mg/kg + Ex-RAD 500 mg/kg).

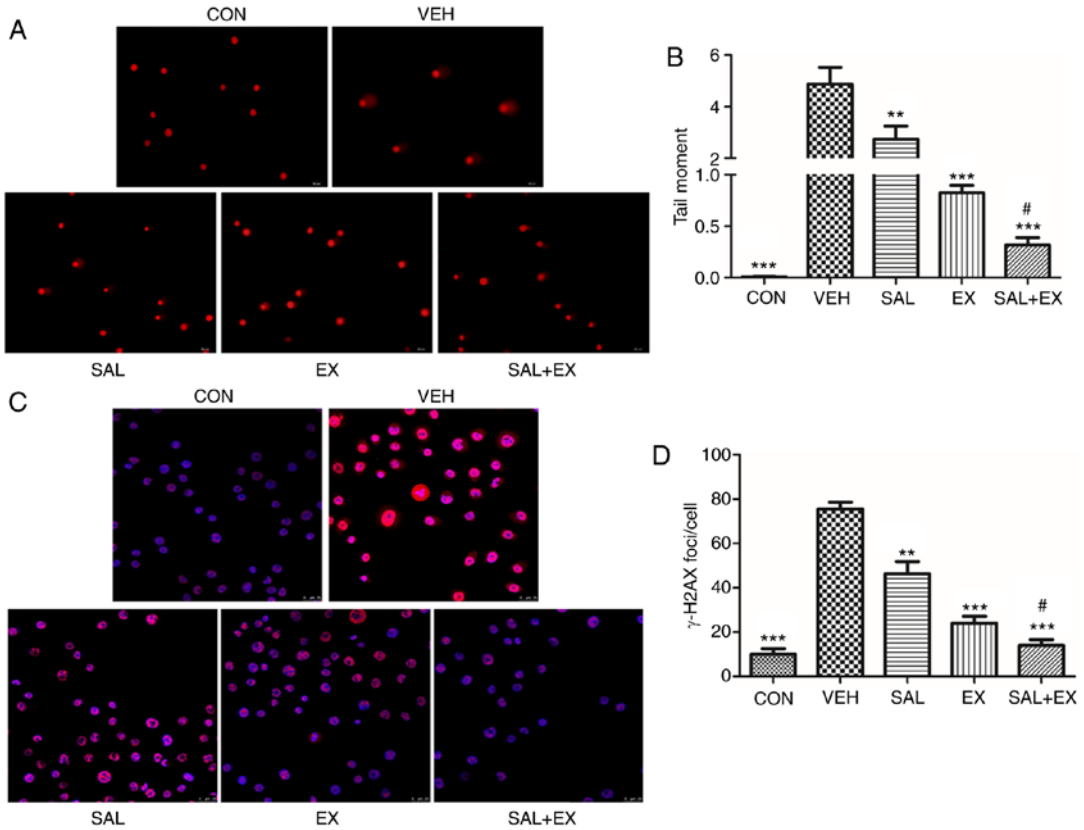


Figure 5. (A) Resolution of radiation-induced DNA breaks in HUVECs. Single cell alkaline gel electrophoresis of HUVECs. Cells were irradiated and treated, or not, with drugs. At the indicated times, the cells were electrophoresed, and cellular DNA was analyzed using propidium iodide staining. (B) The quantification of the tail moment of irradiated cells is graphically displayed. (C) Immuno-localization of phospho-S139 H2AX in HUVECs 1 h after irradiation. Immuno-labeling with antiserum to γ-H2AX (S139) is shown. (D) Quantification of phospho-H2AX foci/cell is shown. Data are presented as means ± SE (n=3); $^{**}$P<0.01, $^{***}$P<0.001 vs. VEH; $^{\#}$P<0.05 vs. EX. Groups: CON (control), VEH (irradiation + vehicle), SAL (irradiation + Sal 100 mg/kg), EX (irradiation + Ex-RAD 500 mg/kg) and SAL + EX (irradiation + Sal 100 mg/kg + Ex-RAD 500 mg/kg).

In the apoptosis assay, radiation-induced apoptosis in the HUVECs was significantly decreased in the SAL and EX groups compared to the VEH group (Fig. 3B and C; P<0.001). Meanwhile, the Sal combination group demonstrated a better protective effect compared with the Ex-RAD-treated group (P<0.05). These results indicated that Sal enhanced the

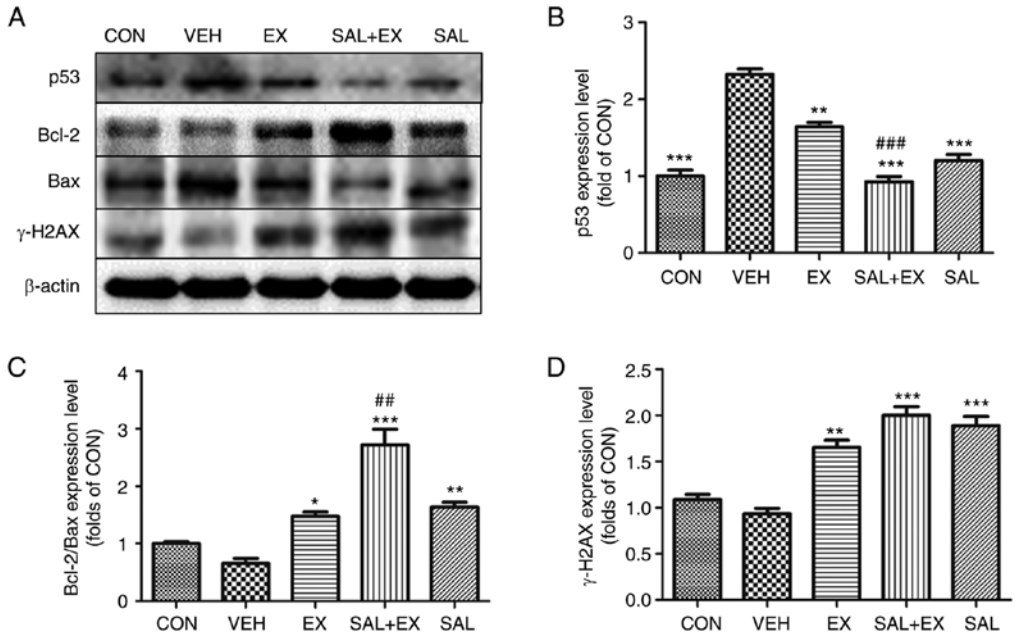


Figure 6. Western blot analysis of DNA damage response in HUVECs. (A) Scanned images showing western blotting of p53, Bcl-2, Bax and γ-H2AX. (B-D) The expression levels of p53, Bcl-2/Bax ratio, and γ-H2AX were quantified by densitometry. The data values are indicated as the relative densitometry of the control. Results are represented as mean ± SE of three independent experiments; $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 vs. VEH; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. EX. Groups: CON (control), VEH (irradiation + vehicle), SAL (irradiation + Sal 100 mg/kg), EX (irradiation + Ex-RAD 500 mg/kg) and SAL + EX (irradiation + Sal 100 mg/kg + Ex-RAD 500 mg/kg).

radioprotective effect of Ex-RAD due to the attenuation of radiation-induced apoptosis.

*Effects of Sal on intracellular ROS generation in HUVECs.* The fluorescence of DCF was used to evaluate the intracellular ROS in HUVECs (Fig. 4). Radiation (5 Gy) significantly increased the level of ROS (5.8-fold) compared with the VEH group. A slight difference was found between the EX and VEH group of cells, whereas the fluorescence intensity of the SAL + EX and SAL group was significantly (almost a half) lower than that noted in the EX group. This indicated that pre-treatment with Sal significantly blocked radiation-induced ROS generation. Sal enhanced the radioprotective effect of Ex-RAD by scavenging intracellular ROS.

*Synergetic effects of the Sal and Ex-RAD combination treatment on radiation-induced DNA damage in HUVECs.* We used an alkaline comet assay to detect radiation damage. The results showed a faster resolution of fragmented tail DNA in the cells treated with Sal and Ex-RAD compared to the untreated cells (Fig. 5A). One-way ANOVA test showed a significant decrease in tail moment (P<0.01), and there was also a significant difference between tail moments of EX and SAL + EX treated cells before irradiation (Fig. 5B; P<0.05).

The kinetics of γ-H2AX foci resolution was used as a biomarker to ascertain the effect of drugs on the repair of double-strand breaks. Immunofluorescence staining of γ-H2AX was assessed 1 h after radiation (Fig. 5C). Both Sal and Ex-RAD obviously reduced the number of γ-H2AX foci in cells compared to the VEH group (P<0.01). Moreover, the combination group showed a markedly decreased γ-H2AX foci number compared with the EX group (Fig. 5D; P<0.05) The results indicated a positive effect of Sal in facilitating repair of DNA damage, and improving the curative effect of Ex-RAD.

*Effects of Sal on the p53 signaling pathway in HUVECs.* In order to elucidate the molecular mechanisms involved in the cell apoptosis induced by γ-radiation in HUVECs, western blotting was used to measure the expression of several apoptosis-related proteins. As shown in Fig. 6A, we found that the expression of the pro-apoptotic protein p53 and Bax was markedly increased after irradiation. As shown in Fig. 6B and C, Sal significantly inhibited the upregulation of p53 (P<0.001), and promoted the increase in the Bcl-2/Bax ratio (P<0.01). The results also showed that combined treatment with Ex-RAD and Sal significantly increased the Bcl-2/Bax ratio (P<0.001), accompanied by a decrease in the p53 level (P<0.01), compared to the levels following treatment with either drug alone. γ-H2AX is a marker to assess the effectiveness of DNA repair by nonhomologous end joining (21). Both Sal and Ex-RAD increased the expression of γ-H2AX (Fig. 6D, P<0.001, P<0.01). There was no significant difference between the EX and SAL + EX group.

## Discussion

In the present study, the results confirmed that salidroside (Sal) significantly enhanced the radioprotective effect of Ex-RAD. The 30-day survival assay showed that the combined administration of Sal and Ex-RAD provided significant protection to mice after 8 Gy irradiation. Acute radiation exposure leads to a drop in circulating blood cells and hemorrhage due to radiation-induced loss of platelets remains a life-threatening issue (22,23). In this initial investigation, we demonstrated that Sal and Ex-RAD significantly increased the peripheral

WBC and platelet counts, mitigating radiation-induced hematopoietic toxicity. In addition, the results demonstrated that hematopoietic recovery was also significantly accelerated in mice treated with the combined agents when compared to mice receiving single-agent treatments.

In the healthy physiological status, ROS remain at low levels and play a normal function. Once the level is enhanced due to radiation, the high levels of ROS may cause severe damage to DNA, which eventually leads to cell death via either apoptotic or other mechanisms (24). Sal, a powerful natural antioxidant, was widely reported for its inhibition of ROS generation in different diseases (25-27). In the present study, intracellular ROS significantly increased after radiation, whereas the level of ROS was obviously decreased in the Sal-treated cells. Moreover, we found that radiation-induced apoptosis was greatly reduced in the Sal and Ex-RAD combined group. Results indicated that Sal promoted the radioprotection of Ex-RAD probably by decreasing ROS generation and protecting against cell death.

Organisms develop various defense mechanisms to protect themselves against ROS, such as antioxidant enzymes. The antioxidant enzymes include SOD, GSH and CAT. SOD catalyzes the dismutation of superoxide ($O^{2-}$) to hydrogen peroxide ($H_2O_2$), while CAT and GSH convert $H_2O_2$ to $H_2O$. MDA is considered to reflect the degree of lipid peroxidation (28-30). In the present study, Sal significantly decreased the MDA level, and significantly increased the activity of SOD, CAT and GSH enzymes in the mouse liver. This indicated that Sal was able to protect against the damage produced by radiation with upregulation of antioxidant enzymes and a decrease in lipid peroxidation.

Radiation-induced damage to DNA is the primary determinant of radiation-induced mutations, carcinogenesis and lethality (31). We used COMET assay and γ-H2AX foci formation to investigate DNA single- and double-strand breaks (DSB), respectively. The intensity of the comet tail relative to the head reflected the number of DNA breaks (17). γ-H2AX molecules appeared in discrete nuclear foci and each γ-H2AX focus represented one DNA DSB (19,32). It was noted that pretreatment of Sal and Ex-RAD significantly reduced the comet parameters compared with the Ex-RAD alone group. γ-H2AX immunofluorescence assay showed that the Sal combination group exhibited significant protection against radiation-induced DNA damage. The results confirmed that the radioprotective effect of Ex-RAD was largely increased by Sal.

To further investigate the molecular radiation protection mechanism of Sal and Ex-RAD, western blot assay was performed to measure the expression of several apoptosis-related and DNA repair proteins. It is well known that pro-apoptosis signaling pathways include activation and stabilization of p53, Bcl-2, Bax, c-Abl, p21 and many more signaling molecules (33-35). Bcl-2 family proteins include pro- and anti-apoptotic molecules. Bcl-2 is an anti-apoptotic protein and Bax is a pro-apoptotic protein. They have been identified as major regulators and the ratio of Bcl-2/Bax plays an important role in cell apoptosis or survival (34,36,37). Both Bcl-2 and Bax are transcriptional targets for the tumor-suppressor protein, p53, which induces cell cycle arrest or apoptosis in response to DNA damage (33,37). Sal downregulated the expression of Bax, p53, and increased the ratio of Bcl-2/Bax. The radiation protection offered by Sal probably involves inhibition of apoptosis through p53-dependent pathways. DNA DSB induced by γ-radiation leads to rapid phosphorylation of H2AX at Ser139 by ATM, ATR and DNA-PKcs, resulting in γ-H2AX. The phosphorylation and dephosphorylation of H2AX are necessary for the DNA damage repair process (38). Our results demonstrated that Ex-RAD and Sal increased the expression of γ-H2AX, Sal is a beneficial natural drug that aids Ex-RAD in DNA repair.

Furthermore, previous investigations have also shown that Sal can inhibit the proliferation of a variety of cancer cells (39-43). We suspected that Sal may have different biological effects in normal cells and tumor cells. We demonstrated that Sal has radioprotective effects on normal cells. However, studies are still needed to further evaluate whether Sal has an inhibitory effect on cancer cells and the different mechanisms of these functions.

In conclusion, the present study indicated that Sal enhanced the radioprotective effect of Ex-RAD. Sal exerted its radioprotective action, largely via scavenging intracellular ROS, reducing DNA damage and upregulating antioxidant enzymes against γ-radiation in HUVECs. The molecular mechanism underlying its radioprotective properties may result from alleviation of radiation-induced apoptosis by inhibiting p53-dependent apoptosis. These data suggest that Sal may be a potential choice for combination treatment against radiation-related damage. However, the detailed *in vivo* radioprotective activities, clinical application, molecular mechanisms and drug targets of Sal warrant further investigation.

## Acknowledgements

We thank the National Natural Science Foundation of China (nos. 81573343 and 21503272) and the Major Science and Technology Projects in Shanxi Province (Xianyang China) (2015SF2-08-01 and 2017ZDJC-05).

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