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Resveratrol attenuates radiation enteritis through the SIRT1/FOXO3a and PI3K/AKT signaling pathways

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ABSTRACT

Radiation enteritis (RE) is the most common radiotherapy complication, and effective RE treatments are lacking. Resveratrol exerts beneficial effects on radiation injury. However, the effect of resveratrol in radiation-induced intestinal injury and the underlying mechanism remain unclear. Here, a C57BL/6 mouse model of RE was established and an intestinal epithelial cell line was used to evaluate the protective effects of resveratrol against radiation-induced intestinal injury and the underlying mechanisms. Resveratrol improved radiation-induced oxidative stress and cell apoptosis via upregulating antioxidant enzymes and downregulating p53 acetylation. *In vivo*, resveratrol-treated mice exhibited longer survival; longer villi; more intestinal crypt cells; upregulated expression of Ki67, catalase, and superoxide dismutase 2; and fewer inflammatory proteins and apoptotic cells. These protective effects were suppressed by inhibition of SIRT1. These results demonstrate that resveratrol can reduce radiation-induced intestinal injury by inhibiting oxidative stress and apoptosis via the SIRT1/FOXO3a and PI3K/AKT pathways.

1. Introduction

Radiotherapy is an important treatment modality for cancer, administered to approximately half of patients with malignancies [1]. However, ionizing radiation (IR) exposure to cancerous regions also inevitably affects normal tissues. Radiation enteritis (RE) is the most common side reaction of abdominal or pelvic radiotherapy [2]. Various complications can occur in severe RE cases, including intestinal obstruction and intestinal perforation, which can be life-threatening [3,4]. Currently, no effective clinical treatment methods are available for RE, except for symptomatic treatment. Therefore, effective methods for preventing and treating RE are urgently needed.

IR-induced oxidative stress is an important factor in radiation-induced intestinal injury [5]. SIRT1 is an NAD + -dependent class III protein deacetylase, mainly localized in the nucleus. SIRT1 can deacetylate a wide range of histone and non-histone substrates, such as p53, forkhead box class O (FOXO), and PPARγ, and thus regulates many cellular processes, including oxidative stress, metabolism, aging, and apoptosis. Accordingly, SIRT1 has been proposed as a potential target for improving radiation-induced damage [6].

Resveratrol, an agonist of SIRT1, exerts several pharmacological effects, including anti-inflammatory, antioxidant, and anti-aging effects, and is considered a natural radioprotector [7,8]. Resveratrol can alleviate radiation-induced hepatic damage [9], bone marrow hematopoietic failure [10], and mouse embryonic stem cell injury [11]. We previously reported that resveratrol reduces radiation-induced intestinal injury by upregulating antioxidant enzyme superoxide dismutase 2 (SOD2), which is associated with SIRT1 [12]. However, the underlying mechanism remains unclear.

FOXO3a is a member of the FOXO transcription factor family, which plays a pivotal role in maintaining redox balance. Activation of the SIRT1/FOXO3a pathway can inhibit oxidative stress primarily by regulating antioxidant enzymes [13–15]. Thus, we hypothesized that resveratrol would ameliorate radiation-induced intestinal

Abbreviations: IR, Ionizing radiation; RE, Radiation enteritis; DCFH-DA, dichlorodihydro-fluorescein diacetate; SOD, Superoxide dismutase; CAT, Catalase; FOXO, Forkhead box class O; SIRT1, Silencing regulator 2-related enzyme 1; ROS, Reactive oxygen species; RES, Resveratrol.

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damage via regulation of the SIRT1/FOXO3a pathway. We tested this possibility using an RE mouse model and the intestinal epithelial cell line IEC-6.

2. Materials and methods

2.1. Cell culture and treatment

The IEC-6 cell line (Peking Union Medical College Cell Bank) was cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA) and 1% penicillin-streptomycin (Gibco) at 37 °C in a constant-temperature cell incubator with 5% CO2. Cells passaged for 2–3 days were used in the experiments.

IEC-6 cells were exposed to a linear accelerator (Varian Clinic 21 ES; Varian Medical Systems, Crawley, UK) that generated high-energy X-rays (6 MeV) at 0.67 Gy/min for final doses of 0, 2, 4, 6, 8, or 10 Gy. Resveratrol (R5010; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide and diluted with culture medium to various concentrations. To inhibit the deacetylation activity of SIRT1, the cells were treated with the selective inhibitor Ex527 (10 μM, E7034; Sigma-Aldrich). The cells were pre-treated with RES and/or Ex527 2 h before irradiation. Control groups were sham-irradiated and incubated in culture medium containing an equal amount of vehicle.

2.2. Cell counting kit-8 (CCK8) assay

Cell viability was assessed with a CCK-8 assay kit (#CA1210; Solarbio, Beijing, China). After trypsin digestion (Gibco) of adherent cells, 3000 cells/well were seeded in a 96-well plate (five wells/group) and incubated for 24 h for adherence to the wells. The culture medium was changed 2 h before irradiation, and each group was pre-treated with resveratrol. After irradiation, the cells were incubated for 24 h and 48 h at 37 °C under 5% CO2; 10 μL of CCK-8 reagent was then added to each well, and cells were further incubated for 2 h in the dark. Cell viability was determined by measuring absorbance at 450 nm in a microplate reader (Bio-Rad, Hercules, CA, USA).

2.3. TUNEL staining

Apoptosis was assessed using the TUNEL apoptosis kit (40307ES20; YEASEN, Shanghai, China) according to the manufacturer's instructions. In brief, cells were fixed with 4% formaldehyde for 30 min and washed with phosphate-buffered saline (PBS) three times for 5 min each. Tissue sections were deparaffinized in xylene and hydrated with a gradient of ethanol solutions, washed with PBS three times for 5 min each, and treated with proteinase K at 37 °C for 5 min. Thereafter, the samples were washed with PBS, fixed with equilibration buffer at 25 °C for 30 min, washed with PBS again, and mixed with Alexa Fluor solution. After washing with PBS, the samples were incubated with 50 μL of TUNEL detection solution for 60 min in the dark at 37 °C. Nuclei were then stained with DAPI for 5 min. After another washing step, the samples were sealed with anti-fluorescence quenching liquid. Images were captured with a fluorescence microscope (OLYMPUS, Tokyo, Japan). The excitation and emission wavelengths were 488 and 535 nm, respectively.

2.4. Determination of reactive oxygen species (ROS) levels

ROS levels were measured using dichloro-dihydro-fluorescein diacetate (DCFH-DA, S0033S, Beyotime, Shanghai, China). In brief, the cells (1 × 107/mL) were incubated with DCFH-DA in the dark at 37 °C for 20 min, washed three times with serum-free medium, and then imaged with a fluorescence microscope (OLYMPUS). The excitation and emission wavelengths were 488 and 535 nm, respectively.

2.5. Western blotting

IEC-6 cells were harvested and lysed in radioimmunoprecipitation assay lysis solution with 1′ Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) for 30 min and centrifuged at 14,000 rpm for 30 min at 4 °C. The cytoplasmic and nuclear proteins were isolated according to the manufacturer's instructions of Nuclear and Cytoplasmic Protein Extraction Kit (P0027, Beyotime Biotechnology, Shanghai, China).

Equal amounts of proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis [10% (w/v) resolving gel] and electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Roth, Roti, 0.45 μm). The PVDF membrane was blocked with 5% milk in 1 × Tris-buffered saline with 1% Tween-20 (Sigma-Aldrich) for 2 h at room temperature. The membrane was then incubated with anti-SIRT1 (1:1000; CST, Danvers, MA, USA), anti-FOXO3a (1:1000; CST), anti-p-FOXO3a (1:1000; CST), anti-AKT (1:1000; CST), anti-p-AKT (1:1000; CST), anti-CAT (1:1000; CST), anti-SOD2 (1:1000; CST), anti-Bim (1:1000; CST), anti-Bax (1:1000; Abcam, Cambridge, UK), anti-Bcl-2 (1:1000, ab59348; Abcam), anti-cleaved-caspase-3 (1:1000; CST), anti-acetyl-P53 (1:1000; Abcam), anti-TNF-α (1:500; Proteintech, Wuhan, China), or anti-IL-1β (1:500; Proteintech) at 4 °C overnight, followed by incubation with anti-rabbit or anti-mouse horseradish peroxidase secondary antibody at 20–25 °C for 2 h. The final protein expression was detected through electrochemiluminescence using the Chemidoc imaging system (Bio-Rad, USA), and the optical density of each band was quantified using the Image J software.

2.6. Co-immunoprecipitation (Co-IP) assay

Co-IP was performed to determine the expression of acetylated FOXO3a using a Pierce Co-IP kit, in accordance with the manufacturer's instructions (Thermo Fischer Scientific, 88804). In brief, the cells were lysed by incubation with 600 μL lysis buffer and 1′ Halt Protease & Phosphatase Inhibitor Cocktail at 4 °C for 40 min, followed by centrifugation at 14,000×*g* for 10 min. The supernatant was harvested and a small aliquot was reserved as the input group. The remaining samples were divided into two tubes: one was incubated with 4 μL anti-FOXO3a (1:50; CST) and the other, acting as a negative control, was incubated with 4 μL of IgG (Beyotime, Shanghai, China). After incubation at 4 °C for 3 h, 30 μL of protein A/G (Thermo Fisher Scientific) was added to the mixture and incubated on a rotator overnight at 4 °C. After washing, bead-bound proteins were eluted by denaturing in an appropriate amount of protein loading buffer at 95 °C for 5 min and centrifuged; the supernatant was used for Western blot analysis using anti-acetyl-lysine antibody (1:1000; CST).

2.7. RE mouse model

Four-to six-week-old male C57BL/6 mice purchased from the Institute of Laboratory Animal Sciences (Peking Union Medical College, Beijing, China). Animals were randomly divided into control, IR, IR + resveratrol (RES), and IR + RES + Ex527 groups, with 13 mice per group. In the IR group, abdominal irradiation was performed using high-energy X-rays (12 Gy, 225 kV) at 1.09 Gy/min. The IR + RES group received the same radiation dose and intraperitoneal injection of resveratrol (R5010; Sigma-Aldrich) (40 mg/

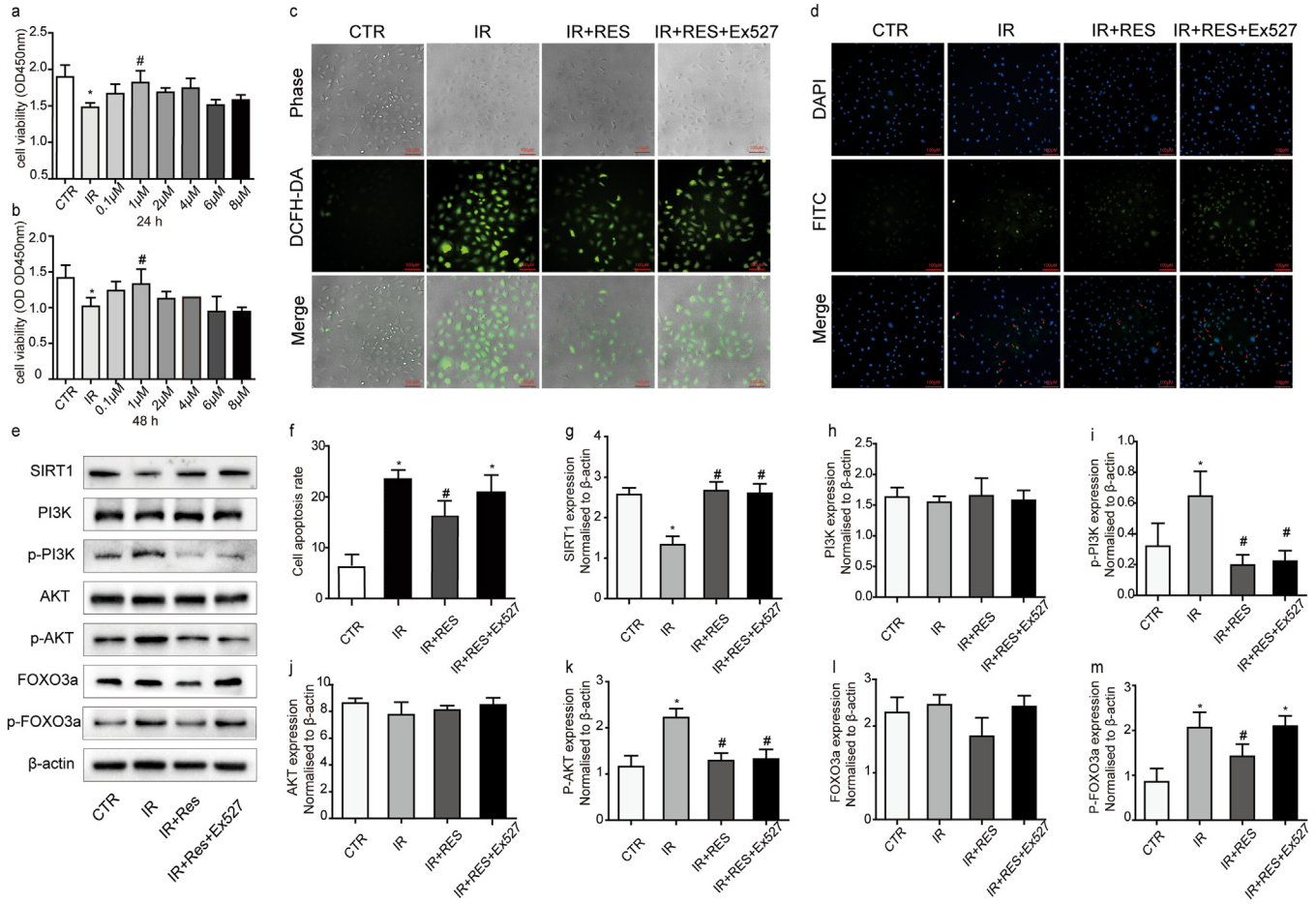

Fig. 1. Resveratrol (RES) protects IEC-6 cells from irradiation (IR)-induced injury by activating the SIRT1 pathway. (a) CCK-8 assay for measuring cell viability at 24 h after IR. (b) CCK-8 assay for measuring cell viability at 48 h after IR. (c) DCFH-DA assay for measuring ROS levels 6 h after irradiation. (d) TUNEL assay evaluating apoptosis at 24 h after irradiation. (e) Western blotting analysis of the expression of SIRT1, p-PI3K, PI3K, p-AKT, AKT, p-FOXO3a, and FOXO3a. (f–m) Data are presented as the mean ± SD of three independent experiments. $*P < 0.05$, compared with the control group; $\#P < 0.05$, compared with the IR group.

kg per day) from 3 days before irradiation to death. The IR + RES + Ex527 group received an intraperitoneal injection of Ex527 (E7034; Sigma-Aldrich, 10 mg/kg per day) 3 days before irradiation. The remaining treatments were the same as those used for the IR + RES group. The control group was subjected to sham irradiation. In each group, eight mice were used to record the general condition and mortality daily, and the other mice were euthanized to harvest the small intestinal tissue 3 days after IR.

All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Tianjin Union Medical Center.

2.8. Histological analysis

Small intestinal tissues from each mouse were fixed with formalin for 16 h and embedded in paraffin. Paraffin-embedded sections were cut into 4-μm sections, deparaffinized in xylene, and dehydrated with a series of gradient ethanol solutions. The sections were stained with hematoxylin (Baso, Zhuhai, Guangzhou, China) for 1 min and eosin for 2 min. For immunohistochemistry, heated citrate buffer solution (pH 6.0) was used for antigen retrieval at 100 °C for 15 min. The slides were rinsed with PBS for 5 min thrice and incubated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity; 10% goat serum was used to block nonspecific binding sites for 1 h. The tissue sections were then probed with anti-Ki67 antibody (ab16667, Abcam) overnight at 4 °C, washed with PBS, and treated with biotinylated IgG and horseradish peroxidase-labeled streptomycin for 20 and 15 min, respectively. Finally, the slides were stained with 3,3′-diaminobenzidine for 1–5 min, followed by hematoxylin staining, rinsing with PBS, and restaining with hematoxylin for 3 min. The samples were then dehydrated using an alcohol gradient (50%, 70%, 95%, and 100%) and cleared in xylene. Images were captured using a light microscope (OLYMPUS) after sealing with neutral gum.

For morphological analysis, six circular transverse sections were analyzed per mouse in a blinded fashion to determine the mean length of the ten longest villi, number of crypt cells per villus section, number of crypts per circumstance, and length of the basal lamina, using the Image-Pro Plus 5.1 software (IPP, Media Cybernetics, Rockville, MD, USA).

2.9. Statistical analysis

All data were analyzed using SPSS 21.0 (IBM Corp Armonk, NY, USA). The Kolmogorov-Smirnov method was used to test the data distribution. Data are expressed as mean ± standard deviation. Two-group comparisons were performed using Student's t-test,

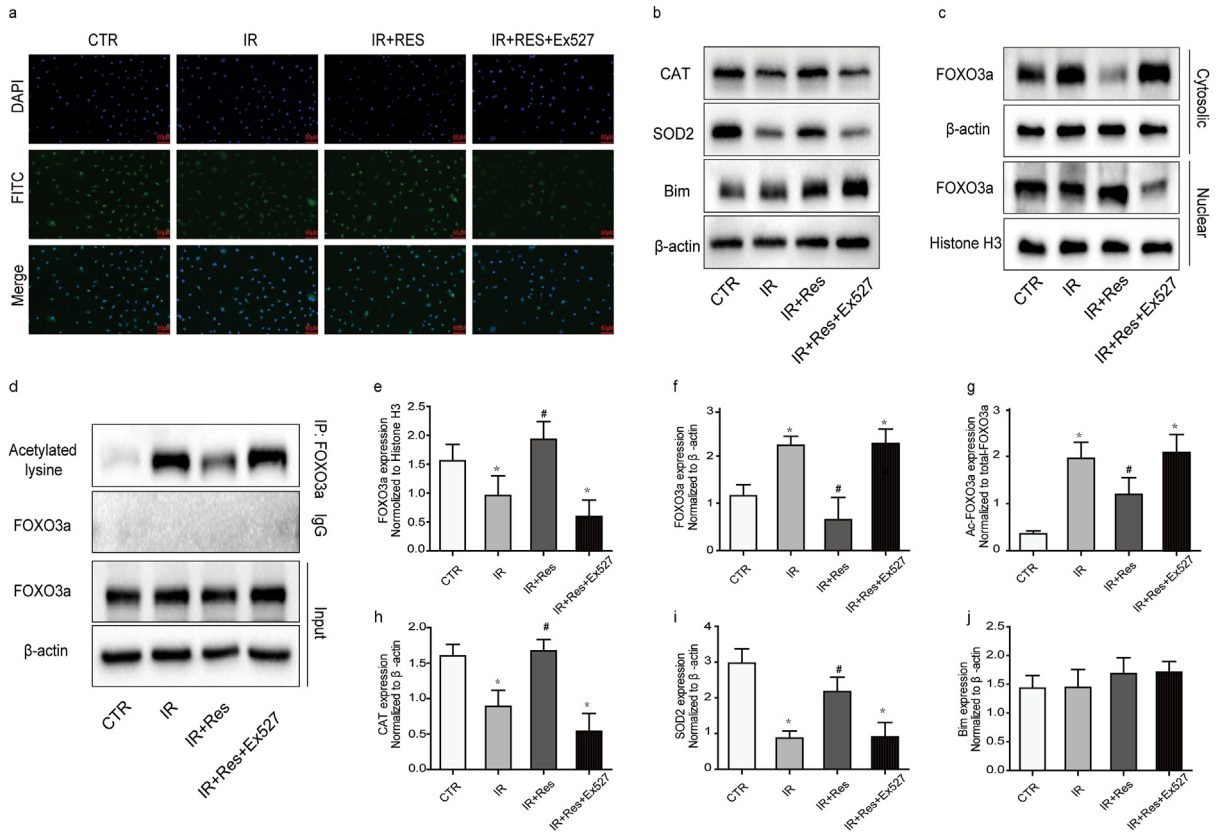

Fig. 2. Resveratrol (RES) promotes the expression of antioxidant enzymes after irradiation (IR) by regulating FOXO3a nuclear translocation. (a) Immunofluorescence analysis showing the subcellular localization of FOXO3a. (b) Western blotting for the expression of CAT, SOD2, and Bim. (c) Cytoplasmic and nuclear FOXO3a expression detected by western blotting. (d) Co-immunoprecipitation assay determining FOXO3a acetylation levels. (e–j) Data are presented as the mean ± SD of three independent experiments. *$P < 0.05$, compared with the control group; #$P < 0.05$, compared with the IR group.

and multiple groups were compared using one-way or two-way analysis of variance. The least-significant difference test was used when variances were homogeneous, and Dunnett's T3 test was used when variances were not homogeneous. The Kaplan–Meier method was used for survival analysis. Values of $P < 0.05$ (two-sided) were considered significant.

3. Results and discussion

3.1. Resveratrol protects IEC-6 cells from IR injury through inhibition of ROS generation and apoptosis via SIRT1 activation

CCK-8 and plate clone formation assays showed that cell growth was suppressed in a radiation dose-dependent manner (Supplemental Fig. 1a–d). Resveratrol, at 0.1–5 μM, exhibited no cytotoxicity toward IEC-6 cells; however, it showed cytotoxicity at 10 μM (Supplemental Fig. 1e and Supplemental Fig. 1f). Within the non-toxic resveratrol concentration range, cell viability was higher in irradiated cells pre-treated with low concentrations (0.1–1 μM) of resveratrol than in the irradiation group. However, higher concentrations of resveratrol (2–8 μM) did not have a significant effect on the viability of IEC-6 cells after irradiation (Fig. 1a, b). Therefore, 1 μM resveratrol was selected for subsequent experiments.

The apoptosis rate and ROS levels were significantly lower in the cells pre-treated with resveratrol than in the cells treated with irradiation. However, after treatment with the SIRT1 inhibitor Ex527, ROS levels and apoptosis rates increased again, suggesting that resveratrol protects IEC-6 cells from IR injury by activating the SIRT1 pathway (Fig. 1c, d).

3.2. The SIRT1/FOXO3a pathway is involved in the protective effect of resveratrol on IR-induced IEC-6 cell injury

The PI3K/AKT pathway is upstream of FOXO3a. AKT phosphorylates FOXO3a in the cytoplasm, thereby inhibiting its nuclear translocation [16]. A study demonstrated that resveratrol alleviates intestinal inflammation following irradiation through regulation of the PI3K/Akt/mTOR pathway [17]. Therefore, we investigated whether the protective effect of resveratrol against oxidative stress and radiation-induced intestinal injury involves the SIRT1/FOXO3a pathway. Resveratrol significantly attenuated the IR-induced upregulation of p-PI3K, p-AKT, and p-FOXO3a and the downregulation of SIRT1 expression (Fig. 1e). Downregulation of p-FOXO3a induced by resveratrol was significantly inhibited by Ex527, suggesting that resveratrol may effectively attenuate IR-induced oxidative stress by activating the SIRT1/FOXO3a pathway.

However, the expression levels of p-PI3K and p-AKT did not significantly differ between the cells pre-treated with resveratrol and cells co-treated with Ex527. One potential reason for this is that FOXO3a phosphorylation is co-regulated by the PI3K/AKT signaling pathway and autoacetylation. The acetylation of FOXO3a increases its translocation to the cytoplasm and promotes phosphorylation, which could inhibit transcriptional activity [18]. Alternatively, resveratrol is a non-specific compound with multiple physiological targets [19]. Thus, the ability of resveratrol to regulate the PI3K/AKT pathway may depend on its activity against molecular targets other than SIRT1.

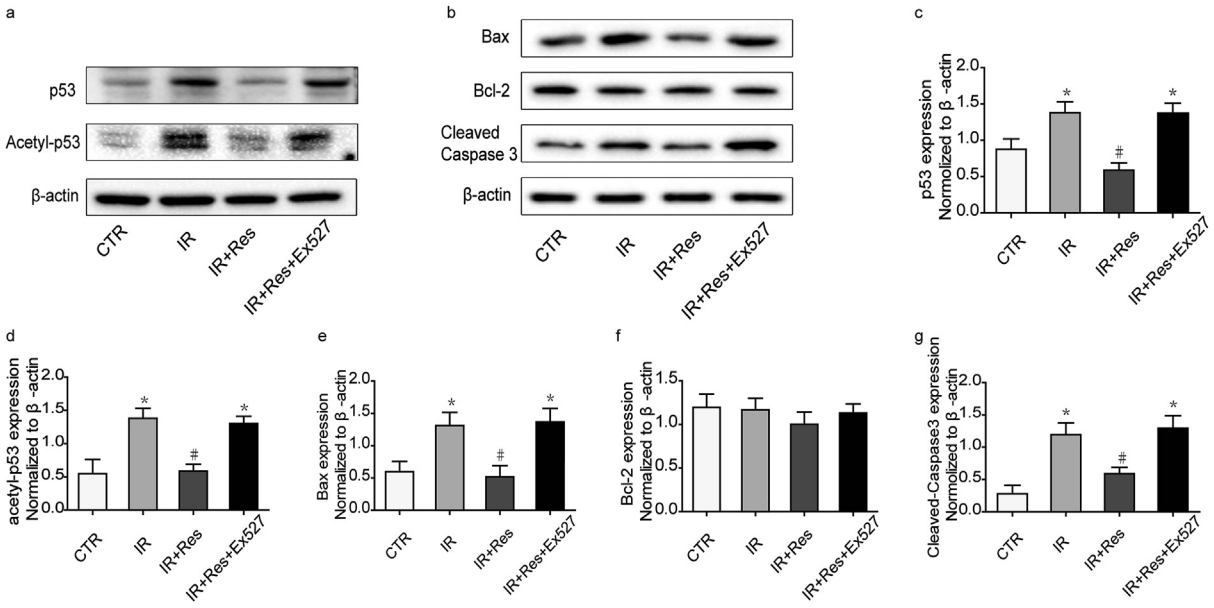

Fig. 3. Resveratrol (RES) inhibits radiation-induced apoptosis via the SIRT1/p53 pathway. (a) Western blotting for the expression of p53 and acetyl-p53. (b) Western blotting for the expression of Bax, Bcl-2, and cleaved caspase-3. (c–g) Data are presented as the mean ± SD of three independent experiments. *$P < 0.05$, compared with the control (CTR) group; #$P < 0.05$, compared with the irradiation (IR) group.

3.3. Resveratrol reduces ROS levels by regulating the nuclear translocation of FOXO3a after irradiation

To investigate the mechanism underlying the regulatory effects of SIRT1, we further examined the subcellular localization and acetylation of FOXO3a. Immunofluorescence analysis revealed significantly increased FOXO3a nuclear localization in the cells pre-treated with resveratrol, which was significantly decreased after

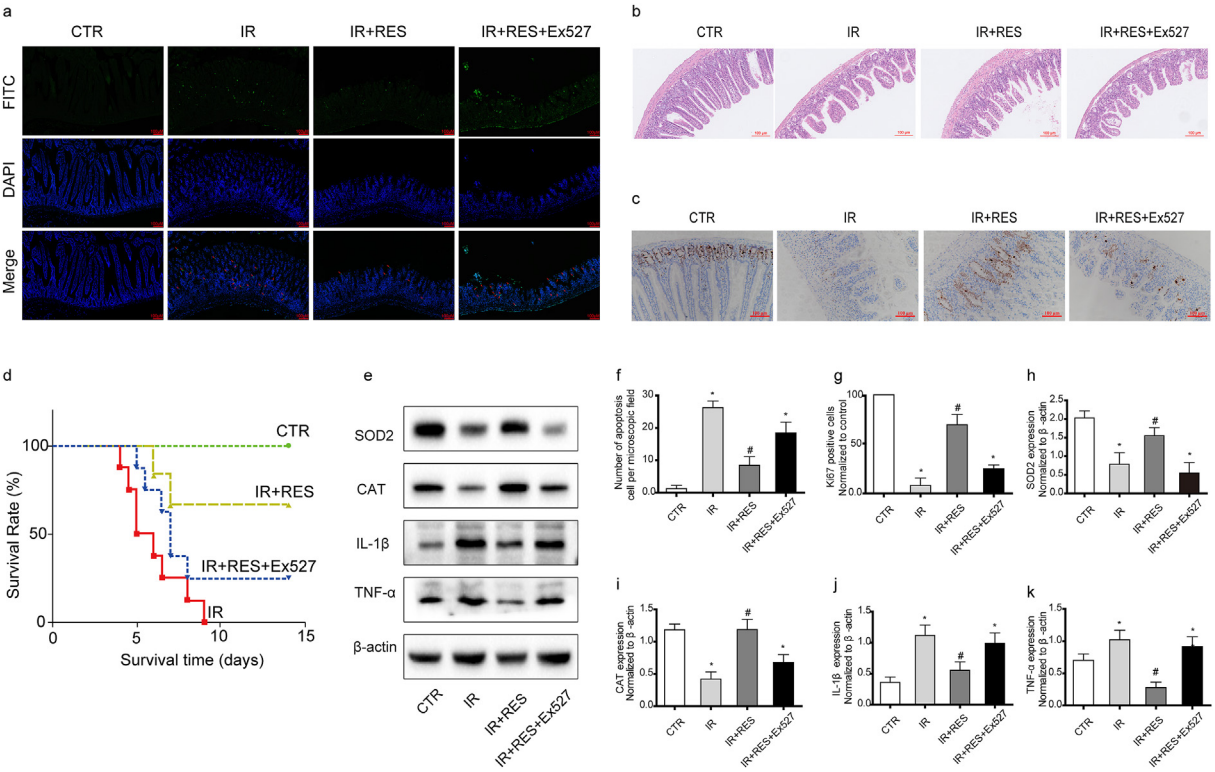

Fig. 4. Protective effect of resveratrol (RES) against radiation enteritis in C57BL/6 mice. (a) TUNEL assay for detecting apoptosis in intestinal crypt cells. (b) Hematoxylin-eosin staining for determining the length of the villi and the number of crypts. (c) Immunohistochemistry for Ki67 expression. (d) Survival curves for mice (n = 8/group). (e) Levels of SOD2, CAT, IL-1β and TNF-α determined through western blotting. (f–k) Data are presented as mean ± SD from three independent experiments (n = 5 mice/group). * Compared with the control group, P < 0.05; #Compared with the IR group, P < 0.05.

Ex527 treatment (Fig. 2a). Western blotting showed that IR reduced the FOXO3a expression level in the nucleus, which significantly increased in the cells pre-treated with resveratrol and then decreased significantly after treatment with Ex527 (Fig. 2c). The expression of acetylated FOXO3a was assessed after immunoprecipitation. FOXO3a acetylation was significantly decreased in the cells pre-treated with resveratrol and increased after Ex527 treatment (Fig. 2d).

The pathogenesis of RE has been largely attributed to the generation of ROS [5], and elimination of ROS can effectively alleviate radiation-induced intestinal injury [20]. SOD2 and CAT are important regulators of anti-oxidant stress [21]. FOXO3a is the upstream regulator of *SOD2* and *CAT* transcription, which are widely involved in antioxidant activity and apoptosis. FOXO3a activation protects cells from oxidative stress by directly binding to the *SOD2* promoter and enhancing its expression [22]. SIRT1 has a dual effect on FOXO3a function: SIRT1-mediated FOXO3a deacetylation prolongs the FOXO3a-dependent transcription of antioxidant enzymes to combat oxidative stress [22], whereas, under stress, SIRT1 suppresses the ability of FOXO3a to induce apoptosis by inhibiting the pro-apoptotic proteins Bim and FasL [14]. This bidirectional regulatory effect is beneficial for cell survival under stress [23]. We found that the expression of CAT and SOD2 was significantly downregulated after irradiation, whereas pre-treatment with resveratrol upregulated the expression of these proteins; these effects were reversed in cells treated with Ex527. However, Bim expression levels did not differ significantly among the groups (Fig. 2b). Therefore, resveratrol increased FOXO3a acetylation by activating SIRT1 and upregulating CAT and SOD2 but did not markedly influence the expression of Bim.

Overall, these results suggested that resveratrol promoted the nuclear translocation of FOXO3a via the PI3K/AKT pathway and deacetylated FOXO3a via SIRT1 activation, thereby enhancing its transcriptional activity. However, regulation of the PI3K/AKT signaling pathway by resveratrol may occur independently of SIRT1.

3.4. RES inhibits radiation-induced apoptosis via the SIRT1/p53 pathway

Apoptosis commonly occurs after cell injury. Following radiation exposure, the acetylation of p53 is rapidly increased and contributes to p53 stability, activity, and apoptosis induction [24,25]. After IR, the expression of p53, acetyl-p53, Bax/Bcl-2, and cleaved caspase-3 was significantly upregulated, and resveratrol pretreatment reversed these effects (Fig. 3a and b). However, improvements in these indicators were significantly inhibited upon treatment with Ex527, suggesting that resveratrol reduces radiation-induced cell injury by suppressing apoptosis through the SIRT1/p53 pathway.

3.5. Protective effect of resveratrol on RE in C57BL/6 mice

High doses of IR can cause gastrointestinal syndrome and death in mice [26]. Therefore, an RE animal model was established using C57BL/6 mice to observe the protective effect of resveratrol on IR. The survival rate was higher in the IR + RES group than in the IR group and was lower in the IR + RES + Ex527 group than in the IR + RES group (Fig. 4d). Compared with the IR group, the IR + RES group exhibited longer villi, more crypts, and more Ki67, a proliferative marker of maintained intestinal regeneration, positive cells (Fig. 4b, c) [27]. In addition, the expression of CAT and SOD2 was upregulated, and there were fewer TUNEL-positive cells following resveratrol treatment, indicating that resveratrol also exerts protective effects *in vivo,* and Ex527 inhibited these positive effects (Fig. 4a, e).

Moreover, the expression of inflammation-related proteins, such as IL-1β and TNF-α, was downregulated in the IR + RES group. However, compared with the IR + RES group, these changes were significantly less prominent in the IR + RES + Ex527 group (Fig. 4e). The accumulation of ROS following radiation can upregulate the expression of inflammatory factors and thereby leading to damage post-radiation [28,29]. Therefore, these results confirmed the protective effects of resveratrol against radiation-induced intestinal injury in C57BL/6 mice via the activation of SIRT1. However, the critical inflammatory factors IL-1β and TNF-α are regulated by multiple signaling pathways, such as the NF-κB, JAK/STAT, and NLRP-3 signaling pathways, and there is a complex crosstalk between these signaling pathways. Thus, the protective effect of resveratrol may be co-regulated by these mechanisms.

4. Conclusion

Overall, the findings of this study confirm the protective effect of resveratrol against radiation-induced intestinal injury by inhibiting oxidative stress and apoptosis via activating the SIRT1/FOXO3a pathway and suggest that the PI3K/AKT pathway is also involved in this process.

However, the present study has some limitations. Importantly, only one cell line was used for evaluating the mechanism underlying the effects of resveratrol, which might not be representative. Therefore, these results should be validated in additional cell lines. In addition, because of the off-target effects of resveratrol, the mechanism underlying the protective effects of resveratrol against radiation damage requires further exploration.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.03.122.

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