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Protective effect of quercetin on lung epithelial cell bystander-effect in the conditioned medium model of lung cancer metastasis induced by radiation

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ABSTRACT

To investigate the protective effect of Quercetin (Que) on lung epithelial cells (BEAS-2B) induced bystander effect (RIBE) after heavy ion irradiation of A549 cells. A549 cells were irradiated with 2 Gy X heavy ion rays to obtain a conditioned medium. BEAS-2B was incubated with a conditioned medium or Que. CCK-8 assay was used to screen the optimal effective concentration of Que and detect cell proliferation. Cell number was measured by cell counter and apoptosis rate was measured by flow cytometry. HMGB1 and ROS levels were measured by ELISA. Western blot was used to detect the protein expression of HMGB1, TLR4, p65, Bcl-2, Bax, Caspase3 and Cleaved Caspase3. The growth and proliferation rate of BEAS-2B decreased while the apoptosis rate increased after conditioned medium stimulation, and Que intervention inhibited this effect. The expression of HMGB1 and ROS increased after conditioned medium stimulation, and this effect was inhibited by Que intervention. In addition, the conditioned medium increased the levels of proteins of HMGB1, TLR4, p65, Bax, Caspase3 and Cleaved Caspase 3, and decreased levels of Bcl-2 protein, but Que intervention decreased the levels of HMGB1, TLR4, p65, Bax, Caspase3 and Cleaved Caspase 3proteins, and increased levels of Bcl-2 protein. The RIBE of BEAS-2B induced by irradiation of A549 is associated with HMGB1TLR4/NF-κB signaling pathway in conditioned medium inducing apoptosis by activating ROS, and Que may block RIBE-induced apoptosis by regulating HMGB1/TLR4/NF-κB pathway.

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Introduction

Radiotherapy remains an indispensable treatment modality for many malignancies (1). Heavy ion radiotherapy has unique biological and physical advantages, improving the efficacy of target organs but also reducing the radiation exposure of non-target organs (2). However, RIBE is one of the major problems in radiation therapy (3). RIBE is a phenomenon that exhibits irradiation effects due to signals received by non-irradiated cells from nearby irradiated cells. Therefore, neighboring or distant cells will present the same radiobiological endpoints such as apoptosis. Interestingly, in vitro studies have shown that RIBE involves mechanisms that closely resemble those of directly irradiated cells (4,5). Recent experimental evidence suggests that HMGB1, which is involved in cell-cell signaling communication, is also involved in the pathogenesis of radiation injury (6), and it has been shown that radiation-induced soluble cytokines promote the release of ROS (7), while high levels of ROS can lead to apoptosis (8-10). Que is a typical flavonol compound and is also considered a potential inhibitor of HMGB1 (11). Several studies have shown that Que has a wide range of biological effects, such as antioxidant, anti-inflammatory, and anti-apoptotic (12,13). Que decreased oxidative stress and apoptosis by inhibiting HMGB1 and its translocation (14). However, the effect of Que in reducing RIBE-induced oxidative stress and apoptosis by inhibiting HMGB1 and its translocation has not been reported.

Materials and Methods

Instruments and Reagents

Cell counter (Beckman, NO: Z2 Coulter); Lanzhou Heavy Ion Accelerator Research Device, Institute of Modern Physics, Chinese Academy of Sciences; Microplate Reader (BIO-RAD, NO: CYTAION1); Light Microscope (Nikon); 0.25% Trypsin (Hyclone, NO: J150049); CCK-8 Kit (Shanghai Dongren Company, batch number: GH8021), Que (Shanghai Yuanye Biological Co., batch number: s25563); FITC/Annexin-V Apoptosis Kit (BD, USA, batch number: 556547); HMGB1 Detection Kit (German IBL, batch number: REHM120); ROS Detection Kit (Shanghai Xitang Biological Co., Ltd., batch number: F02471); HMGB1 Antibody (GeneTex, GTX101277); TLR4 Antibody (GeneTex, GTX64330); p65 Antibody

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(Immunoway, YM3111); β-actin Antibody (Abcam, 8227).

Cell Culture

BEAS-2B (Shanghai Institute of Biological Sciences); A549 (Shanghai Institute of Biological Sciences); DMEM/F-12 medium (Gibco); cells were cultured in 35-mm dishes and placed in a 37 °C, 5% CO2, saturated humidity incubator. The medium was changed every 2 ~ 3 days, and after the cell fusion reached about 80%, 0.25% trypsin was digested and passaged, and the fifth passage of cells was used for the experiment.

Establishment of transfer conditioned medium model

In this experiment, the BEAS-2B bystander effect model induced by radiation of A549 cells was established by transfer conditioned medium mediated method. A549 cells were inoculated and cultured in a CO2 incubator for 24 h. Then the supernatant was discarded and 2 mL BEAS-2B medium was added, followed by heavy ion beam radiation treatment at a radiation dose of 2 Gy. After discarding the medium, 2 mL BEAS-2B medium was added again and placed in the incubator for another 3 h. Then the supernatant of A549 cells was collected and centrifuged at 1500 rpm for 10 min, and the supernatant was used as a conditioned transfer medium. The conditioned medium was transferred to pre-treated BEAS-2B and subsequent experiments were performed after 24 hours of intervention.

Irradiation Conditions and Experimental Groups

Irradiation was performed by Lanzhou Heavy Ion Accelerator Research Unit, Institute of Modern Physics, Chinese Academy of Sciences at a dose rate of 2 Gy/min (20 keV/um). The experiment was divided into a blank control group (Ctrl), transfer conditioned medium group (TCM), Quercetin group (QU), and transfer conditioned medium + Quercetin group (TCM + QU).

Screening the optimal effective concentration of Que by CCK-8 method

BEAS-2B and A549 in the logarithmic growth phase were inoculated into 96-well plates (5×10^3 per well, 100 μL) and transferred to a cell incubator until the cells adhered. 100 μL of Que drug was successively added at concentrations of 0, 6.25, 12.5, 25, 50, and 100 μM. After 1 day of culture, 10 μL of CCK-8 solution was added to each well and the culture was continued for 4 h. After termination of the culture, the optical density (OD) values of the cells in each group were measured with a full-wavelength microplate reader at 490 nm. The experiment was repeated three times. Cell growth curves were plotted with time as abscissa and proliferation rate as ordinate. Cell proliferation rate = (OD value of drug group - OD value of zeroing group)/(OD value of control group - OD value of zeroing group) × 100%.

Cell Counter Detects Number of BEAS-2B Cells

BEAS-2B in each group was cultured for 3 days, observed and photographed under an inverted microscope. When cells became smaller and rounded, digestion was terminated with 1 ml of DMEM dedicated medium. After the cells were repeatedly blown and mixed, 200 μL of cell suspension was drawn and diluted in 5 ml of cell buffer solution (PBS), mixed well and placed in a cell counter for counting, and the experiment was performed in three biological replicates.

CCK-8 assay was used to detect BEAS-2B proliferation

After 3 days of culture in the transfer-conditioned medium, BEAS-2B in each group was prepared into a single cell suspension, and the cell concentration was counted and adjusted with a cell counting plate, which was planted in a 96-well plate (3×10^3 cells/well) with an inoculum size of 100 μL in each well and the edge was filled with 200 μL PBS. After the cells adhered, the medium was discarded, zero-setting wells (serum-free medium) were set, 6 duplicate wells were set for each group, and DMEM medium was re-added and placed in the incubator for culture. After 24 h, 48 h and 72 h of culture, 10 μL of CCK-8 solution was added to each well, and 10 μL of CCK-8 solution was added to each well and placed in the incubator to continue incubation for 4 h. After termination of culture, the absorbance OD values of each group were measured directly at the wavelength of 490 nm, and the experiment was repeated three times.

HMGB1 and ROS levels were measured by ELISA

According to the manufacturer's instructions, the levels of HMGB1 and ROS in the culture supernatants of each group were detected by ELISA kits, and the experiment was performed in three biological replicates.

The apoptosis rate of BEAS-2B detected by flow cytometry

BEAS-2B in each group was cultured until day 3 to prepare single-cell suspensions, prepared into 5×10^5 cells, washed twice with PBS, added with 100 μL Binding Buffer and FITC-labeled Annexin-V (20 μg/mL) 10 μL, protected from light at room temperature for 30 min, added with PI (50 μg/ml) 5 μl, protected from light for 5 min, added with 400 μL Binding Buffer, and quantitatively detected by flow cytometry within 1 h.

Western blot for protein expression

Cell proteins were extracted from each group, protein concentration was measured by BCA assay, and protein concentration was adjusted with RIPA. In each group, 5 μL of protein was loaded, and after electrophoresis with 5% stacking gel and 12% separating gel, it was transferred to the PDVF membrane, and 5% skimmed milk was blocked at room temperature for 2 h. After incubation with the primary antibody in a freezer at 4 ° C overnight, the membrane was washed with PBS three times the next day for 10 min, and the secondary antibody was incubated. After visualization with the developer, the X-ray machine was used for visualization, and the corresponding protein bands were scanned with a scanner, and finally, the bands were analyzed grayscale with Image J software, and β-actin was used as an internal reference to calculate the relative expression of cellular proteins in each group.

Statistical analysis

Statistical analysis was performed using SPSS 23.0 software. Measurements conforming to a normal distribution were expressed as standard deviations of the mean and compared between multiple groups using a one-way ANOVA with a completely randomized design. $P < 0.05$ was considered statistically significant.

Results

Effect of Different Concentrations of Que on Bystander BEAS-2B Proliferation

Que was screened for its effect on A549 and BEAS-2B, and the concentration that could inhibit A549, but not BEAS-2B, was selected. Que was found to inhibit the proliferation of A549 but not the concentration of BEAS-2B at 12.5 μM ($P < 0.01$) (Figure 1), so 12.5 μM was chosen for subsequent experiments.

Inhibition of transfer conditioned medium on bystander BEAS-2B viability and protection of Que

After the conditioned medium intervention of BEAS-2B, BEAS-2B cell viability and count rate were decreased compared with the Ctrl group ($P < 0.05$), and the cell viability (Figure 2A, C) and count rate (Figure 2B) of BEAS-2B were increased after Que addition compared with the TCM group ($P < 0.05$, $P < 0.01$). It was demonstrated that a conditioned medium could inhibit BEAS-2B proliferation, while Que could effectively promote BEAS-2B proliferation.

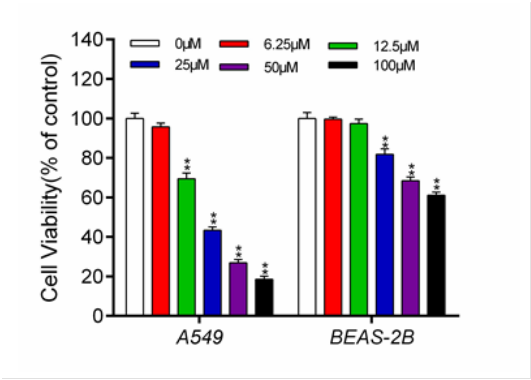

Figure 1. Effect of different concentrations of Que on 24h OD values of A549 and BEAS-2B. All data are presented as mean ± SD (n = 3), and comparisons between groups showed statistical significance ($p < 0.05$ or $p < 0.01$).

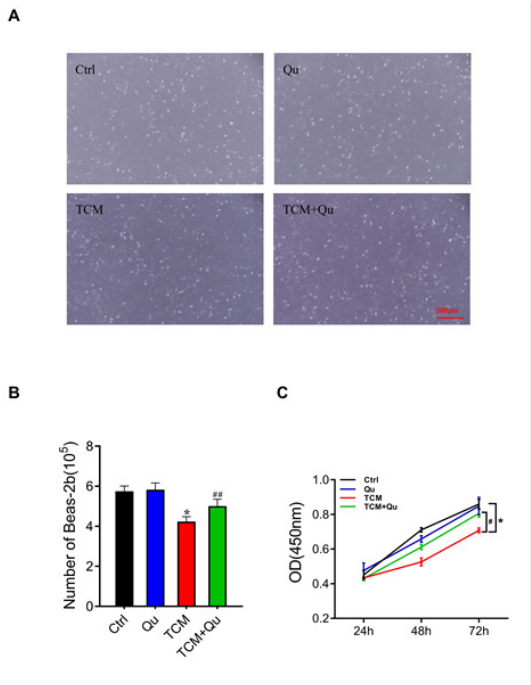

Figure 2. Que promote proliferation of bystander cells. **(A)**The morphology of Co-cultured after radiation and Que-treated BEAS-2B for 72 h. Images were taken using a microscope. **(B)** Count the number of BEAS-2B at 72h in each group using a cell counter. **(C)** CCK-8 assay was used to detect the proliferation rate of BEAS-2B in each group for 72 hours. All data are presented as mean ± SD (n = 3), and comparisons between groups showed statistical significance ($p < 0.05$ or $p < 0.01$).

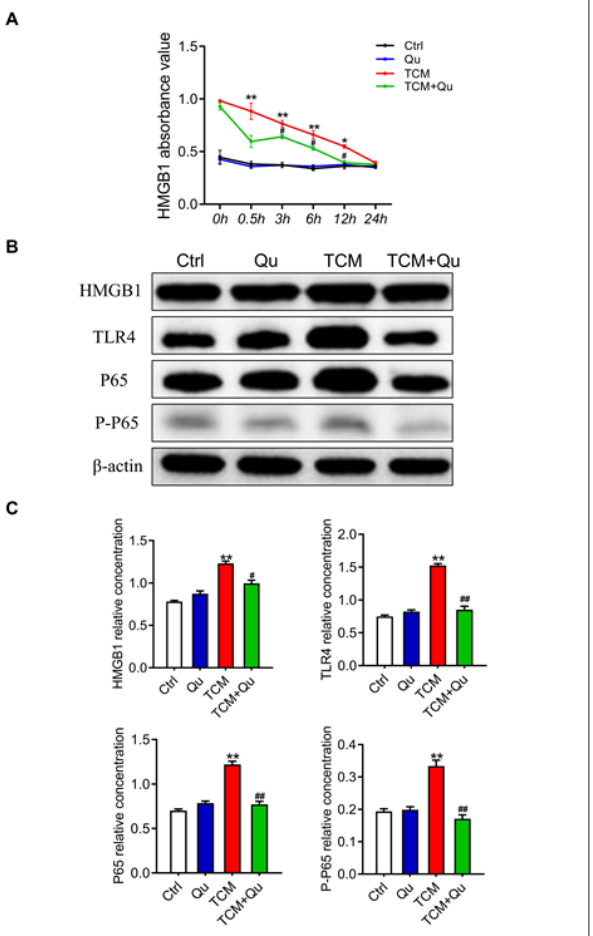

Figure 3. Que downregulates HMGB1/TLR4/NF-κB pathway-related protein expression in bystander BEAS-2B.(A) Relative concentrations of HMGB1 were determined by ELISA for each group from 0 to 24 hours. **(B)** The western blotting bands of HMGB1, TLR4, P65 and P-P65. **(C)** The protein statistical result of HMGB1.

The HMGB1/TLR4/NF-κB signaling pathway is activated in bystander BEAS-2B and Que regulates protein expression of this pathway

After conditioned medium intervention with BEAS-2B, HMGB1 in the medium increased from 0 h to 12 h compared with the Ctrl group. Compared with the TCM group, HMGB1 in the medium decreased from 0.5 h to 12 h after the addition of Que (Figure 3A), demonstrating that Que could effectively reduce the level of HMGB1 in the conditioned medium ($P < 0.05$, $P < 0.01$).

At the protein level, HMGB1, TLR4, and NF-κB protein expression in BEAS-2B were increased compared with the Ctrl group ($P < 0.01$); HMGB1, TLR4, and NF-κB protein expression in BEAS-2B was decreased after Que addition compared with the TCM group ($P < 0.01$) (Figure 3B, C). These findings suggest that HMGB1/TLR4/NF-κB signaling pathway is aberrantly activated in BEAS-2B and Que can downregulate the abnormal elevation of these proteins.

RIBE promotes ROS production by bystander BEAS-2B, and Que inhibits its development

After conditioned medium intervention with BEAS-2B, ROS in the medium increased from 0 h to 12 h compared with the Ctrl group. Compared with the TCM group, ROS in the medium decreased from 0.5 h to 12 h after the addition of Que (Figure 4), demonstrating that Que could effectively reduce the level of ROS in the conditioned medium ($P < 0.01$).

RIBE promotes apoptosis in bystander BEAS-2B and Que inhibits its apoptosis

Compared with the Ctrl group, the apoptosis rate of BEAS-2B increased after conditioned medium intervention ($P < 0.05$), and decreased after Que addition ($P < 0.01$) compared with the TCM group (Figure 5A, B). demonstrated that a conditioned medium could induce BEAS-2B apoptosis, while Que was able to effectively reduce bystander BEAS-2B apoptosis.

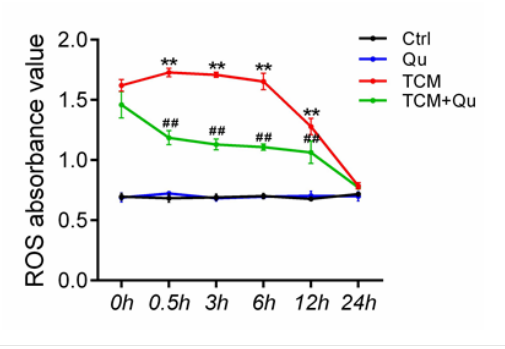

Figure 4. Relative concentrations of ROS were determined by ELISA for each group from 0 to 24 hours. All data are presented as mean ± SD (n = 3), and comparisons between groups showed statistical significance ($p < 0.05$ or $p < 0.01$).

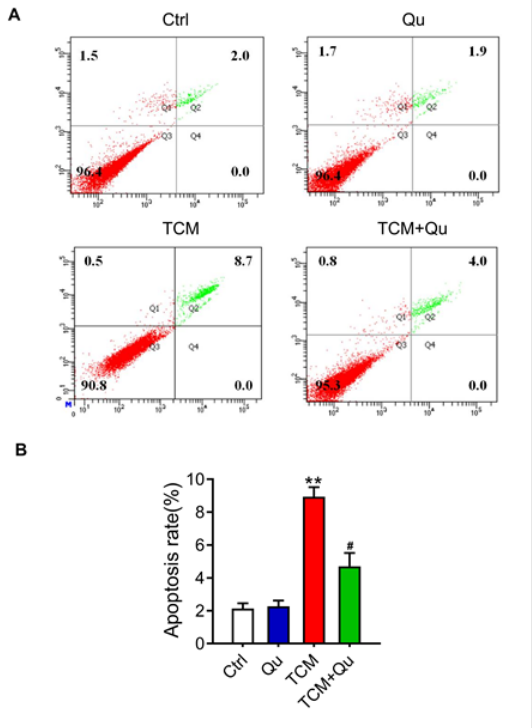

Figure 5. Que inhibits apoptosis of bystander BEAS-2B. (A) Flow cytometric analysis of cell apoptosis was conducted using Annexin V-FITC+PI staining. **(B)** Percentage of apoptotic cells in each group. All data are presented as mean ± SD (n = 3), and comparisons between groups showed statistical significance ($p < 0.05$ or $p < 0.01$).

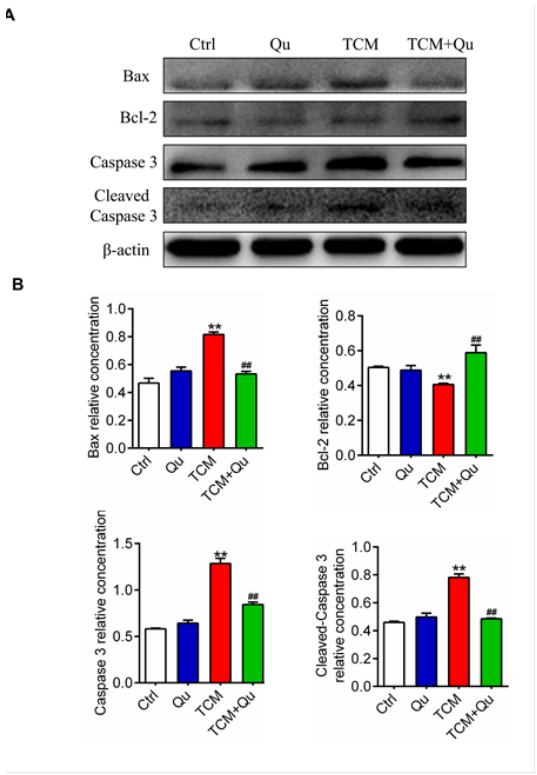

Figure 6. Que reduces the expression of apoptotic proteins of bystander BEAS-2B . **(A)** The western blotting bands of Bax, Bcl-2, Caspase3 and Cleaved Caspase 3. **(B)** The protein statistical result of Bax, Bcl-2, Caspase3 and Cleaved Caspase 3. All data are presented as mean ± SD (n = 3), and comparisons between groups showed statistical significance ($p < 0.05$ or $p < 0.01$).

Effect of Que on Bystander BEAS-2B Apoptotic Protein Induced by RIBE

Compared with the Ctrl group, the anti-apoptotic protein Bcl-2 decreased, the pro-apoptotic protein Bax increased, and the apoptotic endpoint proteins Caspase 3 and Cleaved Caspase 3 increased in BEAS-2B after the conditioned medium intervention; after Que addition, the anti-apoptotic protein Bcl-2 increased, the pro-apoptotic protein Bax decreased, and the apoptotic endpoint proteins Caspase 3 and Cleaved Caspase 3 decreased in BEAS-2B, and the differences were statistically significant ($P < 0.05$) (Figures 6A and B). demonstrated that Que was able to effectively reduce bystander BEAS-2B apoptotic protein levels and protect BEAS-2B from undergoing apoptosis.

Discussion

Radiation therapy is currently one of the main treatments for lung cancer and can significantly improve the survival rate and local tumor control rate of lung cancer patients (15). Radiation not only kills tumor cells but also damages unirradiated cells. RIBE can cause DNA fragmentation, growth inhibition, apoptosis and even carcinogenesis in cells (16, 17) . It has been found that irradiated lung cancer cells can cause damage to BEAS-2B due to the effect of RIBE (18). In the study of RIBE, the transfer-conditioned medium model is one of the most common biological models, which can transfer signaling molecules such as cytokines produced by irradiated cells

into unirradiated cells. Many RIBE signaling factors have been identified in a model of transfer radiation conditioned medium, such as IL-6, IL-1, TNFα, TGF-β, ROS, and NO (19). Que is a polyphenol bioflavonoid with a wide range of pharmacological effects, including antioxidant, anti-inflammatory, and anti-apoptotic properties (12, 13). In the radiation-conditioned medium model, we found that BEAS-2B proliferation was inhibited and the apoptosis rate was increased, and Que intervention was able to effectively reduce the inhibition of BEAS-2B proliferation induced by RIBE and had a protective effect on BEAS-2B apoptosis.

At present, the basic mechanism of RIBE remains unclear. The release and transmission of RIBE injury factor are mainly through three ways: gap junction, exosome action and release of soluble signaling molecules. There are many soluble signaling molecules involved in cell-cell signaling communication, such as TNFα, IL-6, ROS and HMGB1 (20). HMGB1 is a multifunctional protein secreted mainly by monocytes and macrophages (21), and organisms can lead to translocation and release of HMGB1 after exposure to ionizing radiation (22, 23), while HMGB1 protein is involved in radiation pathogenesis, and blocking its release can alleviate radiation injury (6). HMGB1 activates the downstream NF-κB signaling pathway by binding to the transmembrane receptor TLR4, inducing an inflammatory response in the body and further aggravating tissue damage (24, 25). NF-κB signaling is involved in the pathogenesis of various radiation-induced radiation injuries (26-28) and activated NF-κB promotes cytokine expression and elicits systemic and distant effects in tissues after irradiation (29). In this experiment, the expression of HMGB1, TLR4 and NF-κB was significantly increased in the medium model of transfer radiation-conditioned medium after heavy ion irradiation, so the activation of HMGB1/TLR4/NF-κB pathway is one of the factors causing RIBE. However, after Que intervention, it could effectively reduce the expression of HMGB1 and TLR4 and inhibit and NF-κB p65 activation in the model. Activated NF-κB has been shown to regulate ROS generation (30, 31), while ROS can activate intrinsic mitochondrial pathway, extrinsic death receptor pathway, and ER stress to induce apoptosis (32), which is a potent stimulator of apoptosis (8-10), and effective inhibition of RIBE by scavenging reactive oxygen species has been reported in many previous studies (33-35). In this experiment, the ROS level in the conditioned medium of radiation transfer was significantly increased, and decreased after the addition of Que, demonstrating that Que could effectively reduce the ROS level in the conditioned medium. Our data showed that Que could down-regulate HMGB1, TLR4, and NF-κB expression, and inhibit the level of downstream signaling ROS.

ROS can cause activation of mitochondrial pathways (36), and previous studies have shown that ROS generated by RIBE can cause mitochondrial damage. When the damage cannot be repaired, some cells will initiate the apoptotic pathway, most commonly the mitochondrial apoptotic pathway (37). The mitochondrial pathway is essential in the mechanism of apoptosis, and Bax, Bcl-2, and caspase-3 are its main regulatory proteins. Bcl-2 family is an important regulator of apoptosis, Bax is the earliest proapoptotic protein found in the Bcl-2 family, and Bcl-2 is a tumor suppressor protein, which can neutralize proapoptotic factors or inhibit the release of proapoptotic factors. When Bax expression increased and Bcl-2 expression decreased, apoptosis was promoted, Bax expression decreased, and Bcl-2 expression increased, apoptosis was inhibited (38). Caspase-3 is one of the central proteins involved in the execution of apoptosis, which leads to apoptosis by inducing mitochondrial membrane channel opening and permeability changes (39). Bax and Bcl-2 can act as upstream regulators of caspase-3, and Bax expression can further activate the caspase cascade in mitochondria when it increases, ultimately activating the apoptotic endpoint protein caspase 3 to induce apoptosis (40). When Bcl-2 expression is reduced, it prevents the downstream caspase cascade, thereby inhibiting apoptosis (41). From the results of this experiment, Bcl-2 expression was decreased, while Bax, Caspase3 and Cleaved Caspase 3 expression was increased in a radiation-transferred conditioned medium. Bcl-2 expression in the medium increased, while Bax, Caspase3 and Cleaved Caspase 3 expression decreased after Que addition, demonstrating that Que can effectively regulate the levels of apoptosis-related proteins in the conditioned medium.

Conclusions

In summary, the HMGB1/TLR4/NF-κB signaling pathway plays an important role in RIBE-induced apoptosis and regulates the expression of apoptosis-related proteins (Bcl-2, Bax, caspase3, and cleaved caspase 3) by inhibiting the level of downstream signaling ROS. In this paper, the mechanism of Que intervention was explored from the apoptotic regulation of the HMGB1/TLR4/NF-κB signaling pathway, indicating that Que may be a promising drug for the treatment of RIBE injury. These antioxidant and anti-apoptotic properties of Que in preventing and treating RIBE warrant further clinical investigation.

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Disclosure statement

The authors reported no potential conflict of interest.

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article.

Author contributions

Jintian Li and Juan Li: Conceptualization, Methodology, Software, Writing- Reviewing and Editing. Tao Hong: Data curation, Writing-Original draft preparation. Miao Su, Shuanggui Du and Jianqing Liang: Data curation, Investigation. Yi Zhang: Visualization, Supervision. Yiming Zhang, Zhiming Miao, and Tianxing Ma: Software, Va-

lidation. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work, ensuring integrity and accuracy.

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Ethics approval and consent to participate

No human or animals were used in the present research.

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