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, 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, Bcl-2, Bax, Caspase3 and Cleaved Caspase3, 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.
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 passed, 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 was induced by irradiation 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 × 10^4 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-wave-length microplate reader at 490 nm. The experiment was repeated three times. Cell growth curves were plotted with absorbance OD as the ordinate and proliferation rate as the abscissa.

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 PVDF membrane, and 5% skimmed milk was blocked at room temperature for 2 h. After incubation with the primary antibody for 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 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.

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 was 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.

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 the 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 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, Bel-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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