





The Protective Effects and Mechanism of Doxepin on Radiation-Induced Lung Injury in Rats

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Abstract

Radiation-induced lung injuries (RILI) is one of the serious complications of radiotherapy posed by the damage of alveolar cells and inflammation over-reaction. We aimed to investigate the potential protective effects of doxepin on RILI (20 Gy total dose at 3 Gy/min of X-ray irradiation), as well as its underlying mechanism. For animal experiments, such parameters as Immunohistochemistry and hematoxylin and eosin (H&E) staining, WBC (white blood cell), CRP (C-reactive protein), Western blot, and q-PCR were detected. The results indicated that both survival status and weight increase of irradiated rats treated by doxepin (3 mg/kg/day, rat) were higher than those of treated with irradiation alone (Dosing started the day before irradiation). Further, histological examinations showed doxepin could attenuate the radiation injury, as indicated as alveolar inflammatory exudation and there was only mild interstitial inflammation infiltration. Western blotting and q-PCR showed that expression of NF- κ B in X group were higher than that in XMD group. For the first time, we reported doxepin functioned as a radioprotectant candidate, which provide a promising application of doxepin for protecting radiotherapy injuries.

Keywords

doxepin, radiation-induced lung injuries, NF- κ B, JNK

Introduction

Radiation therapy, commonly used for malignancies treatment, is hampered by exposure and damage to adjacent normal tissues which lead to radiation-induced lung injury (RILI),¹ this is one of the most serious obstacles to patients with thoracic or breast cancer who undergo therapeutic radiation^{2,3} and hinders the validity of therapeutic radiation doses and generates adverse effect in cancer patients.^{4,5}

Radiation-induced lung injuries has a relatively long latent stage, radiation pneumonia appears in the early stages, as well as radiation-associated lung fibrosis.⁶ Vascular injury and atrophy appear in the late stage.^{7,8} Normally, the obvious acute inflammatory reaction of RILI will be found at 6–12 weeks after radiation. RILI has many complex pathological mechanisms and involves in numerous cell types and signaling pathways.⁹ Moreover, acute inflammation could produce acute respiratory distress syndrome (ARDS) and death. The

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exact mechanism of radiation-induced inflammatory responses in lung remains unclear, hypoxia, oxidative stress, immunocytes, and cytokines might all play crucial roles in the development of radiation-induced pneumonitis. It has been reported that NF- κ B¹⁰ is related to radiation and inflammatory mechanisms, and the pathogenesis of RILI is also related to inflammatory infiltration. Therefore, it might be possible to treat and prevent RILI via the NF- κ B/JNK pathway.

However, there is no effective treatment for RILI in clinical practice. Some experts recommend systemic corticosteroids to treat symptoms of Radiation Pneumonitis (RP),¹¹ while pneumonia may recur after steroids treatment. A single case study also reported the effectiveness of azathioprine and cyclosporine.^{12,13} Amifostine is a free radical scavenger, which has been shown to significantly reduce the incidence of grade 2 or higher pneumonia in patients with advanced lung cancer receiving radiotherapy.¹⁴ Unfortunately, its use is limited due to its significant side effects and poor tolerability.

The tricyclic antidepressants (TCAs) like doxepin have been proved to function not only as an antidepressant but also for other effects.¹⁵ It is reported that TCAs can make a significantly improved degree of pulmonary fibrosis and lung function^{16,17}; the underlying pharmacological mechanisms have not been fully elucidated. Doxepin, an antidepressant and anti-insomnia drug, may also be a potential anti-inflammatory and anticonvulsant drug. Therefore, the research on the anti-inflammatory and protective effects of doxepin and its molecular mechanism has become an important subject in pharmacology and clinical medicine.

In this study, we aim to find whether Doxepin has a protective effect on radiation lung injury via its potential anti-inflammatory effects, which may provide an economical agent for clinical tumor radiotherapy.

Methods

Experimental Drugs and Rats

Doxepin for experimental use was purchased from Shanghai Qingping Pharmaceutical Co., Ltd Adult, male, Sprague-Dawley (SD) rats (*Rattus norvegicus*) purchased from the Laboratory Animal Technology Center, Vital River (Shanghai, China), weighing about 150 to 200 g.

Experimental Design

The healthy Sprague-Dawley (SD) rats (n=48) were under constant conditions (temperature 25±3°C, 50% humidity), 12/12hour light/dark cycle, housed in an experimental animal room, and were free to drink and eat (feed was purchased from Synergy Pharmaceutical Bioengineering Co., Ltd). After 1 week of acclimation, the rats were randomly and equally divided into 6 groups (n=8/group) using random number table: control group (C group); doxepin without irradiation group (D, group); X-ray irradiation only group (X group); and

irradiation with doxepin (dissolve with double distilled water): with High concentration (XHD group); with medium concentration (XMD group); with low concentration (XLD group). XHD group was treated with a doxepin concentration of 10 mg/kg/day, XMD group with a concentration of 3 mg/kg/day, and XLD group with a concentration of 1 mg/kg/day, starting from the day before irradiation, once a day, until the end of the animal experiment. Doxepin of the corresponding mass was weighed and dissolved in an appropriate volume of ddH₂O (double distilled water) (e.g., .5 mL), and the rats were given intragastric administration with the prepared solution. The dose (3 mg/kg) of the experimental rats is equivalent to about 29 mg of doxepin in a 70 kg human, which is in the range of human treatment for depression. Irradiations were conducted with an X-ray generator (X-RAD 320 ix, Precision X-ray Inc., North Branford, CT, USA) at a dose of 20 Gy and a dose rate of 3 Gy/min.

The rats were anesthetized by intraperitoneal administration of 10% chloral hydrate (200 mg/kg) before radiation. Rats were fixed on an organic glass pedestal and fully stretched. We use a 1 cm thick stereotype to punch holes, only the chest of the rat is exposed, and other parts are covered. The whole thorax received a single dose of 20 Gy (3 Gy/min, X-ray).

In these rats, the left lower lobe of each group was fixed with 4% paraformaldehyde to make paraffin slices, using HE staining to proceeding histological examinations. Hydroxyproline was measured in the homogenate of the left upper lobe to evaluate the extent of radiation-induced lung injury and the improvement of the drugs on the lung. The right lobe of each group was left at -80° for the assessment of inflammatory cytokines and proteins by qRT-PCR, Western blot, and ELISA to study the ameliorative effect mechanism of doxepin on radiation-induced lung injury.

Measurement of White Blood Cell and C-Reactive Protein

After anesthesia, the abdominal cavity was exposed, and 5 mL of blood was collected from the abdominal aorta immediately. Blood samples were placed in a test tube containing EDTA (Ethylene Diamine Tetraacetic Acid) anticoagulant to count the total number of white blood cells. The blood samples were stained with Turk (a cell stain, mainly composed of acetic acid and crystal violet) solution (1:10 dilution), and the total white blood cells (Includes neutrophils, eosinophils, basophils, lymphocytes, and monocytes) were counted repeatedly in the hemocytometer (count/ml). We performed the WBC test (Blood was collected through the tail vein) 5 times during the experiment, 1 day before irradiation, 3 weeks, 6 weeks, 9 weeks, and 12 weeks. We have organized this part of the data in this paper and redrawn the WBC time-varying curve into the text to supplement the previous research results. According to morphological standards, 10 cells were counted under a light microscope for analysis.

A tissue lysis kit was used to extract proteins from the lung samples and all lung tissues were cleared of blood during the analysis. BCA (Bicinchoninic Acid) protein detection kit (P0012, Beyotime, China) was used to determine the protein concentration. Enzyme-linked immunosorbent assay (ELISA) kit (PC188, Beyotime, China) was used to quantify CRP levels (Microplate reader, BIO-RAD).

Histopathological Assessment of Lung Tissue

The lung samples of each group were fixed in 10% paraformaldehyde for 24 h, and the fixed lungs were embedded in paraffin wax and cut into 4 μ m thickness slices using a microtome. Lung tissue sections were stained with hematoxylin and eosin (H&E), for investigation of lung inflammation and collagen deposition. The previous literature¹⁸ used a detailed semi-quantitative method for the study of pulmonary fibrosis in rats, and this study used a semi-quantitative method based on the previous research method.

Western Blot

As previously mentioned, proteins were extracted using a tissue lysis kit (P0013 E, Beyotime, China) from pulmonary samples. Tissues marked C, D, X, and XMD were taken from the lungs with total protein extracted with RIPA (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, .1% SDS, sodium orthovanadate, sodium fluoride, EDTA, and leupeptin). The concentration of each sample was determined by BCA. The samples were then mixed with 2 \times Loading Buffer for SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel electrophoresis, followed by being transferred to NC membrane with a constant current of 300 mA for 110 min. The membranes were blocked in 5% (mass fraction) skim milk for 1h at room temperature. Membranes were washed in TBST (Tris-HCl, NaCl, tween20) 3 times and then incubated with the following primary antibodies (1:1000 dilution) at 4°C overnight: anti-NF- κ B (Abcam, ab16502), and anti-JNK (SANTA, sc-7345). An anti- β -actin (Diagbio, db7283) antibody was used to confirm equal protein loading. After 3 times of wash, the membranes were incubated with secondary antibody (1:10 000 dilution) for 1h at room temperature. Eventually, protein expression was detected using ECL (enhanced chemiluminescence) luminescent liquid.

Q-PCR (Quantitative Real Time PCR) Analysis

The RNA of lung tissues was extracted according to Trizol method, and the purity and concentration of RNA were detected by ultraviolet spectrophotometer. The concentration of RNA was diluted to appropriate concentration by DEPC (diethyl pyrocarbonate) H₂O. The first strand of cDNA was synthesized according to the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (#K1622) instructions (RevertAid Reverse Transcriptase, RiboLock RNase Inhibitor,

5X Reaction Buffer, dNTP Mix, Oligo (dT)18 Primer, Random Hexamer Primer, Control GAPDH RNA, 10 μ M Forward GAPDH Primer, 10 μ M Reverse GAPDH Primer, and nuclease-free water) and stored at -20°C for further use. The primers were designed and produced by Biotech (Shanghai) Co., Ltd Then cDNA was used as a template for qPCR analysis: 6.8 μ L ddH₂O, 10 μ L SYBR, .6 μ L of the upstream and downstream primers, and 2 μ L of the template cDNA. The $2^{-\Delta\Delta\text{Ct}}$ (Ct: cycle threshold) was used to analyze the mRNA level, see Table 1 for primer sequence.^{19,20}

Hydroxyproline Detection

HYP was detected in lung homogenate samples using HYP kit (Nanjing Jiancheng Bioengineering Institute). Samples were weighed and pH adjusted according to the manufacturer's instructions. The volume was diluted to 10 mL and activated carbon was added followed by centrifuge. According to the kit instructions, 1 mL of supernatant was added to the corresponding reagents of the kit and incubated in 60°C water bath for 15 minutes. After centrifuge at 3500 r/min for 10 minutes, the level of hydroxyproline was measured with a microplate reader and the absorbance represented HYP level.

Statistical Analysis

The data were presented as mean \pm SD (Standard Deviation) and statistically processed by using SPSS (Statistical Product and Service Solutions). Graphpad Prism 9 was used to create the artworks. Independent-sample t-test and one-way analysis of variance (ANOVA) were used for comparisons of measurement data. $P < 0.05$ was considered to be statistically significant.

Result

Survival Status, Weight Changes, and Clinical Manifestations of Rats

As seen in Figure 1 (A, B, and C), compared with the radiation-only group (X group), 4 of 8 rats died, only 3 of 8 rats died in XLD group, 1 of 8 rat died in both XMD and XHD group, suggesting the protective roles of doxepin. At the end of the 12 week, XHD and XMD groups showed no difference; meanwhile there were no deaths in the control group (C group) and 1 death in doxepin only group (D group) due to suffocation during the drug administration.

The difference of weight gain (Figure 1(d)) was found in the first 4 weeks after treatments but not significantly in the rest of the process. One week after the treatment, the X group gained the minimum weight, and the C group gained the maximum weight. Additionally, the administration of doxepin, to a certain extent, alleviates the deceleration of increase posed by irradiation. During the middle and late

Table I. Primer sequences for Quantitative Real-Time -PCR.

Gene	Forward prime (5'-3')	Reverse prime (5'-3')	Reference
NF- κ B	ACCTGAGTCTTCTGGACCGCTG	CCAGCCTTCTCCCAAGAGTCGT	[19]
JNK	TGTGTGGAATCAAGCACCTTC	AGGCGTCATCATAAACTCGTTC	[20]
β -actin	CGTGGACATCCGCAAAGACCTG	TGGGAGCCAGAGCAGTGATCTC	None

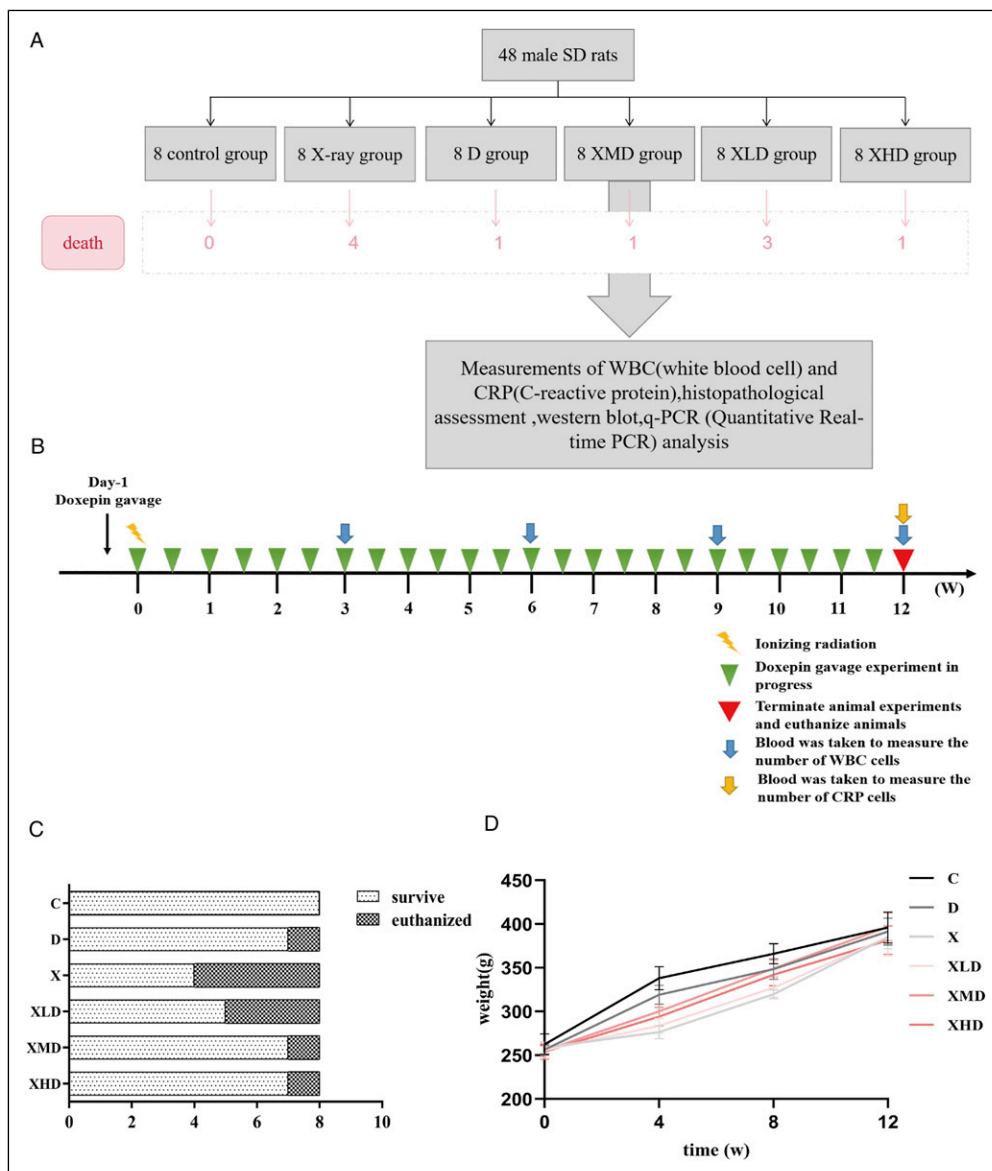


Figure 1. Experiment flow chart, survival and weight change chart of each group. **(A)** Experiment flow chart, grouping situation and experiments done in this paper **(B)** Timeline of irradiation administration in animal experiments. **(C)** Survival status of each group. **(D)** weight changes of each group.

stage of the experiment, the gaps among all groups apparently shrank and tended to be identical. The weight change chart shows that there was no strong difference in weight gain or loss between these groups at 8 to 12 weeks.

During the experiment, the rats in the non-irradiated (C and D) group had normal food intake, no diarrhea symptoms, good mental state without malaise or abnormal hyperactivity. However, rats in the irradiated (X, XLD, XMD, and XHD)

Table 2. Summary and comparison of clinical symptoms of rats in each group (n = 8).

	C group	D group	X group	XLD group	XMD group	XHD group
Attention	N	N	↓↓↓	↓↓	↓	↓
Agility	N	N	↓↓	↓	↓	↓
Appetite	N	N	↓↓↓	↓	↓	↓
Diarrhea	-	-	+++	++	+	+

"-": none; "+, ++, +++": mild, moderate and severe.

"N": normal; "↓, ↓↓, ↓↓↓": mild, moderate and severe decrease.

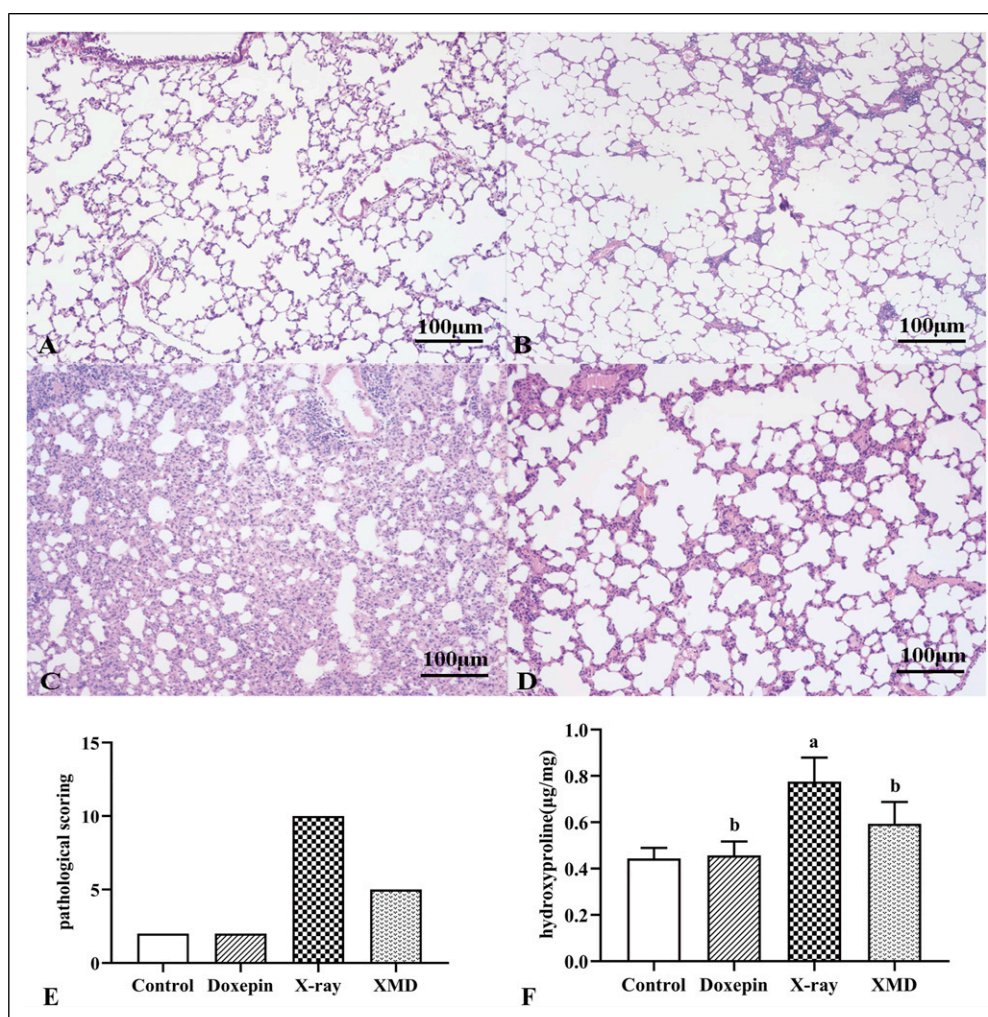


Figure 2. Effect of doxepin treatment on the histological structure of RILI rats. (A) HE staining in C group (B) HE staining in D group (C) HE staining in X group (D) HE staining in XMD group (E) HE staining pathological score in each group (F) Measurement of hydroxyproline content in each group. Scale bars =100 µm.

group experienced decreased appetite, severe diarrhea, low spirits, and slow movement. These symptoms of rats were recovered by doxepin as compared with X group. The symptoms in XMD and XHD groups were the same and showed the protective role of doxepin, and the low-dose doxepin showed little role and the irradiation injuries still existed (see Table 2 for details).

Histological and Pathological Changes of Irradiated Rats

Next, we inspected whether doxepin could demonstrate anti-damage and anti-fibrosis effect in RILI in rats. The H&E staining (Figure 2) shown structure damage of lungs and excessive disordered collagen deposition. The lung tissue of

Table 3. Hematoxylin and eosin staining pathological score.

Pathological Score	Histopathological Features	
Inflammation and fibrosis score (0–15)	Range of inflammatory cells and amorphous material in the alveolar space	0–2
	Extent of inflammatory cells and amorphous material in the alveolar space	0–2
	Interstitial fibrosis	0–5
	Range of bronchial wall fibrosis	0–2
	Extent of bronchial fibrosis	0–2
	Thickening of alveolar walls	0–2

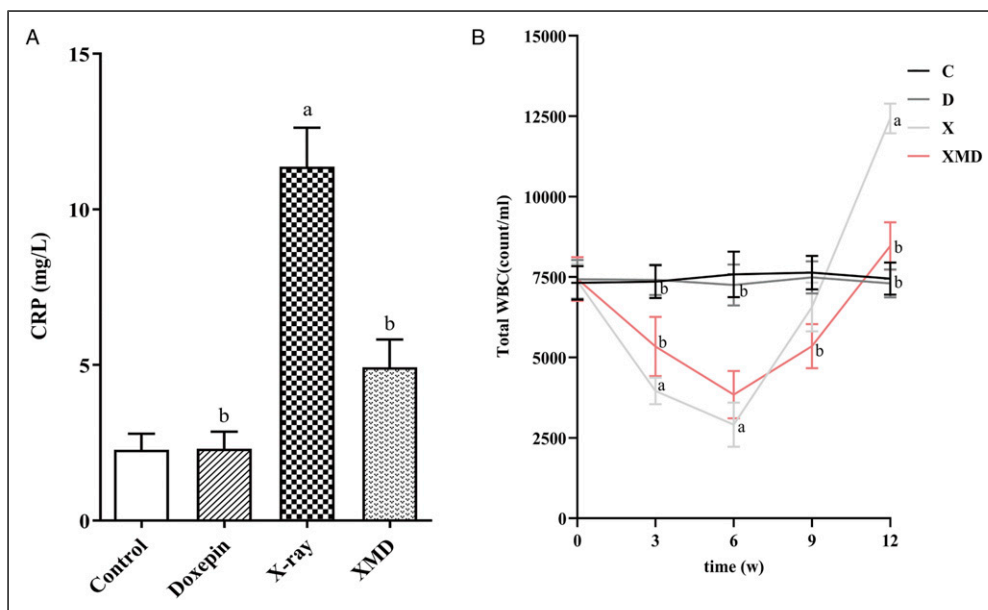


Figure 3. Effects of Doxepin on WBC and CRP Expression in RILI Rats. **(A)** CRP values in groups C, D, X, and XMD at 12 weeks. **(B)** WBC (count/ml of blood) time curve of group C, group D, group X, group XMD. (see Table 3 for details) (^aP < .05 vs control group and ^bP < .05 vs X-ray group).

irradiated rats showed edema, exudation, vascular congestion, thickening of alveolar compartment, obvious swelling of the alveolar interstitium, inflammation infiltration of the interstitium, destruction of alveolar walls, and lung consolidation. XMD group showed less tissue damage, thin alveolar septum, mild blood vessel congestion, no fibrosis tendency, and only mild interstitial inflammation infiltration.

The semi-quantitative method based on the previous research method can be seen from Table 3 that the C group has 2 points, the X-Ray group has 10 points, and the XMD group has 5 points (see Figure 2 and Table 3 for detail).

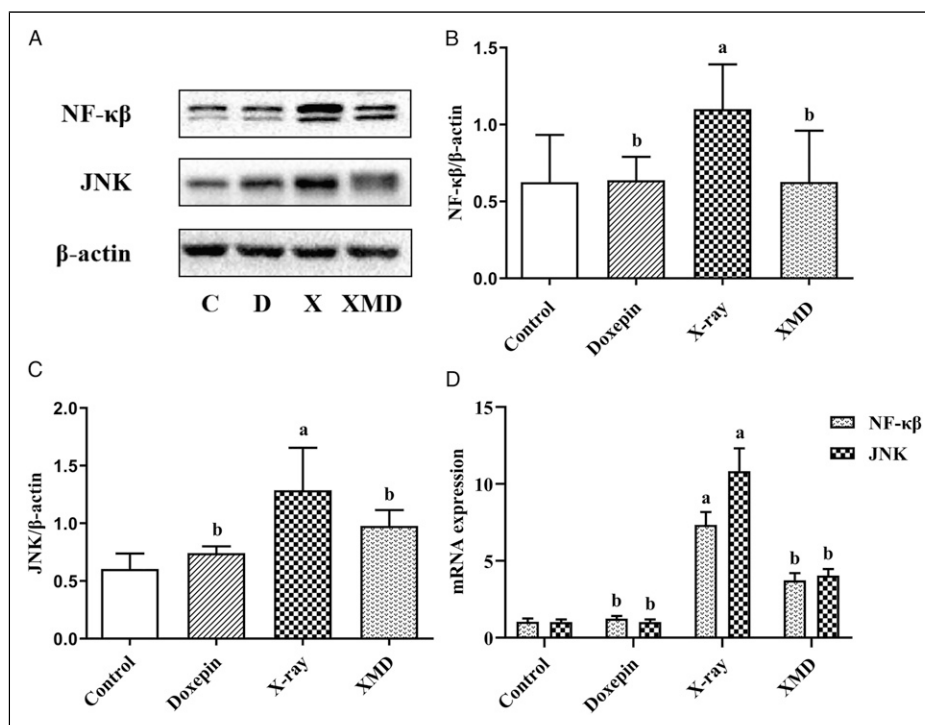
The results showed that the level of hydroxyproline in X group was significantly higher than that in the normal and the XMD group (see Figure 2 for detail).

Doxepin Alleviates the Activation of RILI-Associated Inflammatory Markers

Irradiation can cause changes in inflammatory markers in the blood of rats. The results (Figure 3) showed CRP significantly increased after irradiation, while the level of CRP concentration in the rats protected by doxepin was significantly lower than that of the X group. WBC value was detected at multiple time points (Figure 3), the WBC of XMD group and X group showed transient decrease followed by increase, and the fluctuation of the WBC of XMD group was smaller than that of X group. Table 4 shows the values of WBC and CRP at the end of the animal experiment. This suggests that doxepin might have an anti-inflammatory effect after irradiation.

Table 4. Levels of C-reactive protein and white blood cell in the rats in the C, X, and XMD groups.

	C group	D group	X group	XMD group
WBC (count/ml)	7450 ± 501	7300 ± 438	12 433 ± 463	8467 ± 739
CRP (mg/L)	2.275 ± .520	2.306 ± .551	11.376 ± 1.249	4.925 ± .890

**Figure 4.** Effects of doxepin on the expression of NF-κβ and JNK in RLI rats. **(A)** The expression of NF-κβ and JNK protein in each group increased. **(B)** and **(C)** quantitative analysis of relative protein expression for NF-κβ and JNK detected by Western blot. **(D)** Quantitative analysis of relative mRNA expression for NF-κβ and JNK detected by quantitative real-time PCR (^aP < .05 vs control group and ^bP < .05 vs X-ray group). Each value represents mean ± SEM (n = 8).

Doxepin Ameliorates Radiation-Induced NF-κβ and JNK Expressions

We have found that the medium concentration of doxepin presented protection for RILI, then we chose the medium concentration for molecular biology testing, and we found that doxepin can reduce rat lung tissue NF-κβ and JNK expression induced by irradiation at both protein and RNA levels (Figure 4).

Discussion

Tumor radiotherapy is a local treatment method and has been widely used for lung cancer, esophageal cancer, and various lymphomas.²¹ However, radiation-induced lung injury caused by radiotherapy, is acutely manifested as radiation pneumonitis, and chronically manifested as radiation pulmonary fibrosis. The biological approach used to treat radiation pneumonitis and RIPF (radiation-induced pulmonary fibrosis)

exploits the difference in radiation sensitivity between tumors and healthy tissue for treatment and protection. These measures include the use of radioprotectants, modifiers, or mitigators. These drugs are specifically used to protect normal tissues that have acute or delayed reactions after irradiation and ideally have no protective effect on tumor cells. However, due to the different factors that cause RP (lung sensitivity, cell turnover, dosimetry, and clinical parameters), multiple methods are often used to achieve successful results.⁵

First, we investigated the survival status and manifestations of different group of rats and chose the moderate concentration of doxepin to be the further experimental object. The Clinical Symptom Criteria in this paper come from a new QLQ-C30 questionnaire²² (Assessment of Quality of Life in Cancer Patients), which includes 5 functional scales: physical function, role function, cognitive function, emotional function, social function; 12 Physiological symptoms: fatigue, nausea and vomiting, pain, dysphagia, sleep disturbance, decreased appetite, constipation, and diarrhea. Based on the

experimental needs, several indicators that are easy to observe and representative of rats were selected. In this experiment, due to the small sample size of 8 rats in each group, comprehensive survival analysis and survival rate analysis were not performed.

Next, we used histological and pathological methods to observe whether doxepin had a protective effect on rats after irradiation. The results showed that when the alveolar inflammatory exudation and consolidation generally occurred in the irradiated rats, the alveolar of the irradiated rats administered with doxepin almost remained normal, with only slight inflammatory exudation. Furthermore, we assessed the inflammatory process and degree of inflammation in the lung tissues by measuring CRP and WBC. The results showed that the inflammatory index of rats increased after irradiation, and doxepin could reduce the increase to a certain extent. WBC values were measured at multiple time points, the results showed that WBC showed transient decrease followed by increase after irradiation, and the fluctuation of WBC values in XMD group was smaller than that in X group. This result is consistent with a previous literature.²³ This WBC increase might result from inflammation response caused by irradiation. C-reactive protein (CRP) is a non-specific marker of inflammation, a protein that rises sharply in plasma (acute protein) when the body is infected or damaged. CRP can activate complement and strengthen the phagocytosis of phagocytes to play an opsonizing role, thereby removing damaged, necrotic, and apoptotic cells, and play a protective role in immune response. The results of this paper show that irradiation can induce lung injury accompanied by inflammatory response in vivo, and doxepin can inhibit this response. Finally, the protective effect of doxepin on lung tissue was verified at the protein and gene level.

The results show that doxepin reduces lung damage after irradiation and possibly relates to the modulation of NF- κ B and JNK. The NF- κ B pathway plays a vital role in supporting the initiation and progression of tumor cells and resistance to radiation.²⁴ A previous study²⁵ showed that the most common adverse reaction that occurs after craniocerebral radiotherapy (CRT) is radiation-induced brain injury (RIBI), and the release of pro-inflammatory cytokines and the activation of microglia in brain tissue induced by CRT played important roles in RIBI. This study also indicates that silencing PIDD (p53-induced protein with a death domain) expression can inhibit the transcriptional activation of microglia by down-regulating the PIDD-C/NF- κ B pathway. JNK was reported to be apoptosis-related,^{26,27} but this experiment suggests that it may also play roles in inflammation pathways.

In this study, both WB and real-time PCR showed that NF- κ B and JNK were down-regulated in the XMD group compared with the X group. It can be seen that doxepin has a certain anti-inflammatory effect and can reduce the inflammatory damage of the lungs caused by irradiation. In the rat model of RILI, it is manifested as a decrease of NF- κ B and

JNK, which might be related to its anti-inflammatory effect, but the specific role of doxepin in the NF- κ B pathway needs to be further studied.

Doxepin is of high quality and low price²⁸ has a wide range of clinical applications. Doxepin, as a histamine receptor inhibitor, is involved in physiological and pathological reactions such as allergic inflammation²⁹. We found the protective roles of doxepin for RILI, suggesting the potential of doxepin to develop a novel protective agent for the side effects of radiotherapy. In previous studies, the protective effect of doxepin cream on breast cancer radiation dermatitis has also been reported, which corroborates with our conclusion.²⁸ There are still some limitations in this study, such as limitation of vivo experiments on animals, which is not perfect in human experiments and the expression mechanism in the NF- κ B pathway is still unclear. Thus, further exploration and research are needed.

Conclusion

Doxepin has an unexpected effect on the protection of RILI through its anti-inflammatory effect, which might related to classical inflammatory factors such as NF- κ B and JNK pathways.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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