

Nicaraven mitigates radiation-induced lung injury by downregulating the NF- κ B and TGF- β /Smad pathways to suppress the inflammatory response

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ABSTRACT

Radiation-induced lung injury (RILI) is commonly observed in patients receiving radiotherapy, and clinical prevention and treatment remain difficult. We investigated the effect and mechanism of nicaraven for mitigating RILI. C57BL/6 N mice (12-week-old) were treated daily with 6 Gy X-ray thoracic radiation for 5 days in sequences (cumulative dose of 30 Gy), and nicaraven (50 mg/kg) or placebo was injected intraperitoneally in 10 min after each radiation exposure. Mice were sacrificed and lung tissues were collected for experimental assessments at the next day (acute phase) or 100 days (chronic phase) after the last radiation exposure. Of the acute phase, immunohistochemical analysis of lung tissues showed that radiation significantly induced DNA damage of the lung cells, increased the number of Sca-1⁺ stem cells, and induced the recruitment of CD11c⁺, F4/80⁺ and CD206⁺ inflammatory cells. However, all these changes in the irradiated lungs were effectively mitigated by nicaraven administration. Western blot analysis showed that nicaraven administration effectively attenuated the radiation-induced upregulation of NF- κ B, TGF- β , and pSmad2 in lungs. Of the chronic phase, nicaraven administration effectively attenuated the radiation-induced enhancement of α -SMA expression and collagen deposition in lungs. In conclusion we find that nicaraven can effectively mitigate RILI by downregulating NF- κ B and TGF- β /pSmad2 pathways to suppress the inflammatory response in the irradiated lungs.

Keywords: radiation; DNA damage; lung injury; inflammatory response

INTRODUCTION

Radiotherapy is used for cancer treatment, but exposure to high doses of ionizing radiation also damages the normal tissue cells [1, 2]. Radiation-induced lung injury (RILI), including acute pneumonitis and chronic pulmonary fibrosis, is frequently observed in patients receiving thoracic radiotherapy. It is estimated that RILI occurs in 13–37% of lung cancer patients undergoing curative radiotherapy, which may limit the dose of radiotherapy and affect the quality of life [3]. Currently, the pathogenesis of RILI has not yet been fully understood, and there is no effective drug in the clinic.

It is known that high dose ionizing radiation leads to DNA double-strand breaks [4]. DNA damage contributes to oxidative stress, vascular damage, and inflammation. Pneumonitis develops within hours

or days after high dose irradiation exposure, and is accompanied by an increased capillary permeability and the accumulation of inflammatory cells in lungs [5–7]. The recruited inflammatory cells secrete profibrotic cytokines to activate the resident fibroblasts, which finally leads to an excessive collagen production and deposition in the interstitial space of lungs [8–10].

NF- κ B (nuclear factor kappa B) is an important regulator of inflammatory response. The NF- κ B signaling pathway is known to be activated following radiation exposure [11]. TGF- β /Smad signaling pathway also deeply involves in RILI [12, 13]. Thoracic irradiation causes a continuous increase of TGF- β ₁ in plasma, which is a predictor of radiation pneumonitis after radiotherapy [14]. The activation of TGF- β induces the conversion of fibroblasts into myofibroblasts, the elevated

expression of α -smooth muscle actin (α -SMA), and the synthesis of extracellular matrix proteins such as collagen [15, 16]. Therefore, targeting these pathways can be a potential strategy for mitigating RILI.

Nicaraven, a hydroxyl free radical scavenger [17], has previously been demonstrated to protect against the radiation-induced cell death [18, 19]. We have also recently found that the administration of nicaraven to mice soon after high dose γ -ray exposure attenuates the radiation-induced injury of hematopoietic stem/progenitor cells, which is more likely associated with anti-inflammatory effect rather than radical scavenging [20, 21]. Moreover, nicaraven can reduce the radiation-induced recruitment of macrophages and neutrophils into lungs [22]. Therefore, we speculate that nicaraven may effectively mitigate RILI, at least partly by inhibiting inflammatory response through NF- κ B and TGF- β /Smad signaling pathways [23].

By exposing the lungs of adult mice to 30 Gy X-ray, we investigated the effect and mechanism of nicaraven for mitigating RILI. Our results showed that nicaraven administration significantly reduced the DNA damage of lung tissue (stem) cells, inhibited the radiation-induced recruitment of CD11c⁺, F4/80⁺ and CD206⁺ inflammatory cells in lungs at the acute phase, and also mitigated the radiation-induced enhancement of α -SMA and partly decreased the fibrotic area in the irradiated lungs at the chronic phase.

MATERIALS AND METHODS

Animals

Male C57BL/6 N mice (12-week-old; CLEA, Japan) were used for study. Mice were housed in pathogen-free room with a controlled environment under a 12 h light–dark cycle, with free access to food and water. This study was approved by the Institutional Animal Care and Use Committee of Nagasaki University (No.1608251335-12). All animal procedures were performed in accordance with institutional and national guidelines.

Thoracic radiation exposure and nicaraven administration

The RILI model was established as previously described [24]. Briefly, mice were treated daily with 6 Gy X-ray thoracic radiation for 5 days in sequences (cumulative dose of 30 Gy) at a dose rate of 1.0084 Gy/min (200 kV, 15 mA, 5 mm Al filtration, ISOVOLT TITAN320, General Electric Company, United States) (Supplementary Fig. 1A). Nicaraven (50 mg/kg; n = 6, IR + N group) or placebo (n = 6, IR group) was injected intraperitoneally to mice within 10 min after each radiation exposure, and we continued the daily injections for 5 additional days after the last radiation exposure (Supplementary Fig. 1A). Age-matched mice without radiation exposure were used as control (n = 6, CON group). The body weights of mice were recorded once a week. We sacrificed the mice the next day (Acute phase) or the 100th day (Chronic phase) after the last exposure (Supplementary Fig. 1A). At the end of follow-up, mice were euthanized under general anesthesia by severing the aorta to remove the blood. Lung tissues were excised and weighed, and then collected for experimental evaluations as follows.

Immunohistochemical analysis

The DNA damage in lung tissue (stem) cells was detected by immunohistochemical analysis. Briefly, lungs were fixed in 4% paraformaldehyde, and paraffin sections of 6- μ m-thick were deparaffinized and rehydrated. After antigen retrieval and blocking, sections were incubated with rabbit anti-mouse γ -H2AX antibody (1:400 dilution, Abcam) and rat anti-mouse Sca-1 antibody (1:200 dilution, Abcam) overnight at 4°C, and followed by the appropriate fluorescent-conjugated secondary antibodies at 25°C for 60 min. The nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI) (1:1000 dilution, Life technologies). The positive staining was examined under fluorescence microscope (FV10CW3, OLYMPUS).

The recruitment of inflammatory cells was detected by immunostaining with mouse anti-mouse CD11c antibody (1:150 dilution, Abcam), rat anti-mouse F4/80 antibody (1:100 dilution, Abcam), goat anti-mouse CD206 antibody (1:200 dilution, R&D Systems) overnight at 4°C, and followed by the appropriate Alexa fluorescent-conjugated secondary antibodies (1:400 dilution, Invitrogen), respectively. The nuclei were stained with DAPI. The positive staining was examined under fluorescence microscope (FV10CW3, OLYMPUS).

For quantitative analysis, we counted the positively stained cells in 12 images from two separated independent sections of each lung tissue sample. The number of positively stained cells in each lung tissue sample was normalized by the number of nuclei, and the average value per field (image) from each lung tissue sample was used for statistical analysis.

Masson's trichrome staining

To detect the fibrotic change in lungs, Masson's trichrome staining was performed according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). The stained sections were mounted and then imaged using a microscope (Biorevo BZ-9000; Keyence Japan, Osaka, Japan). The fibrotic area was quantified by measuring the positively stained area using the Image-Pro Plus software (version 5.1.2, Media Cybernetics Inc, Carlsbad, CA, USA), and expressed as a percentage of the total area. The average value from 12 images randomly selected from two separated slides for each lung tissue sample was used for statistical analysis.

Western blot

Western blot was performed as previously described [25]. Briefly, lung tissue sample was homogenized using Multi-beads shocker and added to the T-PER reagent (Thermo Fisher Scientific) consisting of proteinase and dephosphorylation inhibitors (Thermo Fisher Scientific). Total tissue protein purified from lungs were separated by SDS-PAGE gels and then transferred to 0.22- μ m PVDF membranes (Bio-Rad). After blocking, the membranes were incubated with primary antibodies against NF- κ B p65 (1:500 dilution, Abcam), I κ B α (1:1000 dilution, CST), TGF- β (1:1000 dilution, Abcam), pSmad2 (1:1000 dilution, Abcam), α -Tubulin (1:1000 dilution, CST), or GAPDH (1:1000 dilution, Abcam), respectively; and followed by the appropriate horseradish peroxidase-conjugated secondary antibodies (Dako). The expression was visualized using an enhanced chemiluminescence detection kit (Thermo Scientific).

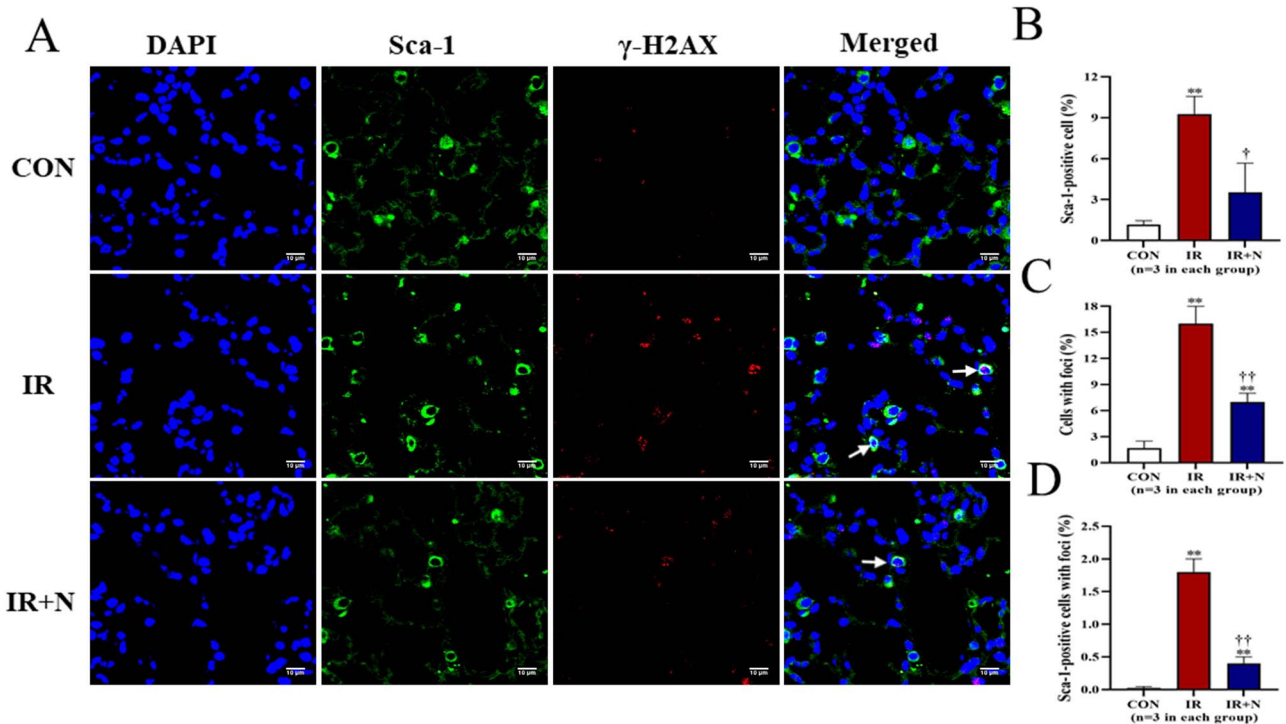


Fig. 1. The DNA damage of lung tissue cells at the acute phase after treatments. (A) Representative confocal images show the expression of Sca-1 and γ -H2AX in lung tissue cells. Quantitative data on the number of Sca-1⁺ stem cells (B), the total cells with γ -H2AX foci formation (C), and the Sca-1⁺ stem cells with γ -H2AX foci formation (D, Arrows) are shown. Scale bars: 10 μ m. The nuclei were stained with DAPI. Data are represented as means \pm SD. ** $p < 0.01$ vs CON group, † $p < 0.05$, †† $p < 0.01$ vs IR group. CON: Control, IR: Radiation, IR + N: Radiation+Nicaraven.

Semiquantitative analysis was done using ImageQuant LAS 4000 mini (GE Healthcare Life Sciences).

Statistical analysis

All the values were presented as mean \pm SD. For comparison of multiple sets of data, one-way analysis of variance (ANOVA) followed by Tukey's test (Dr. SPSS II, Chicago, IL) was used. For comparison of two sets of data, an unpaired two-tailed *t*-test was used. All analysis was carried out with the SPSS19.0 statistical software (IBM SPSS Co., USA). A *p*-value less than 0.05 was accepted as significant.

RESULTS

Nicaraven significantly reduced the radiation-induced DNA damage of lung tissue (stem) cells at the acute phase.

All mice survived after treatments and during the follow-up period. The body weights of the mice were decreased temporarily soon after radiation exposure, but tended to increase approximately 10 days after radiation exposure. Although the body weights of mice between IR group and IR + N group were not significantly different, they were significantly lower than the age-matched non-irradiated mice in the CON group ($p < 0.05$, Supplementary Fig. 1B). Moreover, the lung weights of mice were not significantly different among all groups at either the acute phase or the chronic phase (Supplementary Fig. 1C).

Immunohistochemistry was performed to evaluate the expression of Sca-1 and γ -H2AX in lung tissue cells at the acute phase (Fig. 1A). Compared to CON group, the number of Sca-1⁺ stem cells was significantly increased in the IR group ($9.27 \pm 1.30\%$ vs $1.17 \pm 0.29\%$, $p < 0.01$; Fig. 1B). However, the increased number of Sca-1⁺ stem cells in irradiated lungs was significantly attenuated by nicaraven administration ($3.53 \pm 2.15\%$, $p < 0.05$ vs IR group; Fig. 1B).

The formation of γ -H2AX foci in nuclei of lung tissue cells was dramatically increased in the IR group, but was mildly changed in the IR + N group (Fig. 1A). Quantitative data also indicated that the percentage of γ -H2AX-positive cells was significantly less in the IR + N group than the IR group ($7.13 \pm 0.91\%$, $p < 0.01$; Fig. 1C). Moreover, we tried to evaluate the formation of γ -H2AX foci in Sca-1⁺ stem cells. Interestingly, the number of Sca-1⁺ stem cells with γ -H2AX foci was more effectively decreased in the IR + N group compared to the IR group ($0.35 \pm 0.19\%$ vs $1.93 \pm 0.51\%$, $p < 0.01$; Fig. 1D). These results indicate that nicaraven administration can reduce the radiation-induced DNA damage in lung tissue cells, especially in these Sca-1⁺ stem cells.

Nicaraven effectively decreased the radiation-induced recruitment of inflammatory cells into lungs.

According to our previous studies [20, 21], nicaraven protects tissue (stem) cells against radiation injury by inhibiting

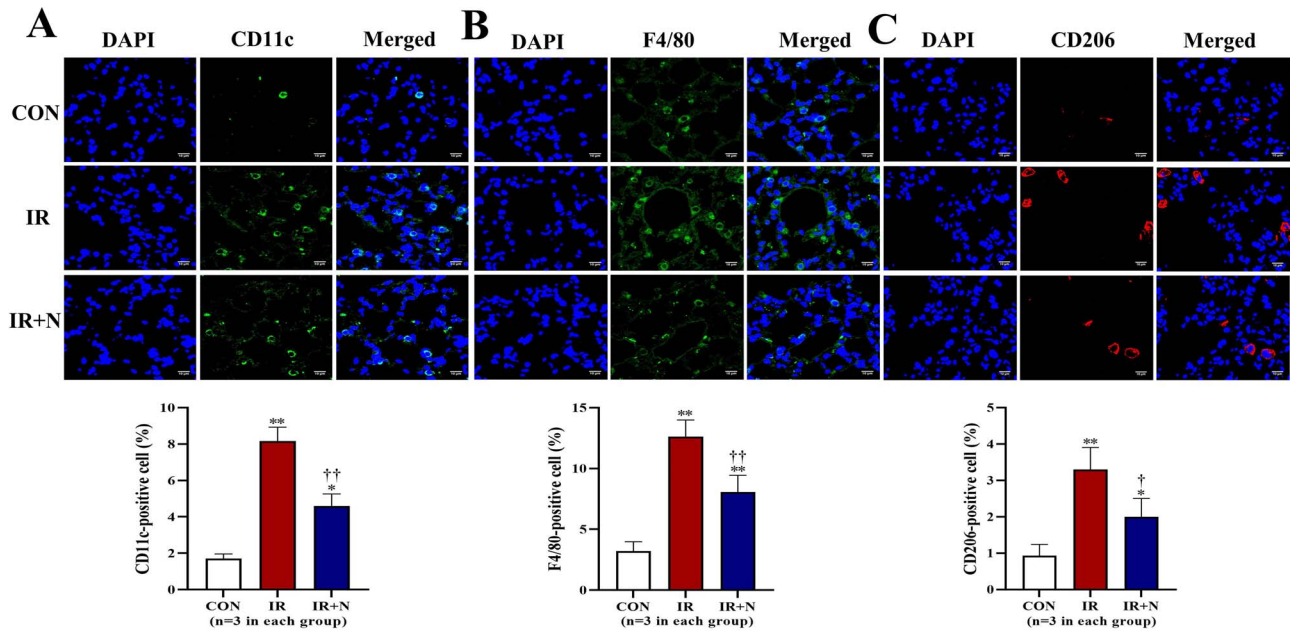


Fig. 2. Immunohistochemical detection of inflammatory cells in lungs at the acute phase after treatments. Representative confocal images (upper) and quantitative data (lower) show the CD11c⁺ cells (A), F4/80⁺ cells (B), and CD206⁺ cells (C) in lungs. Scale bars: 10 μ m. The nuclei were stained with DAPI. Data are represented as means \pm SD. * p < 0.05, ** p < 0.01 vs CON group, † p < 0.05, †† p < 0.01 vs IR group. CON: Control, IR: Radiation, IR + N: Radiation+Nicaraven.

inflammatory response. Therefore, immunohistochemical analysis was performed to detect the inflammatory cells in lungs. The number of CD11c⁺ monocytes and F4/80⁺ macrophages was significantly higher in the IR group than the CON group (p < 0.01, Fig. 2A and B). However, nicaraven administration significantly reduced the recruitment of CD11c⁺ monocytes ($8.23 \pm 0.75\%$ vs $4.61 \pm 0.65\%$, p < 0.01; Fig. 2A) and F4/80⁺ macrophages ($12.63 \pm 1.36\%$ vs $8.07 \pm 1.38\%$, p < 0.01; Fig. 2B) into irradiated lungs. Similarly, the number of M2 macrophages (CD206⁺) was also significantly higher in irradiated lungs than that of non-irradiated lungs (p < 0.01, Fig. 2C). Interestingly, nicaraven administration significantly decreased the CD206⁺ macrophages in irradiated lungs ($3.3 \pm 0.61\%$ vs $2.1 \pm 0.53\%$, p < 0.05; Fig. 2C).

Nicaraven significantly attenuated the radiation-induced upregulation of NF- κ B and TGF- β in lungs

To further understand the molecular mechanism of nicaraven on mitigating RILI, we investigated the expression of NF- κ B and I κ B α (inhibitor of NF- κ B) in lungs at the acute phase. Compared to the CON group, the IR group showed a significant enhancement on the expression of NF- κ B (p < 0.05, Fig. 3A). However, the enhanced expression of NF- κ B in irradiated lungs was effectively attenuated by nicaraven administration (p = 0.09, Fig. 3A). In contrast, the expression of total I κ B α was significantly decreased in irradiated lungs (p < 0.01 vs CON group, Fig. 3B), which was effectively attenuated by nicaraven administration (p < 0.01 vs IR group, Fig. 3B). We also investigated the expression of TGF- β and pSmad2 in irradiated lungs at the acute phase (Fig. 3C and D). The irradiated lungs showed a

significant upregulation of TGF- β and pSmad2 (p < 0.05 vs CON group, Fig. 3C and D), but was effectively attenuated by nicaraven administration (p < 0.05 vs IR group, Fig. 3C and D).

Nicaraven clearly attenuated the radiation-induced enhancement of α -SMA and partially reduced the fibrotic area in irradiated lungs at the chronic phase

We further investigated the expression of α -SMA and collagen deposition in irradiated lungs at the chronic phase. Western blot indicated an enhanced expression of α -SMA in the irradiated lungs (p < 0.05 vs CON group, Fig. 4A), but the enhanced expression of α -SMA in irradiated lungs was completely attenuated by nicaraven administration (Fig. 4A). Similarly, Masson's trichrome staining showed that the fibrotic area in lungs was significantly higher in the IR group than the CON group (p < 0.05, Fig. 4B). However, nicaraven administration tended to only partially reduce the fibrotic area in irradiated lungs ($6.24 \pm 0.64\%$ vs $5.14 \pm 0.51\%$, p = 0.08; Fig. 4B).

DISCUSSION

This study was proposed to investigate the potential effect and underlying mechanism of nicaraven on mitigating RILI. Our experimental data revealed that nicaraven administration not only reduced the DNA damage (γ -H2AX foci formation) of lung tissue (stem) cells, but also inhibited the recruitment of macrophages and neutrophils into irradiated lungs at the acute phase. Nicaraven administration also significantly attenuated the radiation-induced enhancement of TGF- β and NF- κ B, and partially reduced the fibrotic area in irradiated lungs at the chronic phase.

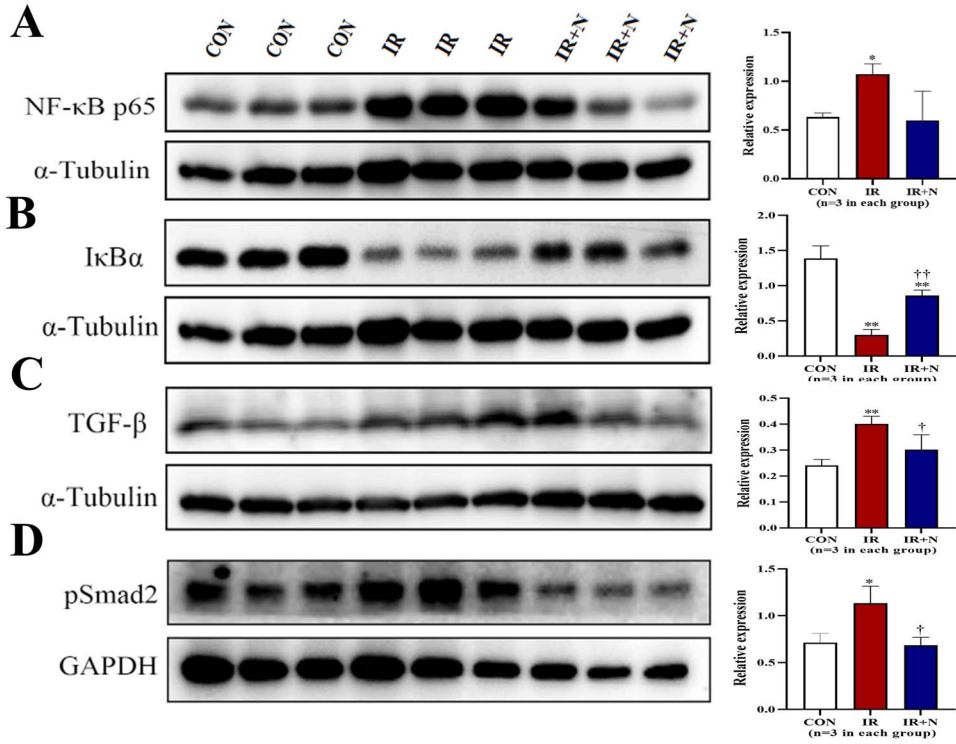


Fig. 3. Western blot analysis on the expression of NF-κB, IκBα, TGF-β, and pSmad2 in lungs. Representative blots (left) and quantitative data (right) on the expression of NF-κB p65 (A), IκBα (B), TGF-β (C), pSmad2 (D) in lungs are shown. Data are normalized to α-Tubulin or GAPDH, and represented as means ± SD. *p < 0.05, **p < 0.01 vs CON group, †p < 0.05, ††p < 0.01 vs IR group. CON: Control, IR: Radiation, IR + N: Radiation+Nicaraven.

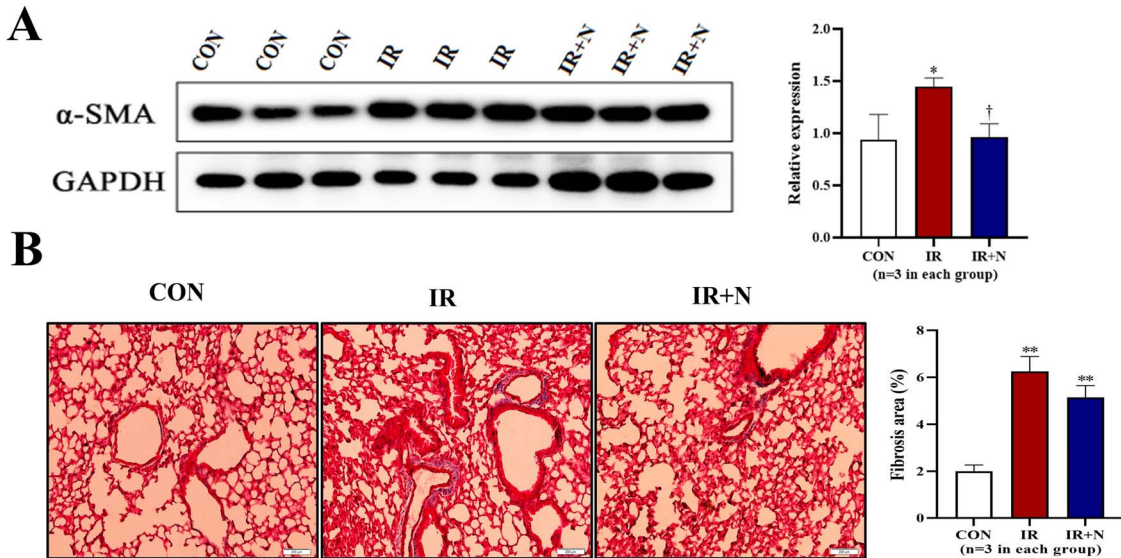


Fig. 4. The fibrotic changes in lungs at the chronic phase after treatments. (A) Representative blots (left) and quantitative data (right) on the expression of α-SMA in lungs are shown. (B) Representative images (left) and quantitative data (right) of Masson's trichrome staining on the fibrotic area in lungs are shown. Scale bars: 200 μm. Data are represented as means ± SD. *p < 0.05, **p < 0.01 vs CON group, †p < 0.05 vs IR group. CON: Control, IR: Radiation, IR + N: Radiation+Nicaraven.

Nicaraven is known as a powerful radical scavenger that effectively protects various tissues and organs against injuries, particularly for ischemia–reperfusion injury in the brain [26–28]. Considering the well-recognized antioxidative property and the potential anti-inflammatory effect of nicaraven, we evaluated the probable role of nicaraven on mitigating RILI.

The exposure to high levels of ionizing radiation leads to DNA double-strand breaks, which elicit cell death or stochastic change [4]. Stem cells are known to play critical role in tissue homeostasis, while ionizing radiation exposure can disrupt the tissue homeostasis. Alveolar epithelium is composed of two cell types: Type I cells account for 95% of the gas exchange surface area, and type II cells can transform into type I cells and have the ability to repair alveoli. Sca-1-positive cells are identified as a population of alveolar type II cells with progenitor cell properties [29]. It has also been demonstrated that the proliferation of Sca-1-positive cells increases during the alveolar epithelial repair phase [29, 30]. In this study, the number of Sca-1-positive cells were exactly increased in irradiated lungs at the acute phase, suggesting the probable role of Sca-1-positive cells for repairing the injured lungs after high dose irradiation. Nicaraven administration showed to reduce the DNA damage of lung tissue cells, especially in these Sca-1-positive cells at the acute phase, which indirectly indicates the protective effect of nicaraven on RILI.

Various chemokines/cytokines are known to be increased in organs/tissues exposed to high dose irradiation, which in turn induces the recruitment of inflammatory cells at the acute phase. The recruited inflammatory cells play a key role in the pathogenesis of RILI [31, 32], because inflammatory cascade is known to promote fibroblast proliferation and collagen deposition [33]. In responding to high dose radiation exposure, the recruitment of monocytes/immune cells (CD11c⁺) into lung tissue plays critical pathophysiological role on RILI. Among the recruited monocytes/immune cells, macrophages (F4/80⁺) represent an important profibrogenic initiator/mediator, but M2 macrophages (CD206⁺) are thought to be an anti-inflammatory phenotype of macrophages. In this study, we found that the recruitment of monocytes/immune cells, including the M2 macrophages was significantly increased in the irradiated lungs. Consistent with previous reports [22, 34], the administration of nicaraven inhibited significantly the recruitment of monocytes into the irradiated lungs. It will be better to understand the precise role of especial subpopulation of inflammatory cells, such as the M2 macrophages in lungs using genetically modified animals. However, the purpose of this study was designed to examine the potency of nicaraven for attenuating RILI through an anti-inflammatory mechanism, we used a wild-type mice rather than the genetically modified mice for experiments.

TGF- β is one of the most critical master regulators on promoting acute inflammation and chronic fibrosis in lungs [12–16]. Previous studies have demonstrated that radiation exposure activates TGF- β /Smad signaling pathway to initiate the inflammatory response, induce the proliferation and activation of fibroblasts, and enhance the synthesis of matrix proteins [35, 36]. Therefore, the inhibition of TGF- β /Smad signaling pathway may effectively mitigate RILI. In this study, nicaraven administration exactly downregulated the expression of TGF- β and pSmad2 in irradiated lungs at the acute phase.

NF- κ B has emerged as a ubiquitous factor involved in the regulation of numerous critical processes, including immune [37], inflammation response [38], cell apoptosis [39], and cell proliferation [40]. While in an inactivated state, NF- κ B is located in the cytosol complexed with the inhibitory protein I κ B α . Radiation exposure can activate NF- κ B signaling pathway. Radiation exposure activates the kinase IKK, which in turn phosphorylates I κ B α and results in ubiquitin-dependent degradation [11, 38, 40]. Dysregulation of NF- κ B signaling can lead to inflammation, autoimmune disease and cancer [41]. In this study, nicaraven administration effectively downregulated the expression of NF- κ B, suggesting the involvement of NF- κ B pathway in the protective effect of nicaraven to RILI.

Generally, although nicaraven administration only tended to partially reduce the fibrotic area in the irradiated lungs, many other parameters, such as the expression of α -SMA was more sensitively and clearly changed. As the activated fibroblasts (α -SMA⁺) are generally thought to be the predominant source of collagen-producing cells, the inhibition on α -SMA expression in irradiated lungs by nicaraven administration suggested the probable benefit of nicaraven for attenuating the development of fibrotic change in irradiated lungs at the chronic phase. In summary, nicaraven administration effectively protected the RILI, likely by suppressing inflammatory response through the NF- κ B and TGF- β /Smad signaling pathways. Nicaraven could be a potential drug for mitigating RILI.

SUPPLEMENTARY DATA

Supplementary data is available at *RADRES Journal* online.

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CONFLICT OF INTEREST

The authors indicate no potential conflicts of interest.

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