

Original Paper

Protective Effects of Myrtol Standardized Against Radiation-Induced Lung Injury

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Key Words

Radiation-induced lung injury • Myrtol standardized • Pulmonary fibrosis

Abstract

Background/Aims: As a major complication after thoracic radiotherapy, radiation-induced lung injury (RILI) has great impact on long term quality of life and could result in fatal respiratory insufficiency. The present study was aimed to evaluate the effects of Myrtol standardized on RILI, and to investigate the underlying mechanism. **Methods:** A mouse model of radiation-induced lung injury was generated by using thoracic irradiation with a single dose of 16Gy. Mice were orally administrated with Myrtol (25 mg/kg/day) for 4 weeks after irradiation, while prednisone (5 mg/kg/day) was used as a positive control. After then, the body weight and lung coefficient were calculated. The severity of fibrosis was evaluated by observing pulmonary sections after radiation and collagen content in lung tissues was calculated following the hydroxyproline (HYP) assay. Pathological changes were observed in all the groups by using HE staining and Masson staining. The serum levels of TGF- β 1, TNF- α , IL-1 β , IL-6, and PGE2 were also measured with an ELISA assay. Western blot assay was used to measure the impact of Myrtol on AKT and its downstream signaling pathway, including MMP-2 and MMP-9. The levels of Vimentin and α -SMA were evaluated with an immunofluorescence assay. **Results:** Treatment with Myrtol standardized, but not prednisone, reduced lung coefficient and collagen deposition in lung tissues, while attenuated histological damages induced by irradiation. Myrtol standardized also reduced the production of MDA, while increased the level of SOD. It was also observed that Myrtol standardized inhibited TGF- β 1 and a series of pro-inflammatory cytokines including TNF- α , IL-1 β , IL-6, PGE2. While in prednisone group, even though the early pneumonitis was ameliorated, the collagen disposition remained unchanged in latter times. Immunofluorescence analysis also revealed elevation of vimentin and α -SMA in the alveoli after a single dose of 16Gy. **Conclusion:** The present results suggest Myrtol standardized as an effective agent for attenuating the lung injury induced by irradiation.

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Introduction

Thoracic radiotherapy (RT) is a common therapeutic modality for treating lung cancer, esophageal cancer, lymphoma and thymoma cancer. RT-induced lung injuries are traditionally divided into early (acute, 1-6 months post-RT) pneumonitis and late (chronic, 6 months post-RT) fibrosis depending on the time after radiation exposure [1, 2].

Previous data showed that radiation therapy induced lung injury was fairly common, occurring in about 5% to 37% of patients with lung cancer [3]. The current clinical treatment for radiation-induced lung injury primarily involved chemical anti-inflammatory agents and immunosuppressive cytokines, antioxidant agents, such as corticosteroids or IFN- γ [4]. These drugs could attenuate acute inflammation for 2-3 months after irradiation, but they could not effectively mitigate fibrogenic processes in latter stage [5]. Furthermore, these agents could cause significant side-effects, including infections, vomiting, and even death which limits their application in clinics [6].

Although the molecular mechanisms of radiation-induced lung injury have not been fully understood, a series of pro-inflammatory cytokines and growth factors are shown to play important roles in the radiation-induced lung injury, because they promote early inflammation and latter phase fibrosis [7], increase the deposition of matrix proteins, and could also distort the pulmonary architecture and respiratory function. Therefore, reducing pro-inflammatory cytokines release and oxidative stress represents possible therapeutic strategies.

Herbal medicine is very popular in Asian and European countries. Among those, Myrtol standardized (CAS-No.8002-55-9) is a phytotherapeutic extract from *Myrtus communis*, such as *Pinus* spp (pine), *Citrus aurantifolia* (lime) and *Eucalyptus globulus*, mainly containing three monoterpenes: 1,8-cineole, α -pinene and dlimonene as marker substances. It has been widely used in the treatment of acute and chronic lung disease [8, 9]. Furthermore, it has been found that it has anti-inflammatory, antibacterial, antioxidant, anti-allergic, antimicrobial and nasal mucociliary clearing effects [10-16]. In addition, the LD50 value of Myrtol is greater than 3500 mg/kg of the body weight, far more than treatment concentration (25 mg/kg/day) when administered orally via gastric intubation. Prolonged administration of the Myrtol was well tolerated and was observed no toxicity.

In the present study, we demonstrated that oral administration of Myrtol could alleviate radiation-induced lung injury. To investigate the underlying mechanism for the involvement of Myrtol in radiation-induced lung injury, we examined the effects of Myrtol treatment on AKT signaling pathway and its downstream MMP-2 and MMP-9 levels in irradiated mice.

Materials and Methods

Animals and treatment

All the protocols were approved by the Animal Ethics Committee of Second Military Medical University, China, in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institute of Health (publication no. 96-01). Fibrosis prone mice 8 week-old female C57BL/6 mice with an approximate weight of 18 – 20 g were obtained from the Experimental Animal Center of Chinese Academy of Sciences, Shanghai, China) were used and randomly divided into four groups as follows: group 1, non-irradiated control (n = 30); group 2, irradiation + saline (n = 30); group 3, irradiation + Myrtol (n = 30, 25 mg/kg/day) and group 4 irradiation+ prednisone (n = 30, 5 mg/kg/day). Mice were kept under standard laboratory conditions (22 \pm 2°C, 55 \pm 10% humidity and 12 - 12 h/light-dark cycle), during which sterilized food and water were supplied ad libitum. Mice were allowed to acclimate from animal center for 1 week prior to treatment. Myrtol standardized (Gelomyrtol) was obtained from G.Pohl-Boskamp GmbH & Co.KG (Hohenlockstedt, Germany). Myrtol standardized was given orally a week before irradiation at a dose of 25 mg/kg/day and maintained (25 mg/kg/day) until 4 weeks after irradiation. Prednisone (SHANGHAI ZZBIOCO., LTD, Shanghai, China) was administered orally 5 mg/kg daily post irradiation for 4 weeks.

Irradiation

Mice were irradiated using ^{60}Co irradiator (Irradiation Center of Second Military Medical University) at a dose rate of $< 1\text{Gy}/\text{min}$. After anesthetization with 10% chloralhydrate (350 mg/kg), the mice were put in a holder designed to immobilize anaesthetized mice so that only the whole thorax was exposed to the beam. Groups of mice ($n = 30$) received a single dose of thoracic irradiation (16Gy). Animals were monitored up to 16 weeks post-irradiation, and the body weight was recorded at each time point (1, 2, 4, 8, 16 weeks post-irradiation).

Sample collection and initial processing

Six mice at each time point (1, 2, 4, 8 and 16 weeks post-irradiation) from treated and control group were sacrificed by cervical dislocation. After measuring body weight, cardiac puncture was performed to obtain about 500 μL blood. The blood was allowed to clot at room temperature for 1.5 - 2 h and then centrifuged at 3,500 rpm, 4°C for 15 minutes. The serum was collected and stored at -80°C for latter enzyme-linked immunosorbent assay (ELISA). The wet weight of the lungs were recorded for each mouse, one part of right lungs were fixed with 4% paraformaldehyde for histological and immunofluorescence analysis. The rest of the lungs were divided into five parts and kept at -80°C for determination of lipid peroxides (MDA), SOD activities, Hydroxyproline, RT-PCR and Western blot measurement, respectively.

Body weight and Lung coefficient

The body weight was measured at 1, 2, 4, 8 and 16 weeks post-irradiation. The ratio of the lung wet weight (mg) to body weight (g) (lung weight/bw) was used as the lung coefficient.

Measurement of lungs collagen content, MDA, SOD activity

The concentration of hydroxyproline was measured by hydroxyproline (Hyp) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. Approximately 50 - 100 mg of lung tissue was hydrolyzed in 600 μL of lysis buffer solution at water bath kettle at 99°C for 20 min blending every 10 minutes. After cooling, the absorbance of colored products was measured at 550 nm using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific Inc. Waltham, MA, USA) to evaluate collagen deposition.

Malondialdehyde (MDA) content and SOD activity in lung tissue were measured using commercialized chemical assay kits according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and the total protein concentration was determined by BCA (Bicinchoninic Acid) protein assay kit (Beyotime Institute of Biotechnology, Nantong, China).

Pathological examination

According to the methodology described by Ashcroft et al. [17] scoring system, lung sections stained with H&E and Masson's trichrome were carried out with Ashcroft score and a quantifying assayication of Masson's trichrome, respectively. Briefly, the severity of fibrosis was graded and scored on a scale of 0–8. Ten fields per section at $\times 200$ magnifications were randomly selected per mouse, and two blinded pathologists carefully and independently examined 60 fields per group using Nikon DS-Fi1-U2 microscope (Nikon, Tokyo, Japan). The mean score of all fields examined was taken as the fibrosis score and positive rate (%) of each animal.

Immunofluorescence staining

Immunofluorescence analysis was performed to identify the expression of $\alpha\text{-SMA}$ and vimentin, mesenchymal cell marker in the lung tissue. Briefly, after deparaffinization, antigen retrieval, and incubation with Rodent Block M as described [18], the sections were incubated with a mixture of anti- $\alpha\text{-SMA}$ (1:500, Abcam, Cambridge, MA) and anti-vimentin (1:1000, Abcam, Cambridge, MA) antibodies at 4°C overnight. After washing with PBS, the sections were incubated with FITC conjugated Alexa Fluor 488 goat anti-mouse (Invitrogen, Carlsbad, CA) and Texas Red-conjugated anti-rabbit secondary antibodies (Abcam) at room temperature for 30 min. Nuclei were counterstained with DAPI dihydrochloride, and the sections were analyzed using a fluorescence microscope (Nikon Eclipse Ti-SR, Nikon, Tokyo, Japan).

ELISA assays

Serum levels of TGF- β 1, TNF- α , IL-1 β , IL-6 and PGE2 were determined by using the commercially available ELISA kit according to the manufacturer's instructions. The OD value was determined at 450 nm using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific Inc. Waltham, MA, USA) and calculated at the linear portion of the curve. Serum cytokines levels were measured using commercial enzyme linked immunosorbent assay Kit, TGF- β 1, TNF- α , IL-1 β , IL-6 from Anogen (Mississauga, Ontario, Canada) and PGE2 from R&D Systems Inc. (Minneapolis, MN, USA) according to the manufacturer's instructions.

Western blot analysis

Frozen lungs were pulverized in a Tissuelyser and lysed in RIPA buffer (Thermo Fisher Scientific Inc. Waltham, MA, USA). Then the lysates were centrifuged at 12,000g at 4°C for 15 min, and the supernatants were collected for total protein concentration analysis by a BCA protein assay kit (Beyotime Institute of Biotechnology, Nantong, China). Equal amounts of protein were separated by SDS-PAGE, transferred to PVDF membrane (Millipore Corp., Billerica, MA, USA), and incubated with 5% skim milk (Becton, Dickinson and Company, Franklin lake, New Jersey, USA) at room temperature for 1.5h to block non-specific binding. The membranes were then incubated with the following primary antibodies at room temperature for 2h or 4°C overnight: AKT(1:1000), p-AKT(1:1000), were obtained from Cell Signaling Technology (MA, USA); MMP2(1:1000), MMP9 (1:1000) were obtained from Abcam (MA, US), E-cadherin (1:1000) or β -actin (1:2000; Cell Signaling Technology, Inc., Danvers, MA, USA). After washing with TBS-T, the membranes were incubated with HRP-conjugated secondary antibodies (Shanghai Biotechnology, Shanghai, China). The protein bands were visualized using enhanced chemiluminescence with a Super Signal west pico kit (Bridgen Biological Technology, Shanghai, China). Films were scanned and analyzed by densitometry using Syngene GeneGenius software (Syngene, Frederick, MD, USA).

RNA isolation and reverse transcription reaction

Total RNA was extracted from the frozen lungs tissue samples (50 - 80 mg) using TRIzol reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's protocol. Total RNA from each sample was quantified using a microvolume spectrometer.

Briefly, the aqueous phase was used for RNA precipitation with an equal volume of isopropanol. The RNA pellet was washed once with 800 μ L 75% ethanol then air-dried and dissolved in 50 μ L RNase free water. RNA was quantified by using a spectrophotometer (Molecular Devices, USA), and the quality was checked by agarose gel electrophoresis. The reverse transcription (RT) reaction was carried out using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Code No. FSQ-301) (Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer's instructions and the cDNA product was stored at -20°C or used immediately for realtime PCR.

Quantitative real-time polymerase chain reaction (qPCR)

We used real-time quantitated PCR to measure the expression of the lung RNA levels, Quantitative real-time Polymerase Chain Reaction (qPCR) was performed by using SYBR Green real time PCR Master Mix, QPK-201(Toyobo Co. Ltd., Osaka, Japan). The 20 μ L standard PCR reaction containing 0.8 μ L of each primer (final concentration 0.4 μ M), 10 μ L of SYBR Green, 2 μ L of cDNA and 6.4 μ L RNase free water. Amplification and detection were performed in a LightCycler 480 (Roche Diagnostics, Penzberg, Germany),

Table 1. PCR Primers

Name	Sense	Antisense
TGF- β 1	CTTCAGCTCCACAGAGAAGAAGTGC	CACGATCATGTTGGACAACCT GCTCC
TNF- α	CATGCGTCCAGCTGACTAAA	TCCCCTTCATCTTCTCTCTT
IL-1 β	TTTAGCATGCCTGCCCTGAA	AGCCCTCCATTCTGAAAAGC
IL-6	GAAGTGGATTCCCACTGCCA	GAGTTGGTGGGTAAGGGAGC
PGES	GAGTTGAAGTCCAGGCCGCTAGCCG	GCTGAGGAGGCTTCAGCTGCTGGTCAC
MMP9	CAGTCCACCCTTGTCTCTTC	TGCCACCCGAGTGAACCAT
MMP2	CCTGATGTCCAGCGAGTG	AGCAGCCTAGCCAGTCC
GAPDH	GGTGCTGTGCTGTGTTC	GCTCCTTCTGGTGTGTTC

primers used in this study (Table 1) were synthesised by Wuhan GeneCreate Biological Engineering Co., Ltd.. The reactions were incubated in a 32-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 5s, 55°C for 10s and 72°C for 15s (data collection). GAPDH was used as a reference for normalization.

Statistical analysis

Data were expressed as the means \pm standard deviations (SDs). Between group differences were tested using a one-way ANOVA. Two-group comparisons were performed by using independent-samples Student's t-tests. $p < 0.05$ was considered significant.

Results

Myrtol rescued body weight decrease caused by irradiation

The body weight gain in mice from control group increased with time. However, the prednisone-treated group and radiation only group were lower than the control group at 2, 4 and 8 weeks post-irradiation. But the Myrtol treatment significantly ($p < 0.05$, $p < 0.01$) rescued the body weight loss caused by irradiation. Even at 16 weeks post-irradiation, body weight loss in prednisone and radiation only group, was still lower than Myrtol group (Fig. 1).

Myrtol reduced bleeding sites and lung coefficient

Lung bleeding sites and edema in Myrtol treated mice were attenuated compared with radiation only animals at 8 weeks post-irradiation (Fig. 2A). But Prednisone can only reduce the severity of acute inflammation in first two weeks. In addition, we also found that the

Fig. 1. Mice body weight alteration from different groups. Average body weight of mice in different group were recorded at 0, 1, 2, 3, 4, 8 and 16 weeks post radiation. Data were expressed as the means \pm standard deviations (SDs) ($n = 6$. * $p < 0.05$, ** $p < 0.01$, IR vs. IR + Myrtol).

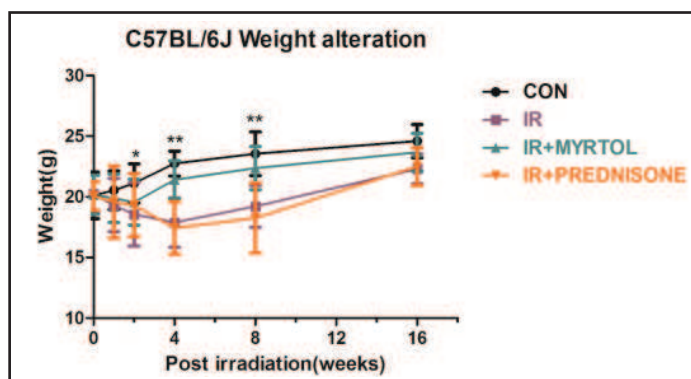
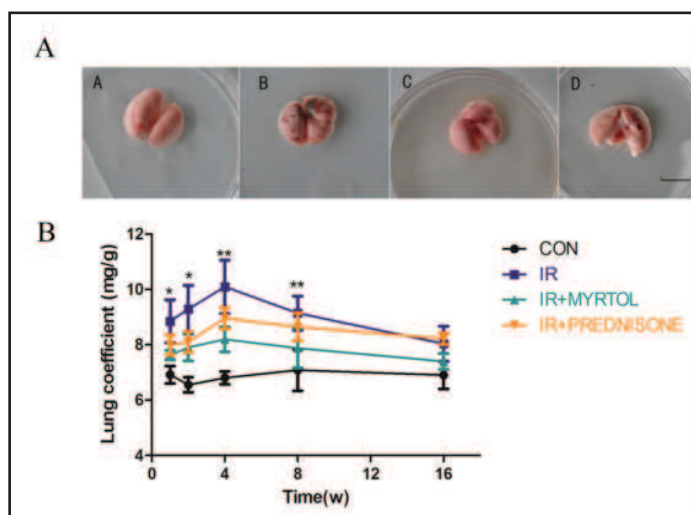


Fig. 2. Lung appearance and lung coefficient. A: Effect of Myrtol standardized on the lung appearance at 8 weeks post gamma-ray irradiation (A) Lung tissue from normal control (B) untreated irradiated (C) Myrtol-treated irradiated (D) prednisone-treated irradiated. Bar = 1cm. B: A bar graph of lung coefficient (lung weight/bw) from different groups. Data were expressed as the means \pm standard deviations (SDs) ($n = 6$, * $p < 0.05$ and ** $p < 0.01$, vs. IR+Myrtol).



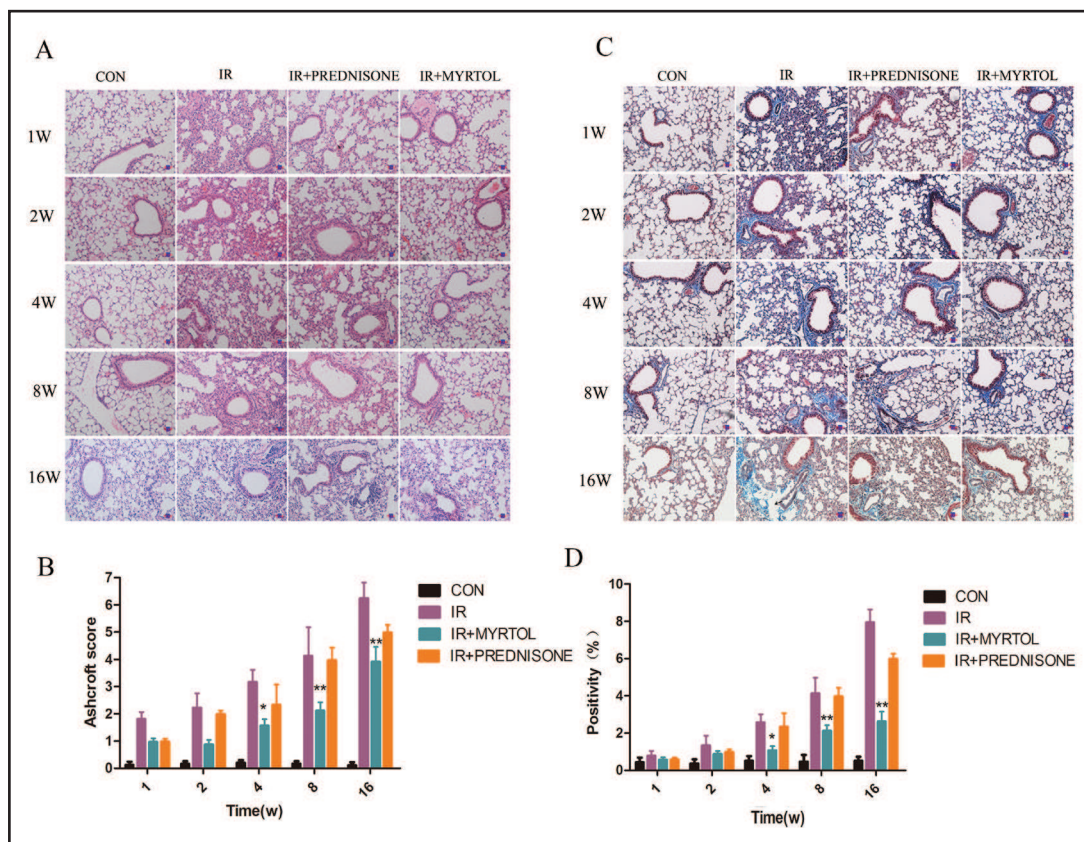


Fig. 3. Histopathological findings of pulmonary inflammation and fibrosis. (A) representative images of H&E staining of lung tissues of mice from different groups at 1, 2, 4, 8, 16 weeks post-irradiation. Bar = 20 μ m. (B) Ashcroft score semiquantitative histological analysis of pulmonary injury and fibrosis from different groups (n=6, * p < 0.05 and ** p < 0.01 vs. IR). (C) Masson's trichrome from mice in the normal non-irradiated (CON), irradiated only (IR), prednisone-treated (PREDNISONE + IR), and Myrtol-treated (MYRTOL+IR) groups. Bar =20 μ m. (D) Quantification of Masson's Trichrome for collagen content from different groups. Data were expressed as the means \pm standard deviations (SDs) (n = 6, * p < 0.05 and ** p < 0.01 vs. IR).

lung coefficient was significantly lower (p < 0.05, p < 0.01) in Myrtol treated mice relative to radiation only mice at 1, 2, 4, 8 and 16 weeks post-irradiation (Fig. 2B). This result correlates with the pulmonary pathological appearance in Myrtol treated animals at different times post-irradiation (Fig. 3A and C). These results indicated that Myrtol attenuated pneumonedema post-irradiation.

Effects of Myrtol on pulmonary inflammation and collagen fibers in vivo

We examined whether Myrtol treatment could exert anti-inflammatory and anti-fibrotic effect in radiation-induced lung injury. We performed H&E stained and Masson-stained assays and observed macrophages infiltration in pulmonary alveoli and fibrosis formation in latter phase (Fig. 3A and C). Severe interstitial pulmonary edema and macrophage accumulation were observed in pulmonary from irradiated group. The alveolitis and fibrosis were significantly reduced in Myrtol groups (p < 0.05, p < 0.01) (Fig. 3B). We confirmed that irradiated mice had marked fibrotic changes in alveolar areas, but irradiated animal treated with Myrtol showed that significantly less damage, as evidenced by Masson-stained sections blue staining rate (p < 0.05, p < 0.01) (Fig. 3D). Morphological observation under inverted microscope, we found that alveolar walls visible thickened, alveoli collapsed, foam

Fig. 4. The effects of myrtol on EMT markers in lung tissues from different groups. Immunofluorescence shows the expressions of vimentin and α -SMA in mice pulmonary ($\times 200$). Notes: vimentin (green), α -SMA (red) and cell nucleus (blue). (A) vimentin, 8 weeks post-radiation. (B) α -SMA, 8 weeks post-radiation. (C) vimentin, 16 weeks post-radiation. (D) α -SMA, 16 weeks post-radiation. DAPI staining (blue) was used to visualize the nuclei ($\times 200$ magnification). Bar, 100 μ m. (E) E-cadherin expression, 8 and 16 weeks post-radiation.

cells around the bronchiole and a large of number of small airway inflammatory cell infiltration. Extensive depositions of collagen and regional fibrotic foci were also observed in the H&E-stained and Masson-stained irradiated pulmonary tissues at 4, 8 and 16 weeks post-irradiation.

We also examined the content of Hyp in lung tissue (Fig. 6F), the major constituent of collagen, from lung tissues. The results showed that less Hyp was found in Myrtol-treated animals than in the irradiation only group and prednisone group. In the first two weeks there was no significant Hyp change observed in Myrtol-treated group compared to controls. However, the amount of Hyp increased in 8 weeks and 16 weeks in the irradiation groups. The Hyp content was significantly lower ($p < 0.05$, $p < 0.01$) in Myrtol-treated animals than in radiation only group at 8 and 16 weeks post-irradiation. Taken together, these results showed marked anti-inflammatory and anti-fibrotic effects of Myrtol *in vivo*.

Effects of Myrtol on mesenchymal markers *in vivo*

Radiation induced EMT is known to promote the processes of fibrosis. So we examined the changes of epithelial marker, E-cadherin, mesenchymal makers, vimentin and α -SMA (Fig. 4). Immunofluorescence staining identified few α -SMA and vimentin positive cells in the lung of Myrtol-treated mice at 8 and 16 weeks post-radiation. In contrast, strong vimentin and α -SMA staining was detected in the pulmonary arterioles of irradiation only and prednisone group in lung tissue. Similarly, we found that E-cadherin expression was upregulated in the lungs of Myrtol-treated animals at 8 and 16 weeks post-radiation. These results

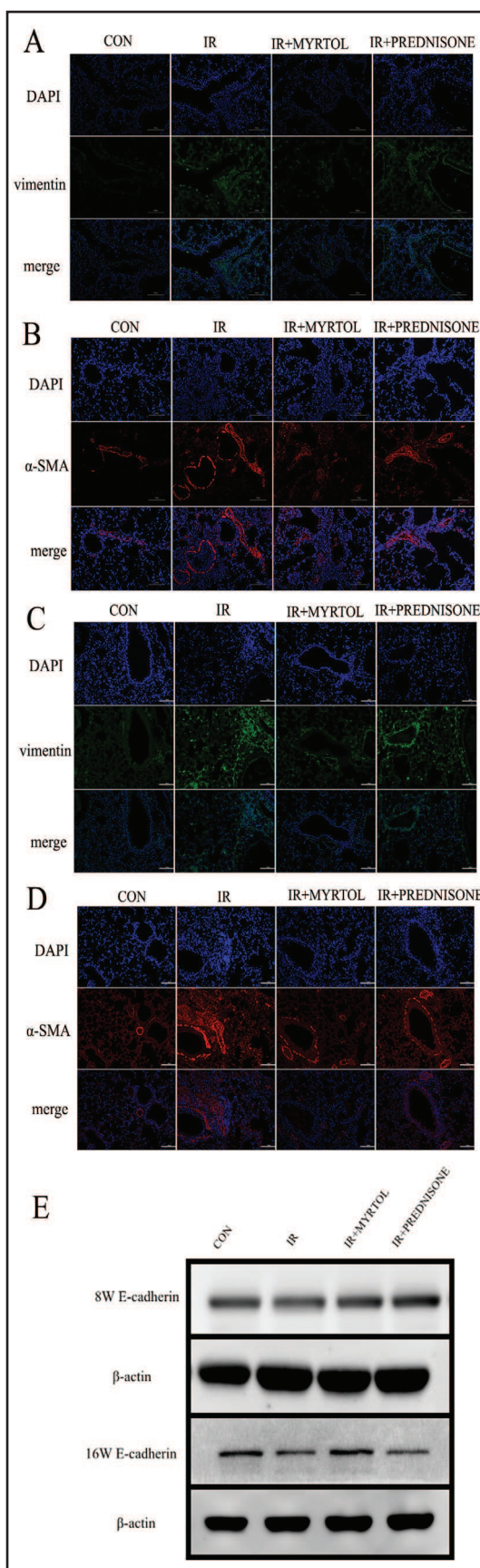
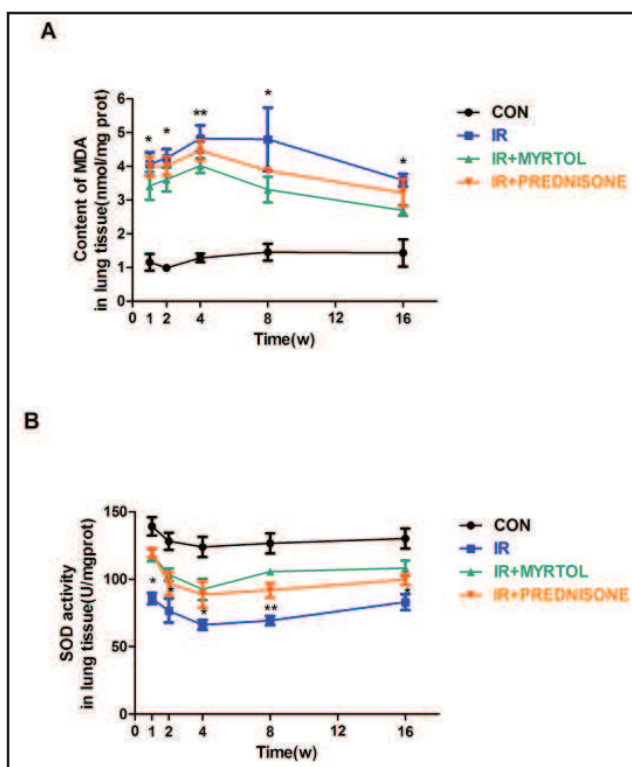


Fig. 5. Effects of Myrtol on the activities of SOD and on the MDA content in the lung tissue. (A) Effect of Myrtol on mice pulmonary MDA content (B) SOD activity at 1, 2, 4, 8 and 16 weeks post-irradiation. Data were presented as mean \pm SD (n = 6, * P < 0.05, ** P < 0.01 vs. IR + MYRTOL).



indicated that Myrtol treatment could inhibit the process of EMT, which might account for some possible mechanism.

Myrtol modulates the lung tissue MDA content and SOD activity

Lung tissue MDA content and SOD activity were measured to assess the oxidative and antioxidant statuses, respectively. The MDA content of lung tissue irradiation only group dramatically increased, which was inhibited by Myrtol treatment ($p < 0.05$, $p < 0.01$) (Fig. 5A). We also detected the lung SOD activity, an indicator of antioxidant capacity, at 1, 4, 8 and 16 weeks after irradiation (Fig. 5B), SOD activity was significantly higher ($p < 0.05$, $p < 0.01$) in Myrtol-treated animals compared with animals in radiation only group at 1, 2, 4, 8 and 16 weeks post-irradiation. SOD activity in the prednisone group was similar with irradiation mice at 4, 8 and 16 weeks post-irradiation. These data demonstrated that Myrtol could relief oxidative damage *in vivo*.

Myrtol regulates serum cytokine levels and mRNA expression levels

As depicted in Fig. 6, we found that the level of both proinflammatory cytokines (TNF- α , IL-1 β , IL-6 and PGE2) and TGF- β 1 in the serum were increased at 2, 4, 8, 16 weeks in irradiation only group. The myrtol treatment significantly ($p < 0.05$, $p < 0.01$) down-regulated the levels of proinflammatory cytokines (TNF- α , IL-1 β , IL-6 and PGE2) and TGF- β 1. Interestingly, PGE2 in latter stage still kept higher levels than that in other groups, indicating a role in preventing radiation-induced fibrosis. These results demonstrated that Myrtol treatment down-regulated the cytokines and the mRNA levels (Fig. 7A-E) in the radiation induced lung injury mice.

Effects of Myrtol on matrix metalloproteinases expression and Akt phosphorylation

AKT signaling pathway plays a critical role in radiation induced lung injury [19]. Our data showed that the phosphorylation of AKT increased in the lung of irradiated mice compared with mice in control group, and Myrtol inhibited the phosphorylation of AKT in lung level in irradiated mice. Myrtol administration inhibited MMP-2 expression and down-regulated

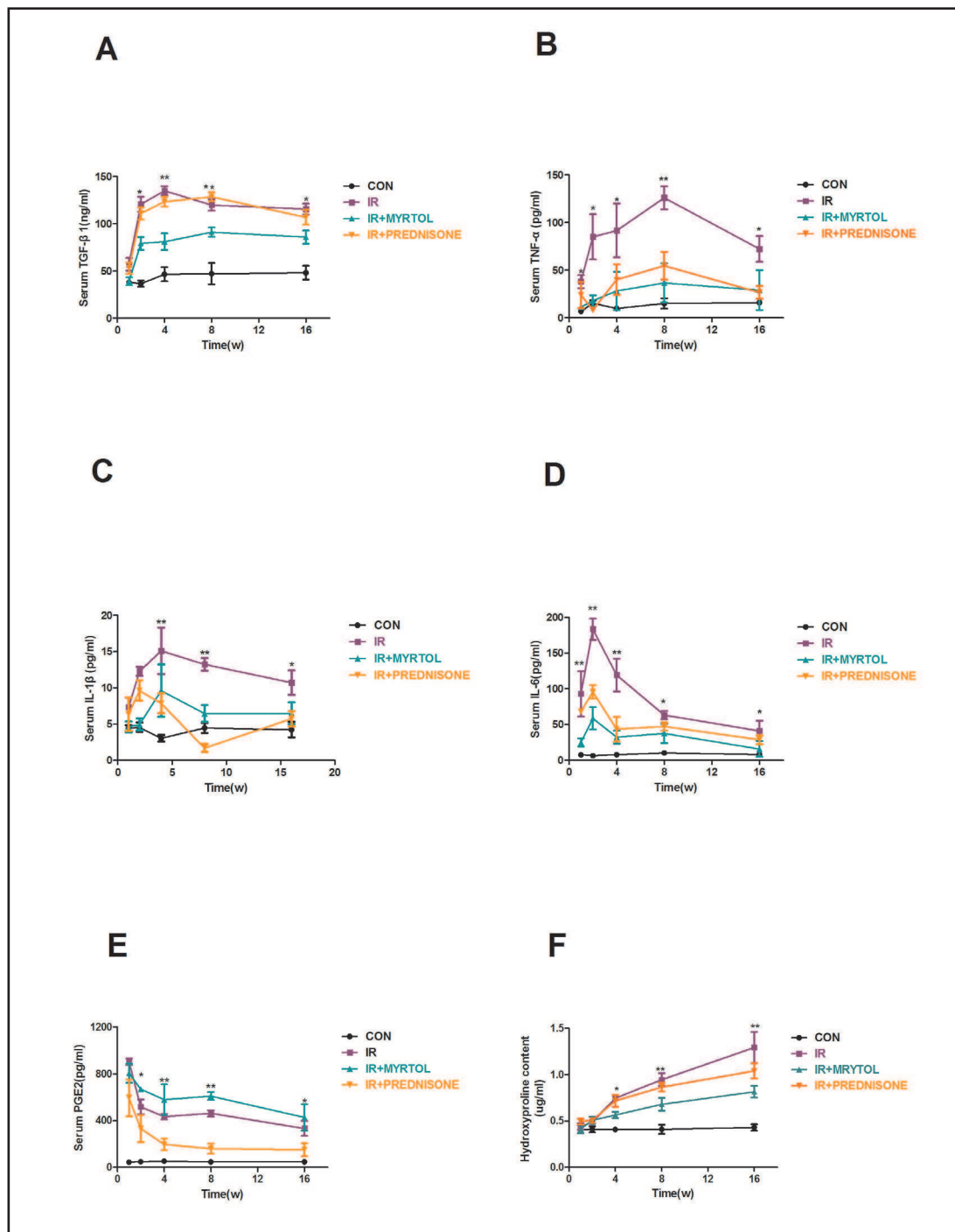


Fig. 6. Expression of serum cytokines and hydroxyproline in lung tissue from different groups in radiation induced injury mice model at 1, 2, 4, 8, 16 weeks post irradiation. (A) The serum levels of transforming growth factor β1 (TGF-β1) (B) tumor necrosis factor α (TNF-α) (C) interleukin (IL)-1β (D) interleukin (IL)-6 and (E) PGE2 and (F) Hydroxyproline (Hyp) content. Data were presented as mean ± SD (n = 6, * p < 0.05, ** p < 0.01, IR vs. IR + Myrtol).

p-AKT protein levels at 2 and 16 weeks post-irradiation (Fig. 8A; 2 weeks B; 16weeks). Real-time PCR analysis also indicated that Myrtol significantly (p < 0.05, p < 0.01) inhibited MMP-2/-9 gene expression in lung tissue at different times (Fig. 7F and G).

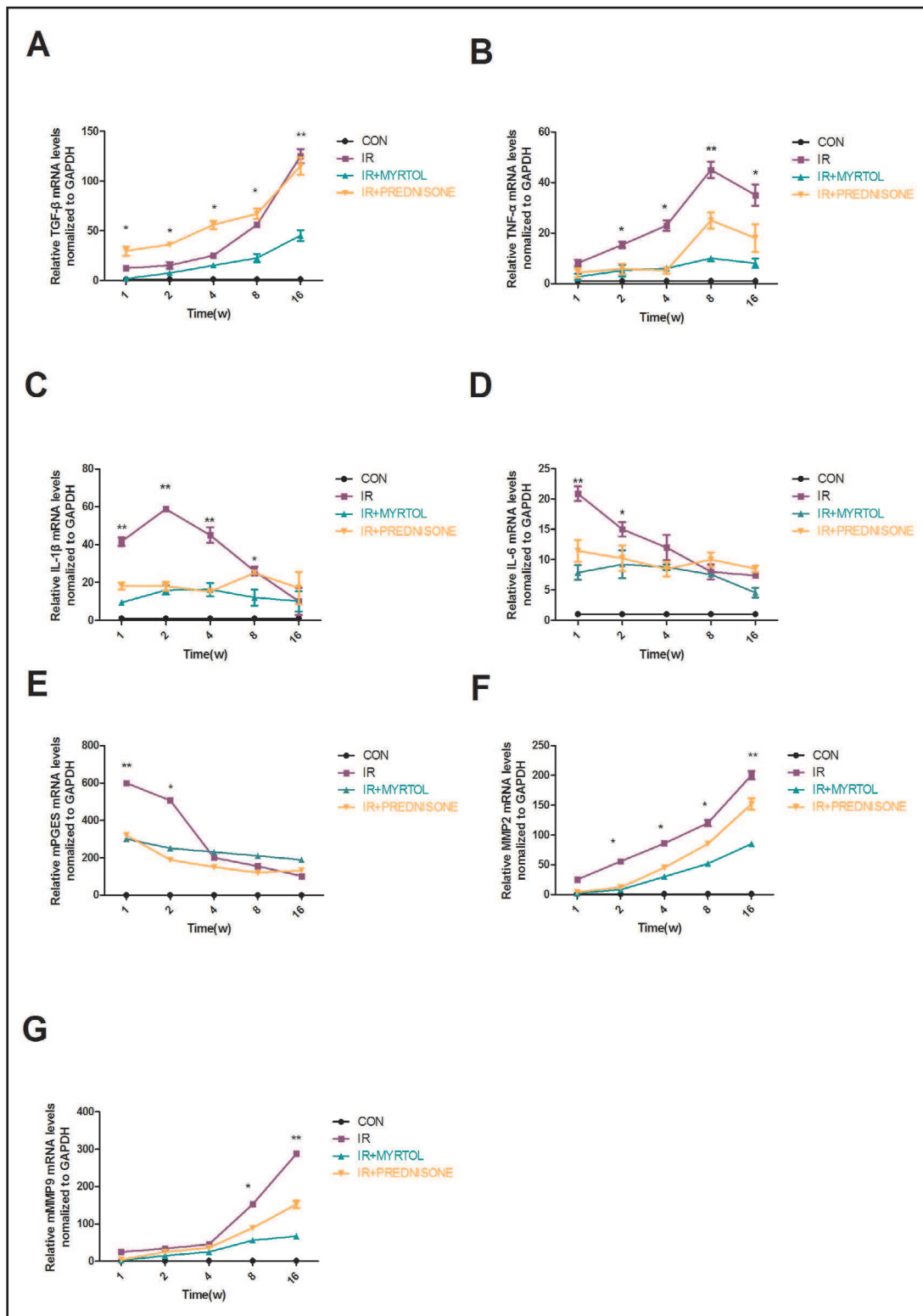


Fig. 7. The mRNA expression of pro-inflammatory cytokines and the tissue growth factor- β 1 (TGF- β 1) in radiation induced injury mice model at 1, 2, 4, 8, 16 weeks post irradiation. (A) TGF- β 1 mRNA, (B) TNF- α mRNA expression, (C) IL-1 β mRNA expression, (D) IL-6 mRNA expression, (E) mPGES mRNA expression, (F) MMP2 mRNA expression, (G) MMP9 mRNA. Data were presented as mean \pm SD and obtained average value for each mRNA was used for statistics, n = 6, * P < 0.05, ** p < 0.01, IR vs. IR + Myrtol group.

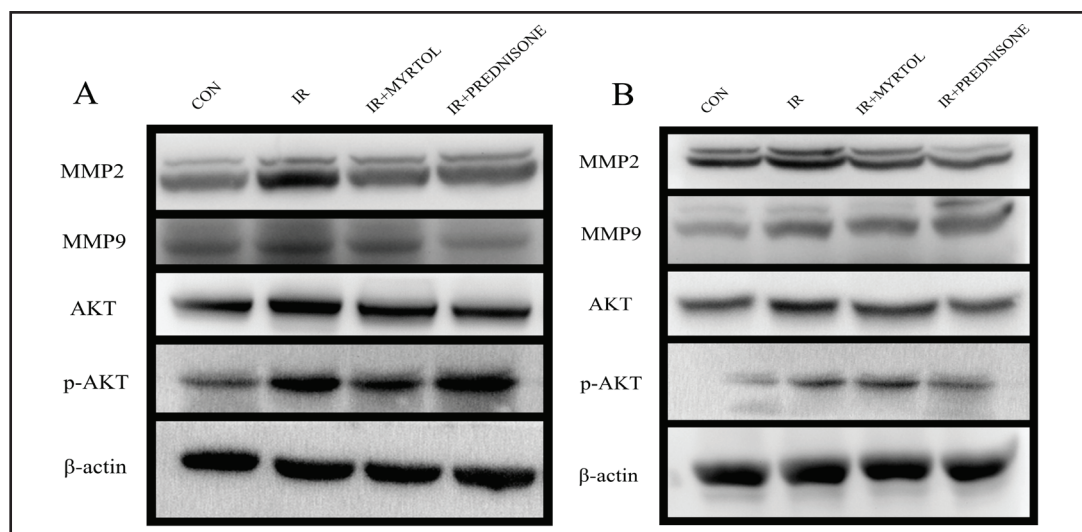


Fig. 8. Myrtol inhibited the phosphorylation of AKT and down-regulated the expression level of MMP-2 and MMP-9. Representative images of the phosphorylation of AKT and expression level of MMP-2 and -9 in different groups after radiation at 2 (A) and 16 weeks (B) were detected by western blot.

Discussion

In this study, we presented experimental evidence demonstrating the protective effects of Myrtol on radiation-induced lung injury. We confirmed that Myrtol oral administration not only ameliorated mice radiation pneumonitis in early phase but also inhibited lung fibrosis formation in latter phase. Myrtol treatment could reduce lung coefficient, improve lung histological change, decrease Hyp content, reduce the radiation-induced oxidative damage in mice pulmonary, and regulate the levels of TGF- β 1, TNF- α , IL-1 β , IL-6 and PGE2. We also showed that Myrtol treatment inhibited AKT activation and down-regulated the matrix metalloproteinase-2(MMP-2) and matrix metalloproteinase-9(MMP-9) expression. Increasing evidence indicates that oxidative stress and ROS contribute directly and indirectly to the progression of radiation-induced injury [20]. The ROS-induced activation of inflammatory cells increased production of ROS [21]. Overbalance of oxidative stress in the lung kept inducing tissue damage, therefore, antioxidant component may be used to remedy radiation-induced lung injury. There are a lot of studies showing that the Myrtol has effective antioxidant activity, which can be used as scavenger of ROS and free radicals [22-26]. It can be shown that Myrtol Standardized ameliorates inflammatory processes by interacting with aggressive oxygen radicals of the \cdot OH and interfere with leukocyte activation [10].

MDA levels reflect mice lipid peroxidation damage and can indicate damage to cell membranes [27]. On the other hand SOD plays a crucial role in antioxidation. After the discovery of SOD in 1969 [28, 29], it was found to protect simple biologic systems from superoxide radical toxicity. Since then, SOD has been found to decrease radiation damage in a wide range of biologic systems. There are some studies describing the antioxidant activities of different extracts of Myrtle [30, 31]. Myrtol has been proved to exert anti-oxidative and antibacterial activities via oligomeric, non-prenylated acylphloroglucinols such as myrtucommulone and semi-myrtucommulone [32, 33]. In another study, black *Myrtus communis* berries contain high levels of anthocyanins and flavonoids shown high radical scavenging activities [34]. In our present study, we also found that the levels of lung tissue MDA significantly decreased ($p < 0.05$, $p < 0.01$) while SOD activity increased along with the treatment of Myrtol ($p < 0.05$, $p < 0.01$) (Fig. 5). We also found that antioxidant activity of Myrtol was preserved in more than 3 months as described by Montoro et al. [35]. At the same time our results showed that

prednisone group reduced MDA level only in first 4 weeks after radiation. These results suggest that Myrtol can reduce oxidative damage, and increase antioxidant proteins and alleviates radiation lung injuries.

At 4-8 weeks after irradiation, the lung tissue had increased deposition levels of hydroxyproline content (Fig. 6F), which was regarded as a marker of the level of collagen content [36]. Hydroxyproline in lung tissue were detected by using a hydroxyproline assay kit. We found that Myrtol dramatically decreased the Hyp content and alleviated the lung tissue fibrosis formation in 4, 8, 16 weeks, even though the treatment of prednisone could reduce early radiation-induced pneumonitis. However, it did not show inhibition of the latter fibrosis formation. Conversely, long term administration of prednisone showed some severe side effects including weight loss, osteoporosis, infection and cardio-cerebrovascular complications.

To investigate the mechanism of Myrtol treatment on inflammation, we measured the serum levels of key inflammatory cytokines including TGF- β 1, TNF- α , IL-1 β , IL-6 and PGE2 (Fig. 6). The ELISA results showed that Myrtol attenuated mice inflammation cytokines. These cytokines were significantly reduced ($p < 0.05$, $p < 0.01$) in the Myrtol-treated animals compared with the untreated radiated treated mice, and these effects lasted for months after treatment ceased.

TGF- β 1 has been shown playing a critical role in the development of pulmonary fibrosis and epithelial mesenchymal transition (EMT) [37-40]. TGF- β 1 was shown to promote fibroblast proliferation and maturation, thereby accelerating the development of pulmonary fibrosis [41]. Blocking TGF- β 1 or its activation could thus be effective treatments for radiation induce lung injury. A number of inflammatory cytokines including TNF- α and IL-1 β are overexpressed in lung tissue from radiation induce lung injury mice model, which is known to sustain TGF- β 1 expression and to promote the progression of the pulmonary fibrosis. Moreover, our *in vivo* results showed that Myrtol decreased the expression of vimentin and α -SMA and increased the levels of E-cadherin (Fig. 4). These indicate that Myrtol could inhibit TGF- β 1 mediated transdifferentiation, potentially via its antioxidant and anti-inflammatory activities.

TNF- α is a pleiotropic cytokine in response to infection or injury [42]. TNF- α mediates lung cytokine networking and eosinophil recruitment in pulmonary fibrosis. TNF- α plays a vital role in pro-inflammatory networks and may be involved in the development and progression of radiation-induced pneumonitis through inducing TGF- β 1 expression in lung fibroblasts [43, 44]. Furthermore, TNF- α polymorphisms indicative of high levels of TNF- α expression correlate with an increased risk of developing interstitial pulmonary fibrosis (IPF) [45, 46]. TNF- α activates fibroblasts proliferation and stimulate secretion pro-inflammatory cytokines, including IL-1 and IL-6 in neutrophils and macrophages [47]. IL-6 also plays an important role in the connective tissue fiberization, potentially by increasing collagen deposition, inhibiting extracellular matrix (ECM) degradation, and stimulating fibroblast proliferation. Previous studies have suggested that IL-6 leads to inflammation and fibrosis associated with hypersensitivity pneumonitis in mice [48, 49]. These results suggest a close relationship in IL-6 and pneumonitis and fibrotic development. Our ELISA results showed that IL-6 levels were reduced by Myrtol in early 4 weeks. Myrtol also effectively reduced IL-1 β , which is one of pro-inflammatory cytokines thought to be involved in many acute and chronic diseases.

Prostaglandin E2 (PGE2) is commonly regarded as a potent pro-inflammatory mediator and is involved in several inflammatory diseases [50]. PGE2 can regulate lymphocyte trafficking into tissue and it can also inhibit Th2 differentiation. In our study, pulmonary irradiation stimulated the production of several cytokines in the early stage and consequently stimulated the release of great amounts of PGE2 by fibroblast. We found that Myrtol could significantly reduce ($p < 0.05$, $p < 0.01$) PGE2 expression in early stage. However, PGE2 also plays a negative feedback role in cytokine production and down-regulate fibroblast metabolic functions including proliferation, collagen synthesis and transition to myofibroblasts [51]. In the later phase (8-16weeks postradiation), there was an downregulation of the expression

of PGE₂, as previously reported in fibrotic fibroblasts and damaged lung epithelial cells [52], thus potentially leading to the persistence of immune activation and chronic inflammation. In our study, Myrtol group preserved high level of PGE₂ in latter fibrosis stage compared with irradiation only group and prednisone group, indicating a role of Myrtol and PGE₂ on lung tissue damages in latent stage, including limiting the immune-inflammatory response as well as tissue repair process.

Matrix metalloproteinases (MMPs) play an essential role in lung injury associated with tissue remodeling structural proteins such as collagens and elastin structural proteins such as collagens and elastin [53]. Moreover, strong evidences show that AKT signaling pathway plays a critical role in promoting MMP-2 and MMP-9 expression [54]. Matrix metalloproteinases (MMPs), a family of Zn-dependent endopeptidases, degrade all kinds of extracellular matrix (ECM) proteins, and participate in tumor progression including growth, angiogenesis, tissue invasion and migration [55]. Selman et al. [56] found that IPF patient MMP-2 and MMP-9 high expression contributes to lung fibrosis. Corbel et al. [57] used MMPs inhibitor batimastat can significantly reduce mice pulmonary fibrosis. Suga et al. [58] reported that MMP-2 might contribute to pulmonary structural remodeling through type IV collagenolytic activity. In this study, we show that Myrtol remarkably down regulates secretion of both MMP-2 and MMP-9. While prednisone having no great effect on MMP-2/-9 secretion compared with radiation only group. Our western blot analysis results revealed that Myrtol enhanced the expression levels of E-cadherin, and reduced AKT, p-AKT, MMP-2/-9 expression in mice lung tissues compared with radiation-only mice. The western blot result showed that the expressions and activities of MMP-2 and MMP-9 were inhibited by Myrtol administration, and inhibited of AKT pathway which played important roles in EMT.

Conclusion

In conclusion, the present results have shown that Myrtol treatment provides persistent antioxidant, anti-inflammatory, and anti-fibrosis effects that protect against radiation-induced injury. These findings suggest that Myrtol might suppress EMT process through inhibiting of AKT activation. These findings suggest that Myrtol has great potential to be used in treating radiotherapy complications in terms of protecting from radiation-induced lung injury.

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Disclosure Statement

The authors declare that they have no interests of any kind that could inappropriately influence (bias) the publication of this paper.

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