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Glycyrrhetinic acid alleviates radiation-induced lung injury in mice

Jinmei Chen[1,2,3], Weijian Zhang[1,2,3*], Lurong Zhang[2,3], Jiemin Zhang[2,3], Xiuying Chen[1,2,3], Meichun Yang[1], Ting Chen[1] and Jinsheng Hong[1,2,3*]

[1]Department of Radiation Oncology, First Affiliated Hospital, Fujian Medical University, No. 20 Chazhong Road, Taijiang District, Fuzhou City, Fujian Province, China
[2]Key Laboratory of Radiation Biology (Fujian Medical University), Fujian Province University, No. 20 Chazhong Road, Taijiang District, Fuzhou City, Fujian Province, China
[3]Fujian Key Laboratory of Individualized Active Immunotherapy, Fujian Medical University, No. 20 Chazhong Road, Taijiang District, Fuzhou City, Fujian Province, China
*Corresponding author. Jinsheng Hong: Department of Radiation Oncology, First Affiliated Hospital, Fujian Medical University, No. 20 Chazhong Road, Taijiang District, Fuzhou City, Fujian Province, China. Fax: +86-591-87981028; Email: hjs703@126.com; Tel: +86-591-87982185
Weijian Zhang: Department of Radiation Oncology, First Affiliated Hospital, Fujian Medical University, No. 20 Chazhong Road, Taijiang District, Fuzhou City, Fujian Province, China. Fax: +86-591-87981028; Email: zwj9090@126.com;Tel: +86-591-87982185
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ABSTRACT

Radiation-induced lung injury (RILI) is a common complication of thoracic radiotherapy, but efficacious therapy for RILI is lacking. This study ascertained whether glycyrrhetinic acid (GA; a functional hydrolyzed product of glycyrrhizic acid, which is extracted from herb licorice) can protect against RILI and investigated its relationship to the transforming growth factor *(TGF)-β1/Smads* signaling pathway. C57BL/6 mice were divided into four groups: a control group, a GA group and two irradiation (IR) groups. IR groups were exposed to a single fraction of X-rays (12 Gy) to the thorax and administered normal saline (IR + NS group) or GA (IR + GA group). Two days and 17 days after irradiation, histologic analyses were performed to assess the degree of lung injury, and the expression of *TGF-β1*, *Smad2*, *Smad3* and *Smad7* was recorded. GA administration mitigated the histologic changes of lung injury 2 days and 17 days after irradiation. Protein and mRNA expression of *TGF-β1*, *Smad2* and *Smad3*, and the mRNA level of *Smad7*, in lung tissue were significantly elevated after irradiation. GA decreased expression of *TGF-β1*, *Smad2* and *Smad3* in lung tissue, but did not increase *Smad7* expression. GA can protect against early-stage RILI. This protective effect may be associated with inhibition of the *TGF-β1/Smads* signaling pathway.

KEYWORDS: glycyrrhetinic acid, thoracic irradiation, pneumonia, TGF-β1, Smad

INTRODUCTION

Radiation-induced lung injury (RILI) is a common complication in radiotherapy inpatients with thoracic tumors [1]. It can be separated into two stages: acute/subacute pneumonia (early stage) and pulmonary fibrosis (late stage). The symptoms of RILI are a cough, asthma, and even respiratory failure. It affects quality of life greatly, and may even lead to death [2].

Drugs to treat radiation-induced pneumonia and radiation-induced pulmonary fibrosis are inadequate. Corticosteroids are the most common therapy for radiation-induced symptomatic pneumonia. Corticosteroids can relieve some symptoms, but cannot prevent the development of RILI [3, 4]. The anti-inflammatory agent celecoxib has been considered for use against chronic lung damage [5], but clinical evidence for its efficacy is lacking. Amifostine has been approved by the U.S. Food and Drug Administration for use as an anti–free radical drug. It has elicited some protective effects against radiation-induced pneumonia [6] but not fibrosis. In addition, the necessity for intravenous injection 30 min before radiation exposure and severe side effects make long-term use for chronic lung fibrosis impossible. Hence, finding effective therapy for RILI with few and non-severe side effects is urgent [7–9].

Glycyrrhetinic acid (GA) is a functional hydrolyzed product of glycyrrhizic acid, which is extracted from herb licorice. In several countries, glycyrrhizic acid is used as a sweetener or flavoring in confectionery, pharmaceuticals, and tobacco products [10, 11]. It can be taken orally and has low toxicity. It has also been used for the treatment of dermatitis [12], chronic hepatitis [13], cough, asthma and pneumonia [14]. Several studies have shown that GA can alleviate inflammatory lung disease [15–18]. Inflammation is the predominant histologic and physiologic feature of early-phase RILI [19, 20]. Hence, this study investigated whether GA can protect against RILI and we have discussed the potential mechanism for this effect.

MATERIALS AND METHODS

Animals

The study was approved by the ethics committee of our institution. Eight-week-old female specific-pathogen-free C57BL/6 mice with weight of 19–22 g (Shanghai SLRC Laboratory Animals, Shanghai, China) were divided randomly into four groups: normal control ($n = 19$); GA without irradiation (IR) (GA group; $n = 20$); IR plus normal (0.9%) saline (IR + NS group; $n = 20$); and IR plus GA (IR + GA group; $n = 20$). Mice were housed in conventional cages under a 12 h–12 h light–dark cycle and controlled temperature and humidity. Sterilized food and water were supplied for the mice.

Radiation procedure

Anesthesia (10 ml/kg 4% chloral hydrate, i.p.) was induced in the mice. Each mouse was placed in an organic glass cage (made by the research team) with its neck fixed and body stretched fully. Groups of 7 or 8 mice were placed on an IR bed with their necks in a straight line and irradiated together. A linear accelerator (Clinac 600 C/D; Varian Medical Systems, Palo Alto, CA, USA) created an X-ray IR field of 38×1.2 cm^2 to encompass the entire thorax of each mouse on the bed (voltage, 6 mV; direction, 0°; total dose, 12 Gy; rate, 3 Gy/min; distance from source to skin, 100 cm). Organs outside the IR field were shielded by the multileaf collimator of the linear accelerator.

Drug administration

Two IR groups and the GA group were treated with the corresponding agents by gavage once a day from 2 h before IR to 7 days after IR, then every 2 days until the 17th day after IR. Mice in the IR + NS group were administered 10 ml/kg NS each time, and mice in the IR + GA and GA groups were administered 40 mg/kg GA (Acros Organics, Pittsburgh, PA, USA) each time.

Histologic examination

Two days after IR, 10 mice from each of the four groups were euthanized, and the remaining mice were euthanized on Day 17. The left upper lobes of the lungs were fixed in 10% neutral-buffered formalin overnight, embedded in paraffin, and cut into sections (thickness, 5 μm). Some sections were stained with hematoxylin and eosin (H&E) for histopathologic analyses and scored in a blinded manner according to a semi-quantitative scale [21]. Five random fields (magnification ×200 for each section) were scored by two clinicians using an optical microscope. The following system was used to score histologic changes in pneumonia: 1, no pneumonia; 2, pneumonia alterations in 1–20% of the field; 3, pneumonia alterations in 21–50% of the field; 4, pneumonia alterations in 51–100% of the field. Some other sections were used for immunohistochemical analyses (see below).

Protein extraction and enzyme-linked immunosorbent assay

The protein in the right lungs was extracted using a Protein Extraction kit (Shanghai Sangon Biotechnology, Shanghai, China). Protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Shanghai Sangon Biotechnology). Concentrations of transforming growth factor *(TGF)-β1* in lung homogenates were quantified with a Mouse *TGF-β1* protein extraction and enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions. Optical density values were detected using a Microplate Reader (iMark; Bio-Rad, Hercules, CA, USA) at 450 nm.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted from the left lower lobes of the lungs of the mice in each group using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNase-free DNase (Invitrogen) was used to remove contaminating genomic DNA according to the manufacturer's instructions. cDNA was synthesized from the total RNA using a Reverse Transcription system (Promega, Fitchburg, WI, USA), following the manufacturer's protocols. RNA extraction and quantitative real-time polymerase chain reactions (qRT-PCRs) were carried out in a total volume of 20 μl using an ABI7500 RT-PCR system (Applied Biosystems, Foster City, CA, USA) with GoTaq® qPCR Master Mix (Promega) under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, then each of 95°C, 15°C and 95°C for 15 s mRNA levels of *TGF-β1*, *Smad2*, *Smad3* and *Smad7* in lung tissue were determined for each group with the mRNA level of β-actin used for standardization. Sequences of mouse primers are listed in Table 1.

Table 1. Primer sequences for qRT-PCR

Gene	Primer	Sequence
TGF-β1	forward	5′-TTTGGAGCCTGGACACACAGTA-3′
	reverse	5′-CGTAGTAGACGATGGGCAGTG-3′
Smad2	forward	5′-CGGCTGAACTGTCTCCTACTAC-3′
	reverse	5′-TATGCGATTGAACACCAGAATG-3′
Smad3	forward	5′-AGGTGTGCGGCTCTACTACATC-3′
	reverse	5′-GTTGGGAGACTGGACGAAAATA-3′
Smad7	forward	5′- AGGCTGTGTTGCTGTGAATCT-3′
	reverse	5′- CTCCAGAAGAAGTTGGGAATCT-3′
β-actin	forward	5′-GAAGGACTCCTATGTGGGTGAC-3′
	reverse	5′-TTCACGGTTGGCCTTAGG-3′

Immunohistochemical analyses

The remaining sections of left upper lung lobes were deparaffinized with xylene and hydrated with ethanol of different concentration gradients. These actions were followed by antigen retrieval with microwave thermal remediation in 0.01 M citrate buffer (pH 6.0; for *Smad2* staining) or TRIS/ethylenediamine tetra-acetic acid buffer (pH 9.0; for *TGF-β1* or *Smad3* staining). Endogenous peroxide activity was inactivated by incubating samples in 3% hydrogen peroxide for 10 min. Sections were incubated with antibodies against *TGF-β1* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), *Smad2* (Abcam, Cambridge, MA, USA) and *Smad3* (Abcam) at 1:100 dilution at room temperature for 2 h, and the Polink-2 Plus® Polymer Horseradish Peroxidase Detection system for rabbit primary antibody (GBI Laboratories, Mukilteo, WA, USA) according to the manufacturer's instructions. A 3,3'-Diaminobenzidine kit (ZSGB-BIO, Beijing, China) was used for signal amplification, and hematoxylin was used as the counterstain. Appropriate positive and negative controls of relevant histologic tissue sections were included for each stain to ensure signal specificity. Stained sections were analyzed in a blinded manner according to the method of Remmele and Stegner [22]. Each section was observed randomly in five fields of magnification (×400) using a system to score staining intensity (scored as A) and percentage of positive cells (scored as B). That is, for A: 0, not stained; 1, light-yellow stain; 2, pale-brown stain; 3, dark-brown stain. Then, for B: 0, no positive cells; 1, fewer than one-third of cells stained; 2, one-third to two-thirds of cells stained; 3, more than two-thirds of cells stained. Scores of each section were the average of five fields (calculated by multiplying A and B).

Statistical analyses

Data are the mean ± standard deviation (SD). Statistical analyses were carried out using one-way ANOVA, followed by Bonferroni's multiple comparison test. $P < 0.05$ was considered significant.

RESULTS

Histologic evaluation of lung injury

Two clinicians evaluated H&E-stained sections of lungs and scored histologic changes after IR. The control group and the GA group both showed no obvious inflammatory changes. However, massive infiltration of inflammatory cells in the alveolae, and alveolar septal edema were observed in the IR + NS group, whereas fewer inflammatory cells and less edema was seen in the IR + GA group (Fig. 1A). Histologic scoring demonstrated that lungs irradiated with a single fraction of 12 Gy had significant changes due to IR-induced injury on Day 2 and Day 17, and that GA administration significantly mitigated IR-induced injury to the lungs as compared with NS administration at each time-point ($P < 0.001$, Fig. 1B).

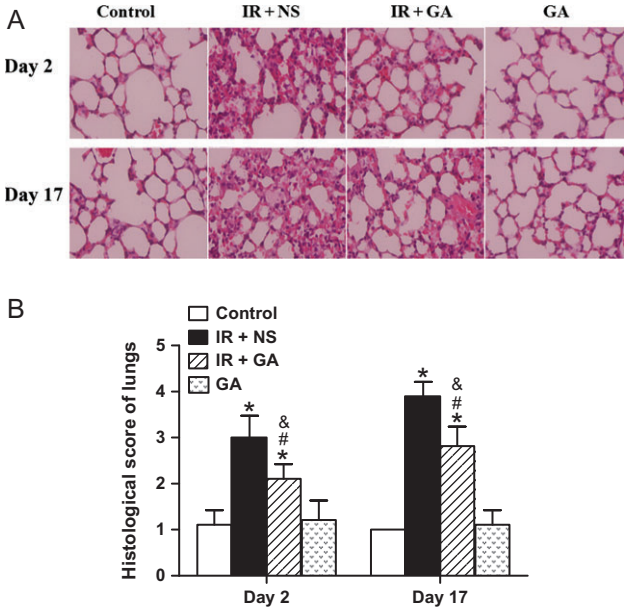

Fig. 1. Histologic detection and semi-quantification of radiation-induced lung injury in mice. Mice of the IR + NS group and IR + GA group were exposed to a single fraction of X-ray irradiation (12 Gy) to the thorax and administered NS or GA. The GA group was not exposed to irradiation. Panel A: Typical hematoxylin and eosin (H&E)-stained sections of mouse lungs from each group 2 days and 17 days after irradiation (magnification × 400). Panel B: Histologic scores of lung injury from H&E-stained sections. Data are the mean ± SD. $^{*}P < 0.05$, *vs* control group; $^{\#}P < 0.05$, *vs* IR + NS group; $^{\&}P < 0.05$, *vs* GA group, at identical time-points, by one-way ANOVA (followed by Bonferroni's multiple comparison test). IR = irradiation, NS = normal saline, GA = glycyrrhetinic acid.

Protein expression of *TGF-β1* in lung tissue

TGF-β1 concentrations (as determined by ELISA) of lung homogenates from irradiated mice administered NS (Day 2, 63.56 ± 3.81; Day 17, 79.48 ± 4.96 pg/ml) were significantly higher than those from mice in the control group (45.51 ± 5.58; 47.41 ± 3.73 pg/ml, respectively) and those from mice in the GA group (46.66 ± 2.72; 49.22 ± 3.74 pg/ml, respectively) ($P < 0.05$). GA administration had no significant effect on *TGF-β1* concentrations of lung homogenates from non-IR mice compared with those from the normal control group on either Day 2 or Day 17 ($P < 0.05$). GA administration significantly reduced *TGF-β1* expression in irradiated lungs at both time-points ($P < 0.05$). *TGF-β1* concentrations in lung tissues of mice from the IR + GA group (Day 2, 49.82 ± 4.81; Day 17, 53.78 ± 3.93 pg/ml) were not significantly different from those of the control group or the GA group on Day 2 ($P > 0.05$), but were significantly different from those on Day 17 ($P < 0.05$, Fig. 2).

Gene expression of *TGF-β1*, *Smad2*, *Smad3* and *Smad7* in lung tissue

Gene expression of *TGF-β1*, *Smad2*, *Smad3* and *Smad7* in lung tissues was determined by qRT-PCR (Fig. 3A–D). IR to the thorax with a single fraction of 12 Gy significantly upregulated the gene expression of *TGF-β1*, *Smad2*, *Smad3* and *Smad7* in lungs on Day 2 and Day 17 ($P < 0.05$); the gene expression of *TGF-β1*, *Smad2*, *Smad3* and *Smad7* for non-irradiated lungs of mice with GA

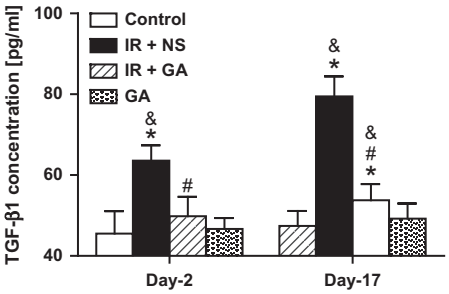

Fig. 2. ELISA detection of *TGF-β1* concentrations in mouse lungs after irradiation. Mice of two irradiated groups, exposed to a single fraction of X-ray irradiation (12 Gy) to the thorax, were treated with NS or GA. The GA group was not exposed to irradiation. Concentrations of the cytokine *TGF-β1* in lung homogenates were determined by ELISA 2 days and 17 days after irradiation. Data are the mean ± SD. $^{*}P < 0.05$, *vs* **control group;** $^{\#}P < 0.05$, *vs* **IR + NS group;** $^{\&}P < 0.05$, *vs* **GA group, at identical time-points, by one-way ANOVA (followed by Bonferroni's multiple comparison test). IR = irradiation, NS = normal saline, GA = glycyrrhetinic acid.**

administration was not significantly different from those with NS administration. Irradiated lungs from GA-administered mice had significantly lower gene levels of *TGF-β1*, *Smad2* and *Smad3* on Day 2 and Day 17 compared with those from NS-administered mice ($P < 0.05$). However, expression of the *Smad7* gene for irradiated lungs was not significantly different between GA-administered and NS-administered mice.

Immunohistochemical examination of expression of *TGF-β1*, *Smad2* and *Smad3*

Expression of *TGF-β1*, *Smad2* and *Smad3* in lung sections 2 days and 17 days after IR exposure was scored. Lung sections of irradiated groups at both time-points had higher staining intensities and more positive cells for *TGF-β1*, *Smad2* and *Smad3* as compared with the non-irradiated control group (Fig. 4A–C). GA administration significantly reduced the expression of *TGF-β1*, *Smad2* and *Smad3* in irradiated lung tissue compared with NS administration on Day 2 and Day 17 ($P < 0.05$), but the levels were not significantly changed in non-irradiated lung tissue on Day 2 and Day 17. Importantly, levels of *TGF-β1* and *Smad2* protein in lungs from irradiated mice administered GA were not significantly different to their baseline levels on Day 2 and Day 17 ($P > 0.05$) (Fig. 5).

DISCUSSION

The present study showed that GA can protect mice against early-phase lung injury and decrease *TGF-β1* concentrations in the lung

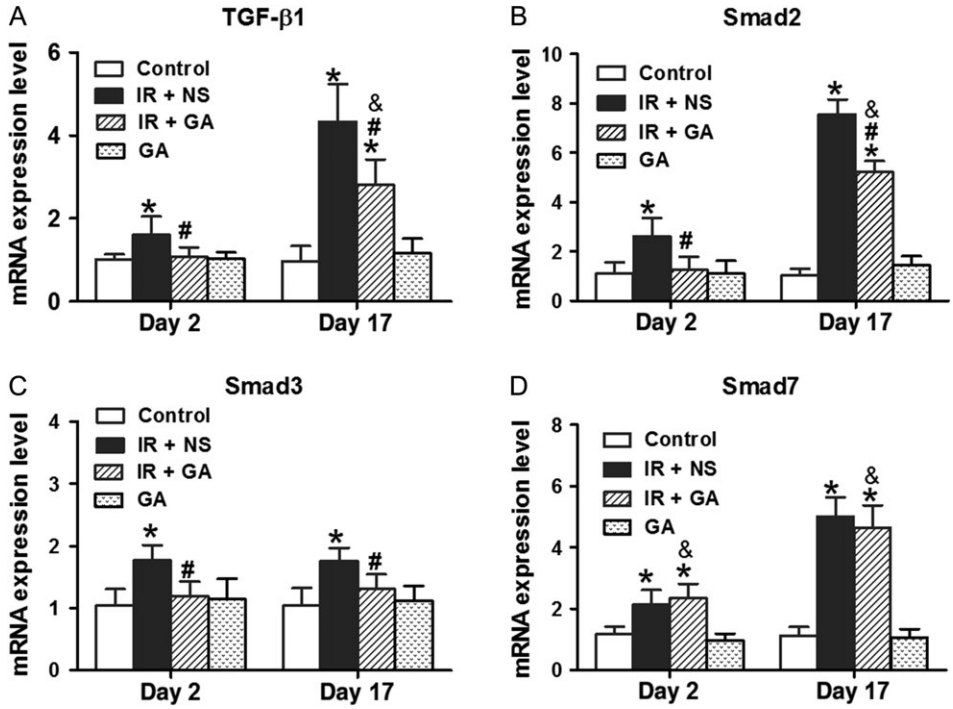

Fig. 3. qRT-PCR analyses of gene expression in mouse lungs after irradiation. Mice were irradiated with a single fraction of 12 Gy and treated with NS or GA (except for the control group and GA group). mRNA levels of *TGF-β1* (panel A), *Smad2* (panel B), *Smad3* (panel C) and *Smad7* (panel D) were analyzed by qRT-PCR 2 days and 17 days after irradiation. Data are the mean ± SD. $^{*}P < 0.05$, *vs* **control group;** $^{\#}P < 0.05$, *vs* **IR + NS group;** $^{\&}P < 0.05$, *vs* **GA group, at identical time-points, by one-way ANOVA (followed by Bonferroni's multiple comparison test). IR = irradiation, NS = normal saline, GA = glycyrrhetinic acid.**

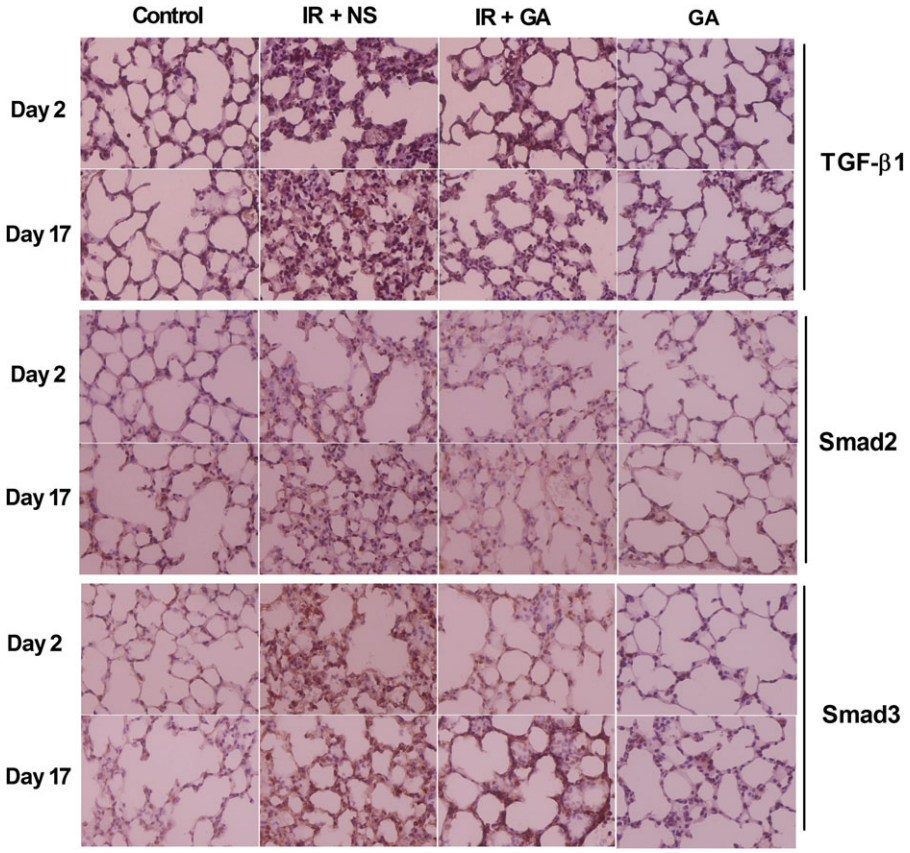

Fig. 4. Immunohistochemical staining of mouse lung tissue after thoracic irradiation. Mice in group IR + NS and IR + GA were exposed to a single fraction of 12 Gy and administered NS or GA. Mice in group GA were not irradiated. Typical *TGF-β1*-, *Smad2*- or *Smad3*-stained sections of mouse lungs from each group 2 days and 17 days after irradiation (×400 magnification). IR = irradiation, NS = normal saline, GA = glycyrrhetinic acid.

after thoracic IR. Also, thoracic IR significantly elevated expression of the mRNAs and proteins of *TGF-β1*, *Smad2* and *Smad3*, as well as the mRNA level of *Smad7* in lung tissue. GA decreased the upregulated expression of mRNA and protein of *TGF-β1*, *Smad2* and *Smad3* in lung tissue, but did not increase the mRNA expression of Smad7.

Recruitment of inflammatory cells into the lung and increased production of inflammatory cytokines are the main features of RILI in the acute phase [23–26]. Evidence suggests a central role for inflammation in the initiation and establishment of RILI [27]. Studies have shown that glycyrrhizin and GA can downregulate expression of inflammatory mediators *in vitro* [28, 29], and that they could be agents for the treatment of inflammatory-mediated lung disease [28]. Some experiments *in vivo* have shown that glycyrrhizic acid and GA alleviate inflammatory lung disease [18, 30]. After ingestion, glycyrrhizic acid is hydrolyzed to GA upon encountering gastric acid (pH 1.5–2) or disaccharidase released from the intestine or bacteria, so only GA can reach the circulation and exerts its effects. Therefore, this research team studied whether oral administration of GA can protect against the lung injury induced by radiation in the acute phase. Histopathologic analyses showed that GA reduced the lung injury induced by radiation. Some studies have also demonstrated the radioprotection afforded by licorice extracts on other tissues. Gandhi *et al.* found that glycyrrhizae and glycyrrhizic acid enhanced recovery of the spleen, thymus and testes from the injury induced by gamma-ray IR in mice [31]. Lin *et al.* reported that glycyrrhizic acid protected the DNA of the leukocytes of peripheral blood and bone marrow cells in mice from radiation-induced strand breaks [32]. Chandrasekharan *et al.* demonstrated that glycyrrhizic acid, silver nanoparticles and their complexes protected the hematopoietic and gastrointestinal systems from radiation, and offered preferential radioprotection to normal cells in tumor-bearing animals [33].

How GA protects against RILI needs to be investigated. Recent studies have revealed some aspects of the underlying mechanism of RILI, but the exact pathogenesis is not known. It is considered that *TGF-β1* (a cytokine that can regulate the growth and differentiation of cells) plays an important part in radiation-induced tissue damage (especially in radiation-induced pneumonia and pulmonary fibrosis) [34–36]. *TGF-β1* signals through cell-surface serine/threonine kinase receptors to activate *Smad* proteins, which modulate the transcription of target genes [37]. Some studies have demonstrated that the *TGF-β1/Smad* signaling pathway has an essential role in RILI [38–40]. In the present study, the research team found some change in *TGF-β1*

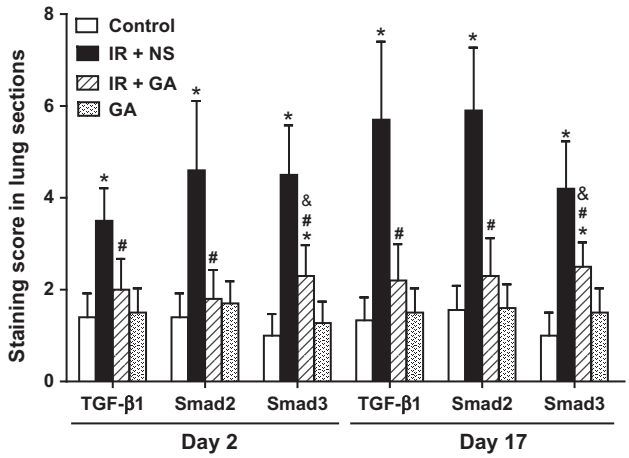

Fig. 5. Semi-quatitative analyses of immunohistochemical detection in mouse lungs after irradiation. Mice in the irradiation groups were exposed to a single fraction of 12 Gy and treated with NS or GA. Protein levels of *TGF-β1*, *Smad2* and *Smad3* in mouse lungs were measured by immunohistochemical detection and analyzed semi-quantitatively with scoring 2 days and 17 days after irradiation. Data are the mean ± SD. $^{*}P < 0.05$, *vs* control group; $^{\#}P < 0.05$, *vs* IR + NS group; $^{\&}P < 0.05$, *vs* GA group, at identical time-points, by one-way ANOVA (followed by Bonferroni's multiple comparison test). IR = irradiation, NS = normal saline, GA = glycyrrhetinic acid.

expression in the lung after radiation exposure. Therefore, this study investigated the role of the *TGF-β1/Smads* signaling pathway in the protection afforded against radiation pneumonia by GA.

This study revealed that the mRNA and protein expression of *TGF-β1* in lung tissue was elevated at the early stage of RILI, and that GA downregulated expression of *TGF-β1*. C57BL/6 mice also showed similar results after thoracic IR with a single dose of 12 Gy or 12.5 Gy; the *TGF-β1* level in lung tissue increased between 12 h and 8 weeks after IR [41, 42]. Of the *Smads*, *Smad2* and *Smad3* mediate receptor-activated signals, whereas *Smad7* mediates inhibitory signals [37]. At the early stage of RILI, the research team observed increasing expression of not only the mediators of signal activation (*Smad2* and *Smad3*), but also that of the mediator of signal inhibitor, *Smad7*. A possible explanation could be that the pro-inflammatory cytokine TNF-α, which was elevated in the bronchoalveolar lavage fluid of mice after thoracic IR, increased expression of *Smad7* by activating nuclear factor-kappa B [43, 44]. Importantly, reduction of the expression of *Smad2* and *Smad3*, but not increase in the expression of *Smad7*, by GA was revealed. Our results suggest that the protection afforded by GA against IR pneumonia is related to inhibition of activation of the TGF-β1/Smads signaling pathway, which has a role in RILI.

Further studies are needed to investigate the effect of GA against lethal whole-body IR-induced mortality and ascertain the relationship between the radioprotective effect of GA *in vivo*. Investigating the dose–effect relationship and protective effect of GA against radiation-induced pulmonary fibrosis will be important.

CONCLUSION

Our results demonstrated that GA can alleviate radiation-induced pneumonia, and that this may be associated with inhibition of activation of the *TGF-β1/Smads* signaling pathway. This research group hypothesizes that GA may be useful for the treatment of RILI.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

FUNDING

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