Int J Clin Exp Med 2017;10(12):16292-16300
www.ijcem.com /ISSN:1940-5901/IJCEM0058309

Original Article

Jiawei Maxing Shigan Decoction (JMSD) attenuates radiation-induced epithelial-mesenchymal transition of primary rat type II alveolar epithelial cells

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Received April 6, 2017; Accepted November 14, 2017; Epub December 15, 2017; Published December 30, 2017

Abstract: Radiotherapy is a common modality in the treatment of thoracic tumors. However, it can cause severe lung complications including pneumonitis and fibrosis. It is known that epithelial-to-mesenchymal transition (EMT) plays an essential role in pulmonary fibrosis. Jiawei Maxing Shigan Decoction (JMSD) has been used to treat radioactive lung injury. In this study, JMSD medicated rat serum and normal rat serum was prepared by intragastrically administered with JMSD and 0.9% sodium chloride, respectively. A total of 10 compounds were identified in JMSD mediated serum by liquid chromatography/mass spectrometry (LC/MS) analysis. Then whether JMSD medicated serum affected the radiation-induced EMT of primary type II alveolar epithelial cells (AEC) was explored and the underlying potential mechanisms were investigated. Primary type II AEC were treated with JMSD medicated rat serum or normal rat serum, and irradiated with ^{60}Co γ-rays. After radiation, epithelial cells converted to a mesenchymal-like morphology, and showed a significant decrease in E-cadherin expression with an accompanied increase in the expression of Vimentin and α-SMA. JMSD medicated rat serum significantly inhibited radiation-induced EMT. Furthermore, we found that transforming growth factor-β (TGF-β) and glycogen synthase kinase 3β (GSK3β) may participate in the roles of JMSD medicated serum on radiation-induced EMT. Collectively, JMSD may therefore a useful radioprotective agent through regulating the EMT of AEC.

Keywords: Jiawei Maxing Shigan Decoction, radiation, TGF-β, ERK/GSK3β

Introduction

Radiation is commonly used in the treatment of thoracic malignancies, including lung cancer, breast cancer and lymphoma [1-3]. However, the efficacy of thoracic cancer radiotherapy can be severely compromised by radiation-induced injury of normal lung tissues. Pneumonitis and fibrosis, two major radiation-induced complications, are limiting factors for the increasing radiation doses and the improving overall survival in patients with thoracic tumors [4]. The injury of alveolar epithelial cells (AEC) and the accumulation of fibroblasts, myofibroblasts and extracellular matrix (ECM) proteins are main features of pulmonary fibrosis [5]. Recent studies suggest that radiation can induce the epithelial-mesenchymal transition (EMT) of AEC, thus contributing to pulmonary fibrosis [6-8]. During EMT, the morphology of AEC undergoes changes from epithelial cobblestone-like phonotype to elongated fibroblastic phonotype. Simultaneously, the expression of epithelial molecular markers such as E-cadherin is decreased and the expression mesenchymal molecular markers such as Vimentin and a-smooth muscle actin (α-SMA) is increased [9, 10]. EMT is regulated by a variety of regulatory networks, such as transforming growth factor-β (TGF-β)/Smad signaling [11] and ERK/glycogen synthase kinase-3β (GSK3β)/Snail signaling [6]. An ideal radioprotective agent, which can protect AEC from EMT and pulmonary fibrosis, will allow higher doses of radiation

to be delivered and enhance the efficacy of radiotherapy.

A growing body of studies have revealed the potential radioprotective effects of many traditional Chinese medicine prescriptions [12], such as bu-zhong-yi-qi-tang [13], si-wu-tang and si-jun-zi-tang [14]. Jiawei Maxing Shigan Decoction (JMSD) has been used to treat pediatric asthma [15], chronic bronchitis [16], infantile mycoplasma pneumonia [17] and radioactive lung injury [18]. However, little is known about the radioprotective effect of JMSD in AEC. In the present study, JMSD medicated serum were collected from rats and the main chemical constituents of JMSD was identified. The effects of JMSD medicated serum on radiation-induced EMT of type II AEC were investigated.

Materials and methods

Preparation of JMSD and medicated serum samples

All Chinese traditional medicines and the JMSD water extract were prepared by School of Pharmacy, Zhejiang Chinese Medicine University. JMSD is composed of 7 herbs: Zhimahuang (honey-fried Herba Ephedrae), 9 g; Shigao (Gypsum Fibrosum), 18 g; Chishao (Radix Paeoniae Rubra), 12 g; Chaoxingren (stir-baked Semen Armeniacae Amarum), 12 g; Sangbaipi (cortex mori from Morus alba L.), 12 g; Yinhua (Flos Lonicerae Japonicae), 9 g; and Gancao (Radix Et Rhizoma Glycyrrhizae), 6 g. The herbs (78 g) were decocted by boiling in distilled water for 1 h twice, and then the water extract was filtered and concentrated to 32 ml.

All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Wistar rats (n=24), weighing 200 ± 20 g, were purchased from Shanghai Experimental Animal Center (Shanghai, China) and randomly divided into two groups: the JMSD group (n=12) and the control group (n=12). Rats in the control group and the JMSD group were intragastrically administered with 4 ml of 0.9% sodium chloride and JMSD, respectively, twice per day (medicine used is 15 times that of the clinical dose for adult) for 3 days. At 2 h after the last time of administration, the blood was drawn from the aorta abdominal, pooled and clotted for 2 hours at room temperature. The serum was isolated carefully by centrifuging the clotted blood at 2000 g for 20 min and stored at -70°C till used.

Liquid chromatography/mass spectrometry (LC/MS) analysis of serum samples

The components of serum samples were analyzed by the Agilent 1100 HPLC system (Agilent Technologies, MA, USA), which equipped with a four-unit pump, an automatic sample injector, a vacuum degasser, an automatic thermostatic column compartment, a diode array detector (DAD) and an LC/MSD Trap XCT ESI mass spectrometer. The separation was performed on a GS-120-5-C18-BIO chromatographic column (5 mm, 250 × 4.6 mm i.d.). The mobile phase was acetonitrile-0.1% formic acid water (25:75, v/v) and the flow rate was 1.0 ml/min. The column temperature was set at 35°C. The injection volume was 10 μl. Mass spectrometric conditions were: collision gas, ultra high-purity helium (He), nebulizer gas (N2), 35 psi, drying gas (N2), 10 L/min, drying temperature, 350°C, HV, 3500 V, mass scan range, m/z 100-2200, target mass, 500 m/z, compound stability, 100%, trap drive level, 100%. All the data were analysis by Chemstation software.

Isolation of primary rat type II alveolar epithelial cells (AEC)

Primary type II AEC were prepared from Sprague-Dawley (SD) rats (6-8 weeks old, Shanghai Experimental Animal Center, Shanghai, China). Dispase was instilled into the lung via a tracheal catheter for 15 min at 37°C. The lungs were removed, carefully teased apart, and treated with DNase I (Sigma, St. Louis, MO, USA) for 5 min at 37°C. The cell suspension was passed through 150-, 15- and 7.5-μM metal strainers, and then centrifuged at 1000 rpm for 8 min. The cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) and plated to a culture dish pre-coated with rat IgG (Sigma). After 1-h of culture, the non-adherent cells were collected and plated to a new culture dish. After culturing for another 20 min, the non-adherent cells were centrifuged at 1000 rpm for 8 min, and the cell pellet was resupended in DMEM supplemented with 20% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and cultured at 37°C in a 5% CO_2 incubator. The purity of isolated cells was more than 90% as determined

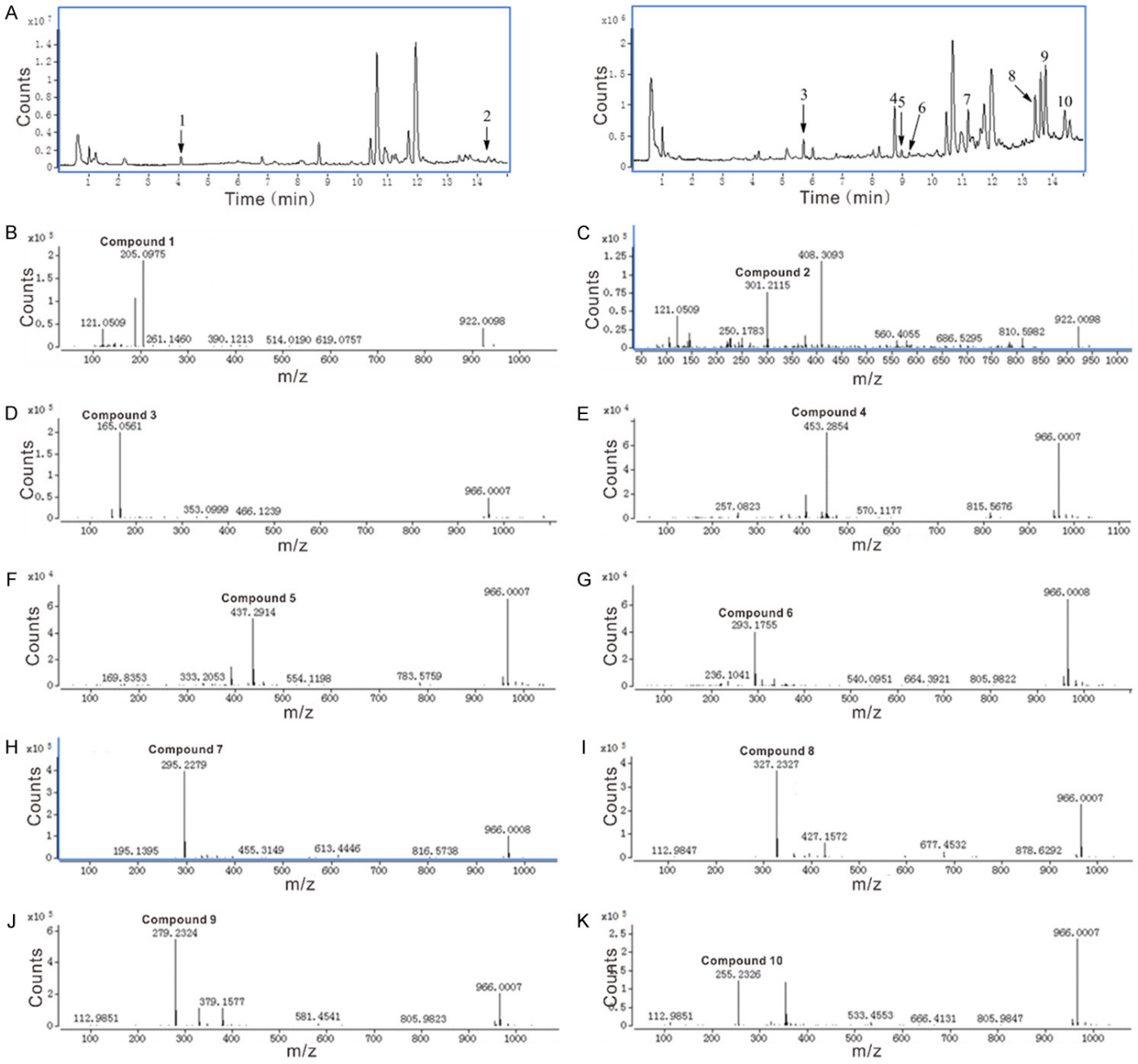
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Figure 1. Screening the components in JMSD medicated serum. (A) HPLC/ESI-MS chromatogram of the aqueous extract in positive and negative mode. (B-K)The product ion mass spectra of farnesene (B), dihydrotanshinone I (C), paeonol (D), emodin (E), schisanhenol (F), tanshinone IIA (G), cryptotanshinone (H), columbianadin (I), uridine (J), and liquiritigenin (K). Conditions: collision gas, ultra high-purity helium (He), nebulizer gas (N2), 35 psi, drying gas (N2), 10 l/min, drying temperature, 350°C, HV, 3500 V, mass scan range, m/z 100-2200, target mass, 500 m/z, compound stability, 100%, trap drive level, 100%.

Table 1. Ten compounds in rat medicated serum extracts

Compound	Name	Retention time	Pseudomolecular ion peak
1	Farnesene	4.12 min	m/z 205.0975 [M + H]+
2	Dihydrotanshinone I	14.35 min	m/z 301.2115 [M + Na]+
3	Paeonol	5.69 min	m/z 165.0561 [M - H]-
4	Emodin	8.22 min	m/z 453.2854 [M + Cl]-
5	Schisanhenol	8.71 min	m/z 437.2914 [M + Cl]-
6	Tanshinone IIA	9.22 min	m/z 293.1755 [M - H]-
7	Cryptotanshinone	11.35 min	m/z 295.2279 [M - H]-
8	Columbianadin	13.40 min	m/z 327.2327 [M - H]-
9	Uridine	13.75 min	m/z 279.2324 [M + Cl]-
10	Liquiritigenin	14.40 min	m/z 255.2326 [M - H]-

by nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) staining.

Cell treatment

Primary type II AEC were divided into five groups. Cells in Group 1 and Group 2 were cultured in the present of 10% normal control rat serum. Group 3 were incubated with 2% JMSD medicated serum (J) and 8% normal control rat serum (N). Group 4 were incubated with 6% J serum and 4% N serum, while Group 5 were incubated with 10% J serum. Cells in Group 2-5 were treated with 8 Gy of ^{60}Co γ-rays (Hangzhou Cancer Hospital) at a dose rate of 3.64 Gy/min at room temperature. After 48-h of culture, Enzyme-Linked Immunosorbent (ELISA) assay and Western blotting analyses were performed.

Western blotting analysis

Cellular protein was obtained by using radioimmunoprecipitation assay buffer with freshly added protease inhibitor cocktail (Solarbio, Beijing, China). Equal amounts of protein were separated on a 10% SDS-PAGE, and then transferred to a nitrocellulose membrane (Millipore, Bredford, USA). After blocking with 5% skim milk, the membrane was incubated with primary antibodies at 4°C overnight, and then with horseradish peroxidase-conjugated secondary antibody (Beyotime, Shanghai, China) at room temperature for 1 h. Finally, the signals of protein bands were detected with an enhanced chemiluminescence substrate mixture (Millipore) and quantified using Image J software (http://rsb.info.nih.gov/ij/, Bethesda, MD, USA). Blots were normalized with signals from GAPDH. The sources of primary antibodies were: antibodies against α-SMA, phosphor-GSK3β, GSK3β, phosphor-Smad2, Smad2, phosphor-Smad3 and Smad3 were from Abcam; Antibodies against E-cadherin, Vimentin, phosphor-ERK, ERK and GAPDH were obtained from Cell Signaling Technology (Danvers, MA, USA).

ELISA

TGF-β concentrations in the culture medium were determined by ELISA kit (Bio-Swamp life science, Shanghai, China) according to the manufacturer's protocol.

Statistical analyses

All experiments were independently performed at least three times and data are presented as the mean ± SD. One-way analysis of variance (ANOVA) followed by a Sidak's test was used for statistical comparisons. Statistical significance was defined as $P<0.05$.

Results

Screening the components in JMSD medicated serum

Rats were intragastrically administered with sodium chloride (normal, n=12) and JMSD (medicated, n=12), respectively, twice per day for 3 days. At 2 h after the last time of administration, serum samples were collected. The serum samples from the same group were

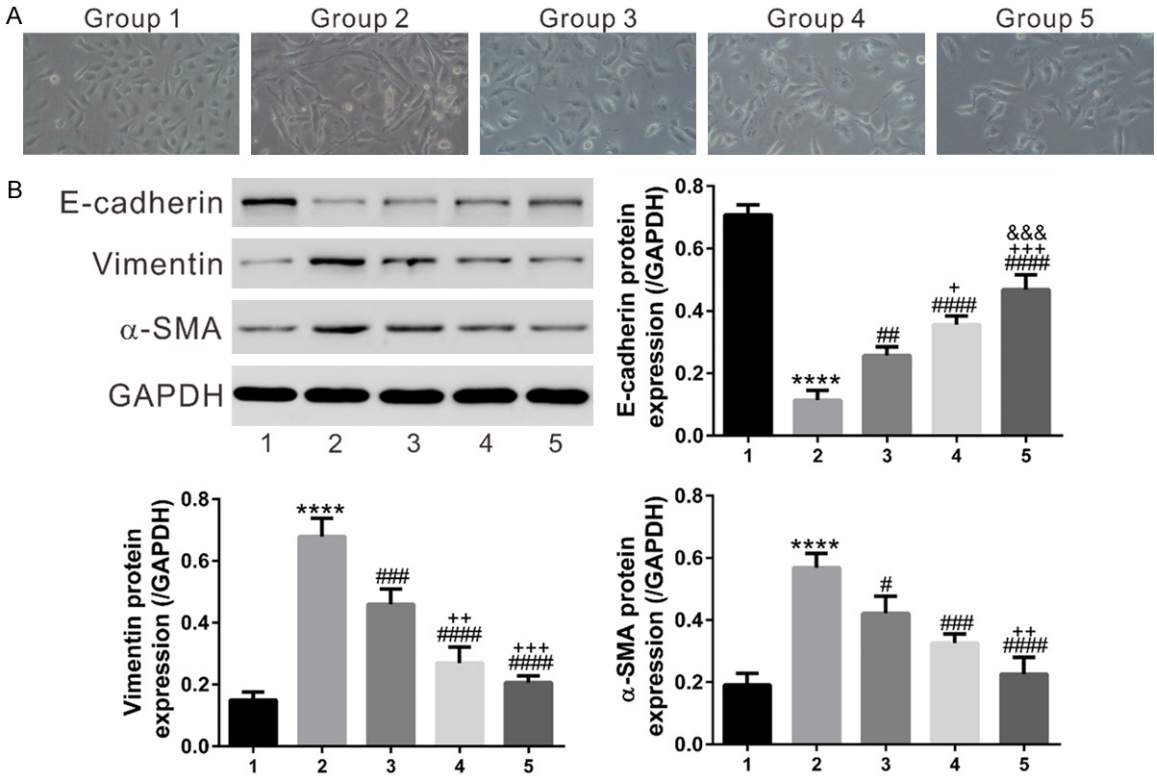

Figure 2. JMSD medicated serum attenuates radiation-induced EMT. Primary type II AEC were divided into five groups. Cells in Group 1 and Group 2 were cultured in the present of 10% normal control rat serum. Group 3 were incubated with 2% JMSD medicated serum (J) and 8% normal control rat serum (N). Group 4 were incubated with 6% J serum and 4% N serum, while Group 5 were incubated with 10% J serum. Cells in Group 2-5 were treated with 8 Gy of ^{60}Co γ-rays at a dose rate of 3.64 Gy/min at room temperature. A. Phase contrast images were taken (original magnification × 200). B. Expression of each protein was evaluated by western blot analysis. GAPDH was used as loading control. $^{****}P<0.0001$ *vs*. Group 1; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $^{\#\#\#\#}P<0.0001$ *vs*. Group 2; $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$ *vs*. Group 3; $^{\&\&\&}P<0.001$ *vs*. Group 4.

pooled and measured by high-performance liquid chromatography coupled with electrospray mass spectrometry (HPLC/ESI-MS) in negative-ion mode (**Figure 1** and **Table 1**). A total of 10 compounds, including farnesene, dihydrotanshinone I, paeonol, emodin, schisanhenol, tanshinone IIA, cryptotanshinone, columbianadin, uridine, and liquiritigenin were unambiguously identified in JMSD medicated serum by comparing the retention times and the MS data with the reference standards. No compounds were identified in normal serum.

JMSD medicated serum decreases radiation-induced EMT

We next assessed the contribution of JMSD medicated serum to radiation-induced EMT. Post radiation treatment, morphology of the primary rat Type II AEC changed from a classical epithelial type to elongated spindle-like type in the present of normal group serum. Co-treatment with 2%, 6% and 10% JMSD mediated serum rescued such morphological alteration (**Figure 2A**). The induced expression of Vimentin and α-SMA by irradiation was attenuated and the decrease in E-cadherin levels was prevented (**Figure 2B**). In a word, our data indicate that JMSD medicated serum attenuates radiation-induced EMT in primary rat Type II AEC.

TGF-β/Smad pathway is suppressed by JMSD medicated serum

Previous research has reported that TGF-β1 concentrations in bronchoalveolar lavage fluid were significantly increased by thoracic radiotherapy [19]. Studies have examined the role of TGF-β in EMT and fibrotic lung disease [20]. Therefore, we identified whether JMSD medi-

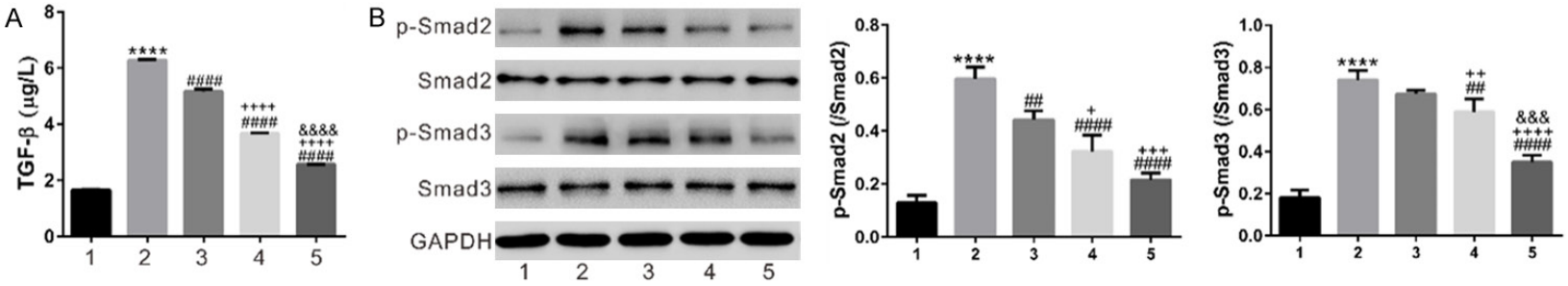

Figure 3. TGF-β/Smad pathway was attenuated by JMSD medicated serum. The Primary Rat Type II AEC were incubated 2% JMSD (J) + 8% normal control rat serum (N), 6% J serum+ 4% N serum, 10% J serum or 10% N serum. Cells were treated with or without 8 Gy of γ-rays. A. At 8 h post irradiation, ELISA assays were performed to measure TGF-β concentration in the cultured medium. B. Western blot analyses were used to detect Smad2, p-Smad2, Smad3 and p-Smad3 at 8 h post irradiation. GAPDH was used as loading control. $^{****}P<0.0001$ *vs*. Group 1; $^{\#\#}P<0.01$, $^{\#\#\#\#}P<0.0001$ *vs*. Group 2; $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$, $^{++++}P<0.0001$ *vs*. Group 3; $^{\&\&\&}P<0.001$ *vs*. Group 4.

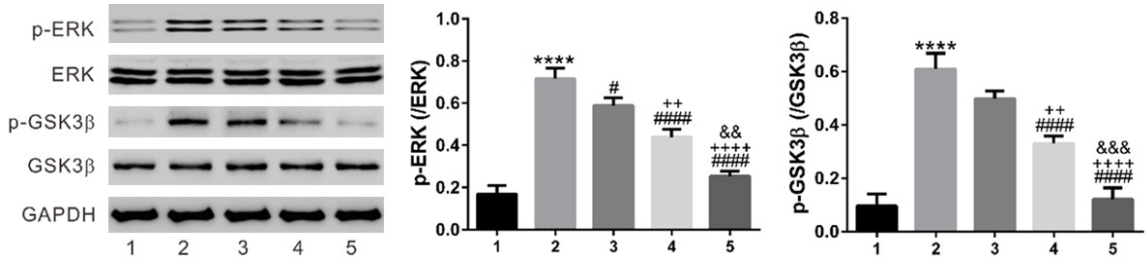

Figure 4. ERK/GSK3β pathway was attenuated by JMSD medicated serum. The Primary Rat Type II AEC were incubated 2% JMSD (J) + 8% normal control rat serum (N), 6% J serum+ 4% N serum, 10% J serum or 10% N serum. Cells were treated with or without 8 Gy of γ-rays. At 8 h post irradiation, Western blot analyses were used to detect ERK, p-ERK, GSK3β and p-GSK3β. GAPDH was used as loading control. $^{****}P<0.0001$ *vs*. Group 1; $^{\#}P<0.05$, $^{\#\#\#\#}P<0.0001$ *vs*. Group 2; $^{++}P<0.01$, $^{++++}P<0.0001$ *vs*. Group 3; $^{\&\&}P<0.01$, $^{\&\&\&}P<0.001$ vs. Group 4.

cated serum modulated TGF-β signaling. We first measured the levels of TGF-β in cell cultured medium by ELISA assay. As shown in **Figure 3A**, the TGF-β concentrations were boosted after irradiation, but reduced by JMSD mediated serum dose-dependently. The phosphorylation levels of Smad2 and Smad3 in primary rat Type II AEC were increased after irradiation, whereas their total proteins remained unaffected (**Figure 3B**). JMSD mediated serum decreased the radiation-induced phosphorylation of Smad2 and Smad3.

ERK/GSK3β pathway is attenuated by JMSD medicated serum

It has been shown that ERK/GSK3β signaling has a modulatory effect on radiation-induced EMT [6]. We hypothesized that JMSD medicated serum may regulate ERK/GSK3β signaling. Western blot analyses illustrated that the phosphorylation of ERK and GSK3β (S9) was significantly elevated in irradiated pneumocytes, whereas the levels of ERK and GSK3β remained unchanged (**Figure 4**). Interestingly, we observed that co-treated with JMSD medicated serum resulted in a significant decrease in phosphorylated ERK and GSK3β in the pneumocytes.

Discussion

Radiotherapy is one of the main modality in the treatment of thoracic tumors. It not only kills tumor cells, but also damages normal tissues and induces pulmonary fibrosis. JMSD composed of 7 traditional Chinese medicines is widely used for the treatment of infantile mycoplasma pneumonia [17] and radioactive lung injury [18]. In the current study, ten main compounds were identified in JMSD medicated serum by LC/MS analysis (**Figure 1**). Inhibitory effects on pulmonary fibrosis has reported for several compounds, such as emodin [21], tanshinone IIA [22] and cryptotanshinone [23]. The effects of other compounds on the radioactive lung injury needs further investigation.

Radiation has been regarded as one of the inducers of EMT of AEC. The down-regulation of E-cadherin represents one critical molecular characteristic of EMT [9, 10]. In our study, after radiation treatment, primary type II AEC changed to spindle-like structure with a reduced expression of E-cadherin, indicating that these cells lost their epithelial phenotype. Conversely, the expression levels of markers of the mesenchymal phenotype, α-SMA and Vimentin [9, 10], were up-regulated by irradiation, further supporting that radiation induced the EMT of type II AEC. More importantly, JMSD attenuated radiation-induced EMT (**Figure 2**). These results suggest that JMSD may protect lung from radiation-induced injury via targeting EMT of AEC.

TGF-β/Smad signal transduction pathways have been implicated in the pathogenesis of pulmonary fibrosis [11]. Thoracic radiotherapy increases the concentrations of TGF-β1 in the bronchoalveolar lavage fluid [19]. TGF-β1 may be closely related with the EMT of AEC and the generation of radiation pulmonary fibrosis. It has been shown that radiation can activate TGF-β/Smad signaling [11]. In line with previous finding, we found an increase in TGF-β, phosphor-Smad2 and phosphor-Smad3 in response to radiation. JMSD effectively decreased such effects, which may contribute to the alleviation of pulmonary fibrosis (**Figure 3**). ERK/GSK3β is

another signaling pathway involved in radiation-induced EMT. Radiation activates the MEK/ERK signaling pathway, which phosphorylates GSK3β. The phosphorylated GSK3β promotes the stabilization and nuclear translocation of Snail, and subsequent EMT induction [6]. Here, radiation stimulated the phosphorylation of ERK and GSK3β, which was weaken by JMSD treatment. These data suggested that JMSD effectively decreased radiation-induced EMT through TGF-β/Smad and ERK/GSK3β signaling.

In summary, co-treatment with JMSD can effectively attenuated the effects of radiation that induced EMT, and increased the release of TGF-β and the phosphorylation of Smad2/3, ERK and GSK3β. This study provides promising insights in the therapy of radiation-induced pulmonary fibrosis.

Acknowledgements

The study was upported by Zhejiang Provincial Natural Science Foundation of China (LY15H290002), National Natural Science Foundation of China (81173246) and Science and Technology Planning Project of Hangzhou City (Key Program, 201450208).

Disclosure of conflict of interest

None.

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