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Original Article

Preventive effect of traditional Chinese medicine (TCM) in the rats with irradiation induced pulmonary fibrosis

Hai-Ming Jiang[1*], Ye-Jia Hu[2*], Yu-Zhu Li[3], Xiao-Feng Du[3], Xin-Lei Hao[3], Wei-Qiang Zhang[3], Li-Hua Yang[3]

[1]Department of Emergency, Affiliated Hospital of Binzhou Medical University, Yantai 264000, China; [2]Department of Physiology, School of Medical Science, Binzhou Medical University, Yantai 264000, China; [3]Department of Critical Care Medicine, Yantai Affiliated Hospital of Binzhou Medical University, Yantai 264000, China. [*]Equal contributors.

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Abstract: In this study the effect of traditional Chinese medicine (TCM) on pulmonary fibrosis caused in rats on exposure to radiations was demonstrated. The radiation source for inducing pulmonary fibrosis in rats was Cobalt-60 irradiator emitting radiations at a dose of 22 Gy. TCM or dexamethasone was administered to the rats at a dose of 12 and 5 mg/kg, respectively. The treatment was continued for a period of 21 days followed by measurement of rate of mortality and lung index values. Results showed that the rate of mortality in the animals treated with TCM was reduced to a marked level compared to the DEX treated group. TCM also led to inhibitory effect on the tissue damage in the pulmonary tissues compared to the untreated rats. In addition the level of MDA was decreased, activity of SOD increased and the alveolar epithelial type II (AE2) cells protected on treatment of rats with TCM. It also improved the expression of transformation factor β1 (TGF-β1), interleukin (IL)-6, IL-10, and tumor necrosis factor-α (TNF-α) along with the activation of Nrf-2. Therefore, TCM exhibits preventive effect on the pulmonary fibrosis in rats caused on exposure to radiations.

Keywords: Pulmonary fibrosis, necrosis factor, preventive, activation, damage

Introduction

Pulmonary injuries induced on exposure to radiations like pneumonitis and lung fibrosis, are associated with the decrease in therapeutic efficiency of tumor treatment methods and affect the quality of life very badly during survival periods [1]. Currently, various malignant diseases in thoracic cavity including lung, esophageal and breast cancers, malignant lymphoma and thymoma are treated using radiation therapy. In humans the complications associated with pulmonary fibrosis start to develop after 6 months of the exposure to the radiations [2]. The rateof pulmonary injury incidence in the cancer patients undergoing radiotherapy has been found to be 20.3-36.9% [3-6]. The pulmonary fibrosis begins with proliferation of the fibroblasts and accumulation of collagen leading to the disruption of pulmonary tissues [7]. Exposure to radiations induces generation of reactive oxygen species (ROS) which lead to harmful effects on DNA structure, membrane lipid peroxidation, signal transduction pathway and transcription factor activation [8]. The stress induced by ROS following exposure to radiations continues during the pulmonary fibrosis through the oxidant-producing enzyme activation, enhanced mitochondrial membrane permeability, and respiratory burst activation in the phagocytic cells [9]. At present the drugs including steroids and other anti-inflammatory candidates are being used for the treatment of pulmonary fibrosis [2]. However, these drugs produce several side effects and also the mitigation of fibrosis is inefficient.

ROS generation leads to translocation of Nrf2 to nucleus where it induces release of antioxidant enzymes [10]. The system of antioxidant enzymes is comprised of heme oxygenase-1 (HO-1), γ-glutamine cysteine synthetase (γ-GCS), NAD(P)H: quinone oxidoreductase-1 (NQO-1) and superoxide dismutase (SOD). The present study demonstrates the effect of TCM on pulmonary fibrosis in rats induced by radiation exposure.

Traditional Chinese medicine (TCM) involves the use of herbal medicines, acupuncture and moxibustion, tuina, dietary therapy and qigong. TCM treatment is primarily associated with the differentiation of the syndrome and the herbal formulas prescription [11]. It is reported that TCM therapy exhibits a promising effect on the inhibition of several types of cancers [12]. Use of TCM in addition to healthy lifestyle has been shown to play a vital role for the treatment of various diseases [13].

Materials and methods

Animals and treatment strategy

Themale Sprague-Dawley rats weighing 200 ± 10 g were obtained from the Experimental Animal Center of Shandong Engineering Research Center for Natural Drugs (Yantai, China). The animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals of Yantai University. The animals were provided with free access to water and food on a 12 h light and dark cycle. For the surgery of the animal's sodium pentobarbital anesthesia was used.

The pulmonary fibrosis rat model was prepared by exposing the thorax cavity of the animals to gamma-rays emitted by ^{60}Co irradiator [Reviss Services (UK), Ltd., Buckinghamshire, UK]. The rats were randomly divided into 4 groups of 10 each; TCM, dexamethasone (DEX) negative control (irradiated and untreated) group and positive control (no irradiation no treatment) group. The animals in the TCM and DEX groups received TCM and DEX at the dose of 20 and 5 mg/kg, respectively. The animals in the positive and negative control groups received normal saline.

Processing of lung samples for histopathological examination

Two animals from each group were sacrificed on the day 20, 40, 80 and 160 after the treatment was started to extract the lungs. Right lung after paraformaldehyde fixing and gradient ethyl alcohol dehydration was paraffin embedded. The samples were cut into 3-μm thin sections and stained with hematoxylin and eosin (H&E), Masson's trichrome (Masson) and Sirius red. The left lung was immediately stored under liquid nitrogen atmosphere for further analyses. From the aortic artery of each animal blood samples were collected, centrifuged at 12000 rpm for 10 min and stored at -70°C.

Calculation of lung index

The body weight of the each animal before sacrifice and wet weight of the lungs from each animal was measured immediately after extraction. From these values, lung index was obtained by dividing the ratio of lung weight with the body weight.

Determination of lung collagen content

The left lung was lysed in the lysis buffer for 30 min at 110°C. The supernatant isolated was subjected to measurement of absorbance at 565 nm. For determination of the collagen content in the lung samples hydroxyproline (Hyp) assay as per the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used.

Serum SOD activity

For determination of the SOD activity suppression of ferricytochrome*c* reduction caused by the sample treatment was measured using xanthine/xanthine oxidase. The method involves suppression in the reduction of nitrobluetetrazolium (NBT). For this purpose MDA content kit available commercially (Nanjing Jiancheng Bioengineering Institute) was used as per the manual protocol.

Serum malondialdehyde (MDA) content

The serum content of MDA was determined according to the manual protocol using the SOD activity kit (Nanjing Jiancheng Bioengineering Institute). For determination of the MDA content production of chromogen in the reaction between MDA and 2-thiobarbituric acid was measured.

Immunohistochemical analyses

The paraffin embedded lung sections after xylene deparaffinization and rehydration were treated with citrate buffer (10 mM of sodium citrate, pH 6.0 for 15 min) followed by H_2O_2. Incubation of the sections was performed with surfactant protein-B B (SPB) or α smooth muscle actin (α-SMA) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA)antibodies. After incubation the sections were PBS washed and then incubated with poly-peroxidase-conjugated

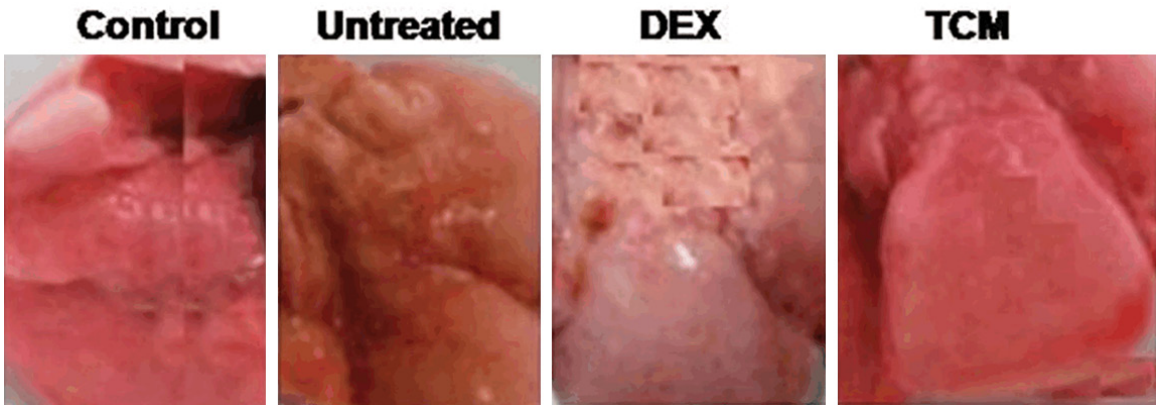

Figure 1. Effect of TCM and DEX on the morphology of the lungs in rats at day 120 after irradiation.

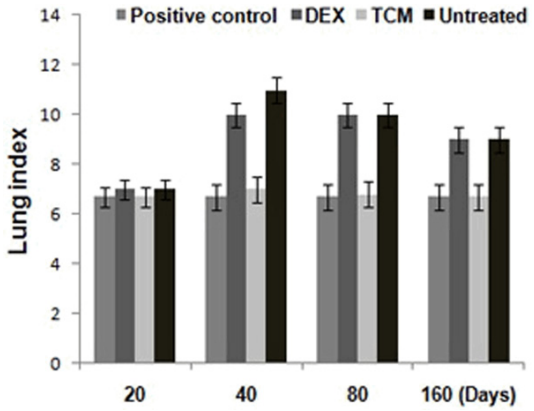

Figure 2. Effect of TCM on the lung index score in the rats on the day 20, 40, 80 and 160 after irradiation.

anti-mouse/rabbit IgG for 45 min using the Polymer-HRP Detection System (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Diaminobenzidine (DAB; Dako, Glostrup, Denmark) visualization of the slides using Mayer's hematoxylin counterstaining and ethyl alcohol dehydration was performed.

Level of cytokine in serum

TGF-β1 ELISA kit (Boster Biological Technology) was used to measure the content of TGF-β1 as per the manual protocol. Optical density was measured at 455 nm and value was calculated from the plot. For the purpose of measuring IL-6, IL-10, and TNF-α content flow cytometry bead assay (BD™ CBA Flex Set; BD, Sparks, MD, USA) was used.

Western blot analysis

Left lung tissues were lysed in radio immunoprecipitation assay (RIPA) lysis buffer and the tissue lysate was centrifuged at 15000 rpm and 4°C for 10 min for 15 min. BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) was employed to measure the content of proteinsin the supernatant. The proteinsafter electrophoresis on 10-12% SDS-PAGE gel in reducing conditions wereelectroblotted onto nitrocellulose membrane (Millipore Corp., Billerica, MA, USA). The non-fat milk blocked membranes were incubated overnight with primary antibodies including NQO-1 (Millipore) Nrf-2, HO-1, or β-actin (Cell Signaling Technology, Inc., Danvers, MA, USA). The membrane was washed with PBS and then incubated with secondary antibody for 45 min. Visualization of the protein bands was performed with enhanced chemiluminescence bearing Super Signal detection kit (Boster Biological Technology).

Statistical analysis

All the data are presented as the means ± standard deviations (SD). The differences between the groups were examined using a One-way ANOVA followed by Dunnet's t-test. $P<0.05$ was takes as statistically significant difference.

Results

Effect of TCMon mortality and lung morphology

The rate of mortality in the positive control, negative control, DEX-treated and TCM treated groups was 0, 40, 30 and 5%, respectively. In the rats treated with TCM, examination of the lungs showed normal morphology without any lesion and bleeding spaces. However, the lungs in the negative control group showed presence

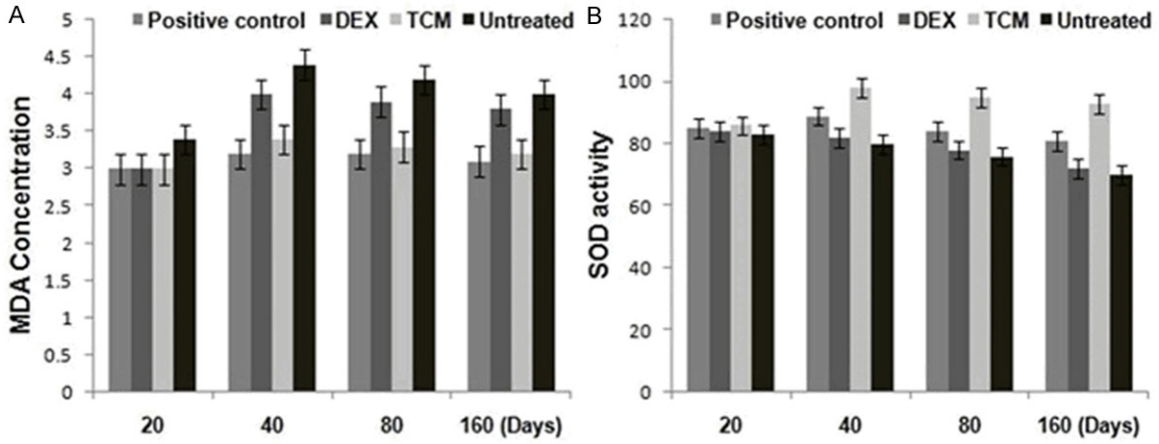

Figure 3. A. The effect of TCM and DEX on the level of malondialdehyde (MDA) level. B. Superoxide dismutase (SOD) activity levels on day 20, 40, 80 and 160 after irradiation.

of excessive lesions and bleeding spaces on the outer surface. DEX treatment on day could only prevent the appearance of lesions on the days 20 and 40 whereas on the days 80 and 160 lung morphology was similar to those of negative control group (**Figure 1**).

TCM reduces the lung index score

Calculation of lung index revealed that in TCM treated rats it was significantly lower compared to negative control group on the day 20, 40, 80 and 120 after radiation exposure. However, in the positive control group lung index was lower and similar to that of the TCM treated rats (**Figure 2**).

TCM modulates the serum redox state

Rats treated with TCM were found to possess markedly reduced content of MDA compared to negative control group on the days 20, 40, 80 and 160 after irradiation. In DEX treated rats MDA content was reduced on day 20 and 40 only but was similar to negative control groups on the day 80 and 160 (**Figure 3A**). In TCM treated rats SOD activity was increased compared to negative control group on the days 20, 40, 80 and 160 after irradiation. DEX treatment increased the SOD activity on the day 20 and 40 after irradiation and the activity was similar to negative control group on the rest of the tested days (**Figure 3B**).

Effect of TCM on α-SMA and SPB

Results from immunohistochemistry showed inhibitory effect of TCM on the expression of α-SMAa (myo) fibroblast marker which was increased on radiation exposure (**Figure 4**). However, TCM treatment increased the expression of SPB which was reduced in the rats after exposure to radiations (**Figure 4**).

Effect of TCM on level of serum cytokine

TCM treated rats showed reduced expression of TGF-β1 on day 20, 40, 80 and 160 after irradiation. In DEX treated rats the level of TGF-β1 was reduced on days 20 and 40 and was similar to those of negative control group on the day 80 and 160 (**Figure 5A**). Therefore, TCM treatment reversed the promoting effect of radiations on TGF-β1 until day 160.

Rats treated with TCM also showed reduced IL-6, IL-10 and TNF-α expression on the day 20, 40, 80 and 160 after irradiation. Comparison of the expression of these three proteins in DEX treated and negative control groups showed similar results on day 80 and 160. In positive control and TCM treated groups the expression of the proteins was almost similar (**Figure 5B-D**).

Inactivation of Nrf-2 by TCM activates Nrf-2

Western blot analysis showed activation of proteins including HO-1, Nrf-2, and NQO-1 in the rats treated with TCM markedly higher compared to DEX treated rats (**Figure 6**). The TCM treated animals showed higher expression of the above proteins on all the tested days (20, 40, 80 and 160) after irradiation. Comparison of the expression of these proteins in DEX treated and negative control groups revealed that DEX only exhibited enhancing effect until day

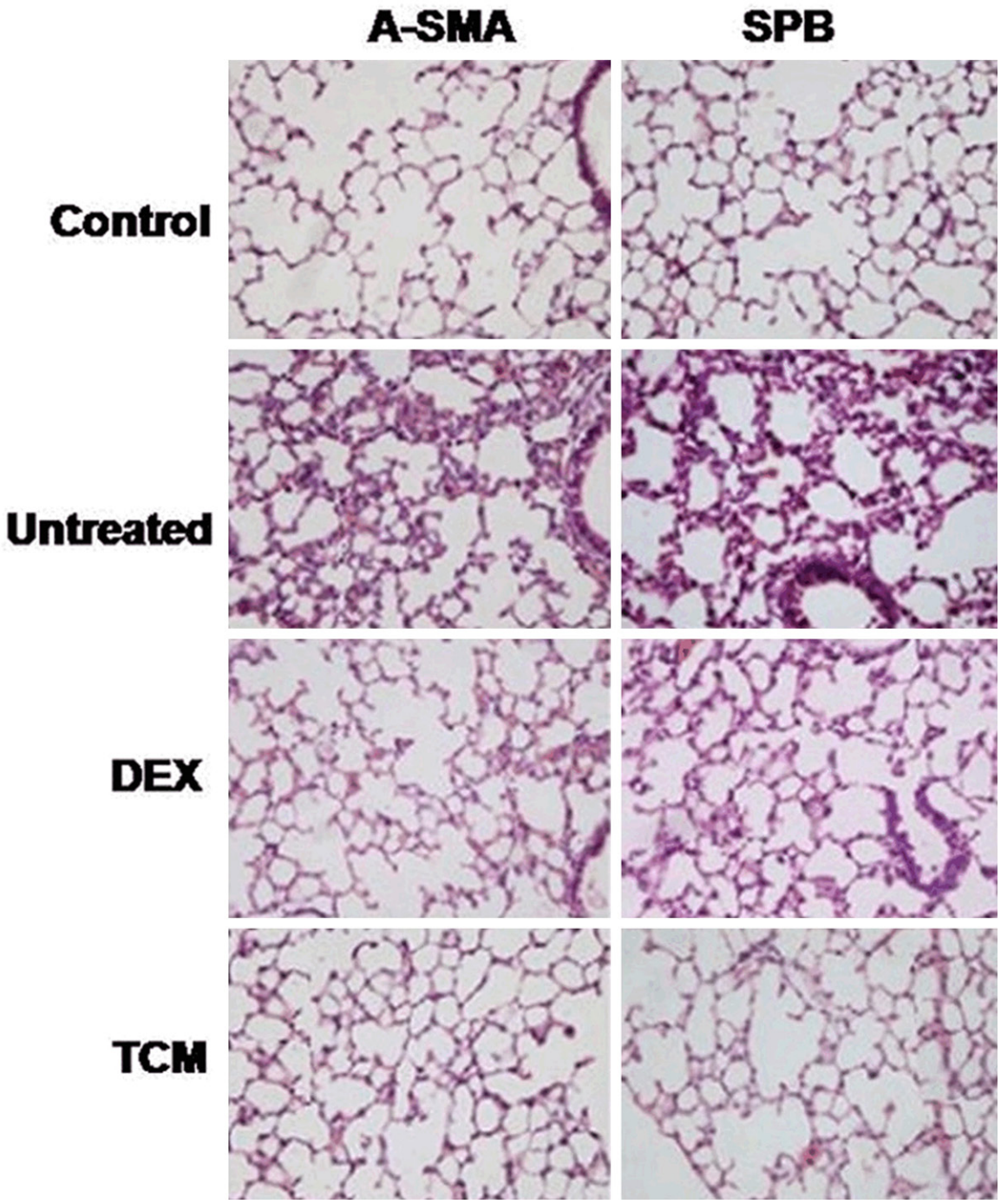

Figure 4. Effect of TCM on the level of α smooth muscle actin (α-SMA) and surfactant protein-B (SPB) level after 120 days of irradiation.

40 there after the expression was similar with those of negative control group.

Discussion

Reactive oxygen species (ROS) produced in the cells on exposure to radiations leads to harmful effects on DNA structure, membrane lipid peroxidation, signal transduction pathway and transcription factor activation [8]. The stress induced by ROS following exposure to radiations continues during the pulmonary fibrosis through the oxidant-producing enzyme activation, enhanced mitochondrial membrane per-

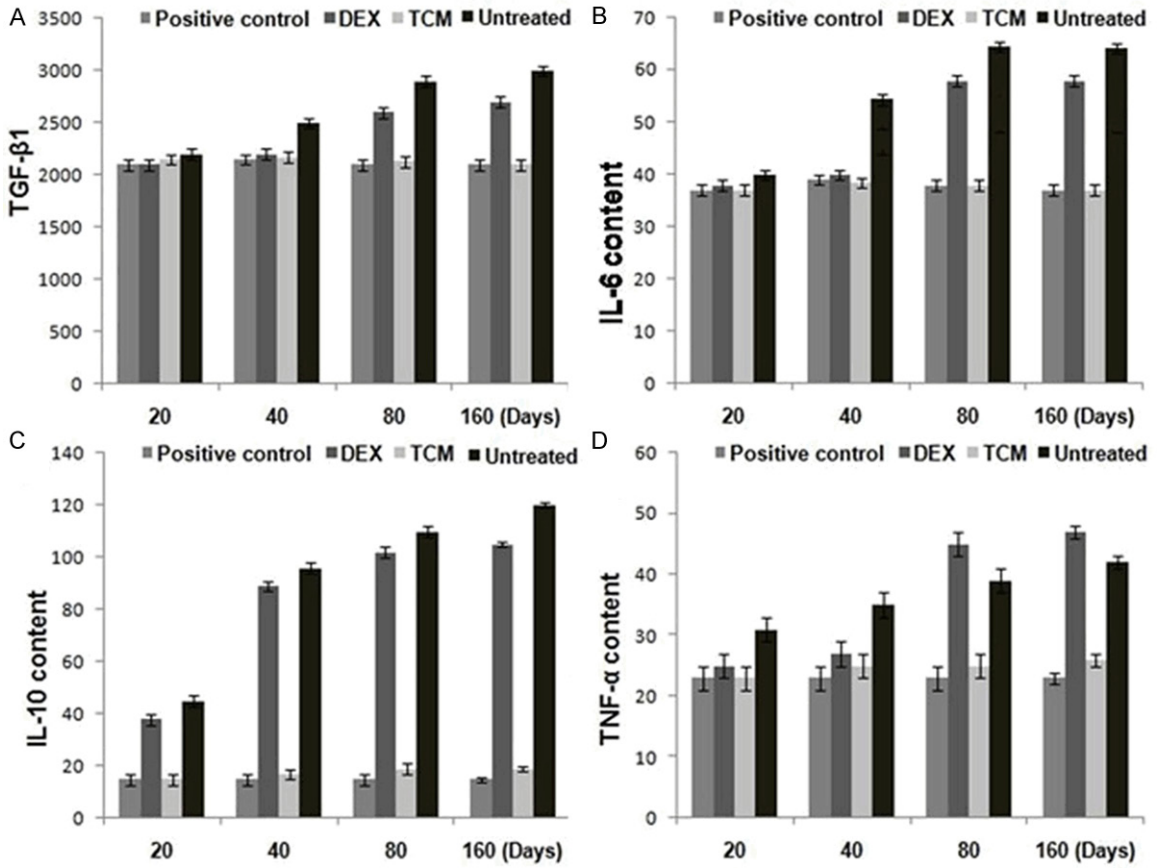

Figure 5. The effect of TCM on the expression of transforming growth factor (A) β1 (TGF-β1), (B) interleukin (IL)-6, (C) IL-10, and (D) tumor necrosis factor α (TNF-α) after 20, 40, 80 and 160 of irradiation.

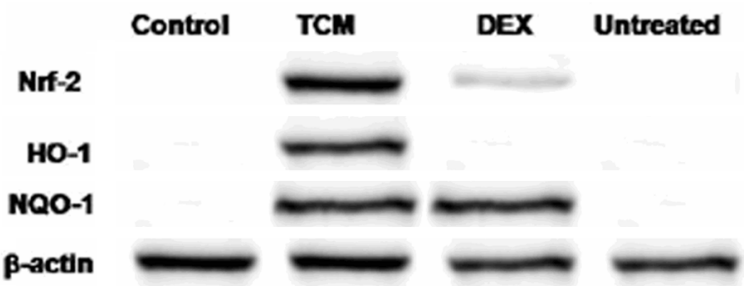

Figure 6. Effect of TCM and DEX on the activation of nuclear transcription factor NF-E2-related factor 2 (Nrf-2), heme oxygenase-1 (HO-1) and NAD(P)H: quinone oxidoreductase-1 (NQO-1) protein expression after 120 days of irradiation.

meability, and respiratory burst activation in the phagocytic cells [9]. The results from the present study revealed that TCM treatment reverses the pulmonary fibrosis induced on exposure to radiations in the rats. The rate of mortality and the lung index score in TCM treated rats was reduced marked compared to the untreated and even DEX treated rats.

TCM treatment also caused activation of the Nrf-2 and the associated antioxidant enzymes, HO-1 and NQO-1. Membrane lipids undergo peroxidation in the presence of ROS resulting in the formation of MDA which is taken as the oxidative damage marker [14]. SOD acts as the scavenger for ROS and thereby prevents the animals from pulmonary fibrosis [15]. The results from the current study showed that TCM treatment reduced the content of MDA along with the inflammatory cytokines and increased the SOD activity in the rats. Therefore, TCM induced decrease in the oxidative stress can partly be due to enhanced expression of antioxidant proteins. Pulmonary fibrosis is induced by the proliferation and maturation of the fibroblasts which in turn is caused by cytokine, TGF-β1 [16]. The degree of expres-

sion of TGF-β1 has been found to correlate with the rate of incidence of pulmonary fibrosis [17].

Other factors found to be associated with the fibroblast proliferation and the induction of pulmonary fibrosis is the TNF-α [18] as well as pro-inflammatory cytokines like IL-1 and IL-6 develop which also develop the fibrous connective tissue. The expression of these cytokines was found to be significantly reduced in the TCM treated rats.

Nrf2 plays a critical role in the regulation of the major antioxidant enzymes HO-1 and NQO-1. Our western blot analysis results revealed that TCM significantly enhanced the expression levels of Nrf-2, HO-1, and NQO-1 in the rat lung tissues compared with radiation only and DEX-treated rats.

In summary the present study demonstrates that TCM induces marked decrease in the rate of morbidity and reverses the radiation induced pulmonary fibrosis in the rats.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Li-Hua Yang, Department of Critical Care Medicine, Yantai Affiliated Hospital of Binzhou Medical University, 717 Jinbu Street, Muping District, Yantai 264000, Shandong, China. Tel: 0086-535-2123456; Fax: 0086-535-2123456; E-mail: yanglh3001@gmail.com

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