

Modulation expression of tumor necrosis factor α in the radiation-induced lung injury by glycyrrhizic acid

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ABSTRACT

To evaluate the ability of glycyrrhizic acid (GLA) to reduce the tumor necrosis factor α (TNF- α), release on messenger ribonucleic acid (mRNA) and protein production in the lungs using GLA in response to irradiation were studied. The animals were divided into four groups: No treatment (NT group), GLA treatment only (GLA group), irradiation only (XRT group), and GLA treatment plus irradiation (GLA/XRT group). Rats were killed at different time points. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used to evaluate the mRNA expression of TNF- α in the lungs (compared with non-irradiated lungs). An enzyme-linked immunosorbant assay (ELISA) assay was used to measure the TNF- α protein level. The TNF- α mRNA expression in the lungs of the XRT rats was clearly higher at all-time points compared to the NT rats. The TNF- α mRNA expression in the lungs of the GLA/XRT rats was lower at all-time points compared to the XRT rats. Release of the TNF- α on protein level in the lungs of the XRT rats increased at all-time points compared to the NT rats. In contrast to the XRT rats, the lungs of the GLA/XRT rats revealed a reduction on TNF- α protein level at 6 h after irradiation. This study has clearly showed the immediate down-regulation of the TNF- α mRNA and protein production in the lungs using GLA in response to irradiation.

Key words: Glycyrrhizic acid, radiation-induced lung damage, tumor necrosis factor α

Introduction

The use of radiotherapy inevitably induces exposure of normal tissues. The pathological processes of radiation changes begin immediately post irradiation, but the pathological and clinical appearance may not become obvious for weeks, months, or even years after radiation.^[1] The lung is a radiosensitive organ of body and therefore imports a problem for radiation exposures to the thoracic region.^[2] The mechanisms involved in the radiation-induced lung damages remain incompletely understood; previous studies have shown a cyclic inflammatory response, related

to inflammatory cells, reactive oxygen species (ROS), and the up-regulation of pro-inflammatory cytokines as major factors in causing lung injuries.^[3-6] Lung injuries occur in two separate phase. Two to three months after irradiation, radiation pneumonitis can occur followed by radiation fibrosis 4 months to 1 year post irradiation.^[7] Radiation has been reported to induce the production of biological mediators, such as cytokines, which modulate diverse aspects of pneumonitis and the fibrogenic response.^[8-10] Tumor necrosis factor α (TNF- α) is a key mediator for the pathogenesis of radiation pneumonitis because it shows the spectrum of biological activities. TNF- α exerts in pro-inflammatory effects by inducing the expression of adhesion molecules, and also it exerts fibrogenic effects by stimulation the growth of fibroblasts and increasing the collagen deposition.^[11] Therefore, a pharmacological modification of the TNF- α production at the initial step could possibly halt the progress of radiation-induced damages. The growth of effective radioprotectors is the large topic in view of their use during radiation exposure.^[12] Some plants and their bioactive constituents, display antioxidant, immunomodulating, and anti-inflammatory properties, and the radioprotective response in many cases is mediated by these effects.^[13,14] Root extracts of the plant *Glycyrrhiza glabra L.*, known as Yashtimadhu, in Ayurveda have been used for healing different maladies because of its

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anti-viral, anti-inflammatory, and immune-modulating, and anti-bacterial activities.^[15] The extract, called licorice, and the active compounds of the extract have been reported to have anti-oxidant, immuno-modulating, and free radical-scavenging activity.^[16-18] Component of the extract is glycyrrhizic acid (GLA, a triterpenoid saponin glycoside) [Figure 1], and *in vivo* and *in vitro* tests have shown that GLA is non-genotoxic.^[19]

The aim of this study is the evaluation of the ability of GLA to reduce the TNF- α release on messenger ribonucleic acid (mRNA) and protein level of lung irradiation.

Materials and Methods

Reagent

GLA powder (Sigma-Aldrich Chemical Co., USA), TriPure RNA isolation reagent (Roche Co, Germany), complementary deoxyribonucleic acid (cDNA) synthesis kit, and primers (Termo scientific, USA), SYBER green I master mix (Takara Co., Japan), and TNF- α rat enzyme-linked immunosorbant assay (ELISA) kit (abnova Co, Taiwan), Ketamine hydrochloride and xylazine (Alfasan, Woerden-Holland). Other reagents were of analytical grade, obtained from local manufactures.

Animals

Male Wistar rats weighing 170–210 g were purchased from the vivarian section of Department of Pharmacology, Tehran University of Medical Sciences, Tehran, Iran. The animals were randomly housed, six together in metal wire netting cages, with room temperature maintained at 20–22°C, relative humidity of 50–70%, and an airflow rate of 15 exchange/h, to 12 h alternate light and dark cycle. Animals had free access to tap water in glass bottles and standard rat chow. The experimental procedures were approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran in accordance with the Standards for the Care and Use of Laboratory Animals.

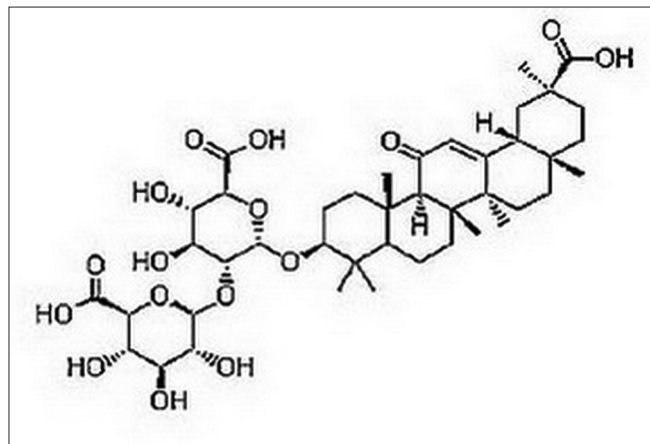


Figure 1: Glycyrrhizic acid (GLA)

The animals were divided into four groups: Rats that received neither irradiation nor GLA (NT = no treatment; group: 9 animals), those that received GLA but no irradiation (= GLA; group: 27 animals), those that underwent thoracic irradiation without GLA application (= XRT; group: 27 animals), and those that received both GLA and thoracic irradiation (= GLA/XRT; group: 27 animals).

Radiation schedule

Prior to irradiation, the animals were anesthetized with an intraperitoneal (i.p.) injection of ketamine hydrochloride 80 mg/kg body weight, and xylazine 5 mg/kg body weight. Positioning was facilitated using a Lucite fixation setup, making it possible to irradiate six animals simultaneously. The rats were in a supine position and the whole thoracic region was irradiated by a Cobalt-60 unit (Theratron 780, AE Canada Ltd, Canada) at a source-thoracic cage distance of 80 cm, and a single doses of 16 Gy (at 1 cm depth), with a dose rate of 99.84 cGy/min. Lead blocks measuring 10-cm thick defined the irradiation fields and prevented primary irradiation of the adjacent tissues. Following irradiation, the rats were maintained six per cage in laminar flow hoods in pathogen-free rooms to minimize pulmonary infections and supplied with standard laboratory diet and water.

Experimental protocol

GLA powder was dissolved in double distilled water (DDW) to reach a final concentration of 1 mg/ml. In the GLA/XRT and GLA groups, 4 mg/kg GLA was injected i.p. 1 h before thoracic irradiation or sham-irradiation, respectively. This dose and schedule of administration of GLA in combination with radiotherapy was designed on the basis of previous animal studies.^[20] All rats underwent actual (XRT and GLA/XRT groups) or sham irradiation (NT and GLA groups).

Foreach treatment modality (XRT, GLA/XRT, GLA groups), three animals were sacrificed at 1 h, 6 h; 1 and 3 days (d); and 1, 2, 4, 8, and 16 weeks (w) post treatment (p.t.). Untreated, sham-irradiated animals were maintained under identical conditions for the course of the experiment; and at each assessment time point (1, 6 h; 1 and 3 d; 1, 2, 4, 8, and 16 w), one of these animals was sacrificed to obtain an age-matched control group of nine animals in total.

Tissue isolation

In all time points after anesthetizes, rats sacrificed and chests were immediately opened for sampling of the lungs. The lungs were immediately perfused via the trachea with 4% buffered formaldehyde. The tissue samples were placed in fixative for histopathologic analysis, and quickly frozen in liquid nitrogen, then kept at -80°C for RNA isolation and subsequent real time reverse transcriptase polymerase chain reaction (RT-PCR) analyses (to quantify the cytokine expression), and ELISA assay.

Histopathological examination

For histopathological analysis, the tissues were fixed in 10% neutral buffered formalin, then embedded in paraffin, and sectioned at an average thickness of 5 μ m. The mounted sections were stained with hematoxylin and eosin (H and E). Finally, slides were investigated by a pathologist blinded to the study groups, under light microscope (BX 50, Olympus Corporation, Tokyo, Japan).

RNA extraction and cDNA synthesis

Total RNA from rat lung tissue was extracted by using TriPure RNA isolation reagent as per the manufacturer's instructions. Then about 1 μ g of total RNA was subjected for cDNA synthesis using MMLV reverse transcriptase and random hexamers.

Real-time quantitative reverse transcriptase polymerase chain reaction

Quantification of mRNAs was carried out by real-time PCR Rotor-Gene™ 6000 (Corbette Life Science™, Germany) using SYBR green I master mix containing HotStarTaq DNA polymerase in final 20 μ l PCR mixture volume consist of 10 μ l PCR Master Mix, 2 μ l of cDNA, 2 μ l forward and reverse primers and 6 μ l ddH₂O. PCR protocol was done in following conditions: 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and elongation at 72°C for 20 s with final melting curve analysis. To normalize target genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The sequences of primers are listed in [Table 1]. Relative mRNA expression was obtained using the relative expression software tool (REST).^[21]

Determination of TNF- α

In order to determine the inflammatory cytokine, tissues were homogenized as follows; first, we prepared a lysis buffer containing 200 mM NaCl, 5 mM EDTA, 10 mM tris, 10% glycerin, 1 mM PMSF. Then, 1 tablet of protease inhibitor cocktail for 10 ml of lysis buffer was added, finally, pH was set at 7.4. Also, 200 μ l lysis buffer was added to 10 mg tissue before homogenization. Tissue homogenates were centrifuged two times at 201 g for 15 minutes at 4°C to remove cell debris. Supernatants were used for the measurement of cytokine level.^[22] Total proteins of

lung tissue were measured using Bradford assay as mg/ml. The level of TNF- α in the lung samples were measured using a commercially available ELISA kit according to the manufacturer's protocol as pg/ml. The level of TNF- α divided to total protein in the lung tissue. TNF- α level is expressed as pg/mg of tissue.

Statistical analysis

Data were expressed as means \pm SE. The distribution of the data in each group was achieved using the Kolmogorov-Smirnov test. Kruskal-Wallis analyses and the Bonferroni post-hoc test were carried out to test for difference in means among treatment groups contained in the Statistical Package of Social Sciences (SPSS) software package (version 11.0) for Windows 98. Probability value less than 0.05 was considered significant.

Results

Histopathology

Histopathological changes of radiation-induced lung injury in the rats of XRT group were severe, and included edema in the alveolar wall and/or air spaces (arrow a), thickening of the alveolar septa by infiltration of mononuclear (arrow b), a large numbers of foam and dust cells (arrow c), congestion of dilated vessels, collagen deposition, and a large fibrous area (arrow d). A decreasing in severity of these alterations was noted in the rats of GLA/XRT group. In contrast, non-irradiated lungs (NT, GLA groups) obtained from the same period of time showed no evidence of pulmonary inflammation or other significant histopathological changes [Figure 2].

Relative mRNA expression

The results of the quantitative evaluation of the TNF- α relative mRNA expression in the lung tissue of the rats in the different study groups are shown in [Figure 3]. Following thoracic irradiation with a single dose of 16 Gy (XRT group), radiation-induced TNF- α relative mRNA expression in the lung tissue revealed a distinguished increase for TNF- α as early as 1 h p.i (5.24 ± 0.3), statistically significant compared to other groups ($P < 0.05$). Subsequently, the relative mRNA expression for TNF- α returned to low levels (0.85 ± 0.06) at 3 days p.i. Radiation-induced relative TNF- α expression reach elevated values at 1, 2, 4, and 8 weeks (statistically

Table 1: Sequences of gene-specific primers used for quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis in rats. Forward (FW) and reverse (RV) primer were always located in different exons. All complementary deoxyribonucleic acid (cDNA) sequences were obtained from the Genbank database

Gene	Accession number	Primer	Sequence (5'→3')	Product size (bp)
TNF- α	Nc_005119	FW	GCGTGTCATCCGTTCTCTAC	176
		RV	TTCTGAGCATCGTAGTTGTTGG	
GAPDH	Nc_005103	FW	CGTATCGGACGCCTGGTTAC	157
		RV	CATTCTCAGCCTTGACTGTGC	

not significant compared to other groups), with the highest value at 8 weeks p.i (6.5 ± 0.53). The lung tissue of the GLA/XRT group, revealed a down-regulation of the TNF- α relative mRNA expression at the all assessment time points (statistically significant compared to the XRT group at 1 h p.i). The TNF- α relative mRNA expression in the lung tissue of these rats reached maximal values at 8 weeks p.i (5.22 ± 0.29). It was interesting to note that rats in GLA/XRT group showed a remarkable decrease for TNF- α as early as 1 h p.i (1.51 ± 0.07). Results in our animal model suggested that GLA played a down-regulatory role in radiation-induced lung inflammation. Rats in GLA group exhibited no TNF- α mRNA expression.

Level of TNF- α protein

The results of the evaluation of the TNF- α protein level in the lung tissue of the rats in the different study groups were demonstrated in [Figure 4]. Following thoracic irradiation with a single dose of 16 Gy (XRT group), the TNF- α protein level in the lung tissue revealed a obvious increase for TNF- α protein as early as 6 h p.i (182.71 ± 10.24 pg/mg, and at 16 weeks p.i (114.27 ± 3.17) pg/mg, statistically significant compared to other groups ($P < 0.05$), with the lowest value (42.24 ± 1.96) pg/mg at 1 week p.i. The lung tissue of the GLA/XRT group, revealed a down-regulation of the TNF- α protein production at the all assessment time points (statistically significant at 6 h compared to XRT group). The TNF- α protein level in the lung tissue of these rats revealed the peak values at 6 h and 16 weeks p.i (79.23 ± 2.06) pg/mg and (83.74 ± 8.15) pg/mg, respectively. Rats in NT and GLA groups exhibited very low TNF- α protein level.

Discussion

Radiation-induced lung damage is a basic difficulty for the lung in thoracic irradiation. Because the mechanism of radiation-induced damage is unknown, little can prevent it, when radiotherapy is the only choice.

At the cellular level, radiation-induced pneumonitis is defined by lympholytic alveolitis, resulting from mononuclear cells infiltration of the vascular compartment into the alveolar spaces and the pulmonary interstitium.^[23,24]

Lung fibrosis is a frequent response to damages to the lung. Although there are varied initiating mechanisms, the final steps of fibrosis are defined by proliferation and progressive accumulation of connective tissue replacing normal parenchyma. The pathogenesis of lung fibrosis includes endothelial and epithelial cell injury influx of the inflammatory cells and production of their chemical mediators leading to the activation of the fibroblasts.^[25,26] In present study, we observed histopathology alterations in the animals that received thoracic radiation (XRT group) as

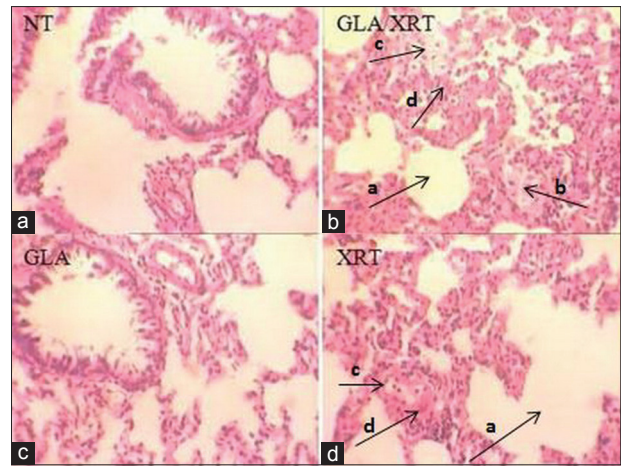


Figure 2: Normal lung architecture showing alveoli separated by delicate interalveolar septa, in the NT, glycyrrhizic acid (GLA) groups rats that sacrificed at 8 weeks p.i. Severe lung alterations showing intra-alveolar edema (arrow a), thickening of the alveolar septa by infiltration of mononuclear (arrow b), a large numbers of foam and dust cells (arrow c), congestion of dilated vessels, collagen deposition, and a large fibrous area (arrow d), in the XRT group rats, and mild to moderate severity of these alterations in the GLA/XRT group rats that sacrificed at 16 weeks p.i, (H and E, $\times 400$)

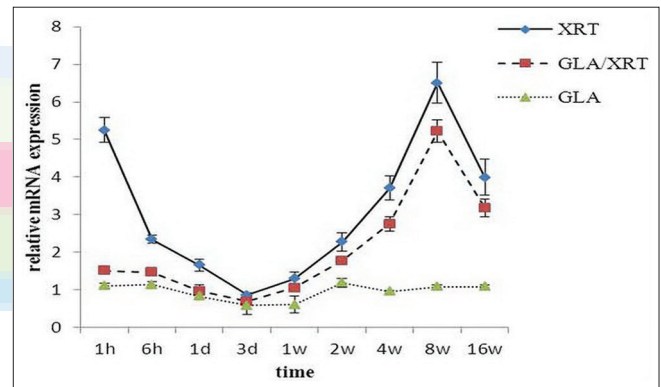


Figure 3: Time course of tumor necrosis factor α (TNF- α) messenger ribonucleic acid (mRNA) expression in the lung tissue of different groups. Data are mean \pm SE of duplicate determinations from three different rats

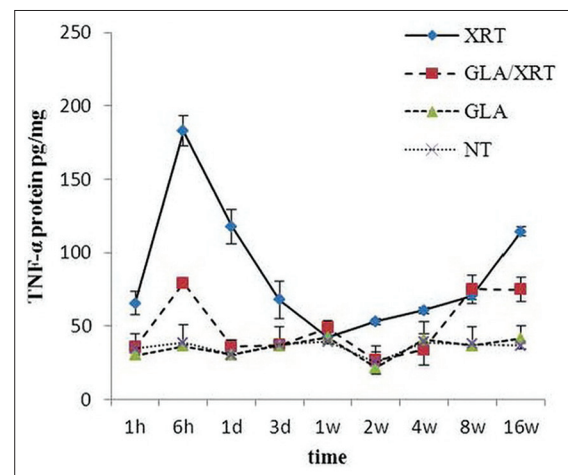


Figure 4: Time course of tumor necrosis factor α (TNF- α) protein levels in the lung tissue of different groups. Data are mean \pm SE of duplicate determinations from three different rats

mentioned in the results section, in keeping with previous reports.^[1,27-29]

Licorice root extract is capable of effectively inhibiting the process of radiation-induced lipid peroxidation and improving the state of the lung surfactant antioxidant system.^[30] Another study showed radioprotective properties of this extract on gamma-radiation-induced strand breaks in plasmid pBR322 DNA and lipid peroxidation in rat liver microsomal membranes *in vitro*.^[31] Recent study showed that feeding with GLA prior to UVB caused delay in tumor appearance, multiplicity, and size.^[32] Use of GLA for prevention and/or treatment of pulmonary fibrosis, in particular, irradiation-induced pulmonary fibrosis reported in last study.^[33]

The results of current study illustrate that administration of GLA 1 h prior to thoracic radiation may have a protective effect against radiation-induced injury, as assessed by edema, hemorrhage, interstitial inflammation, proliferation of fibroblasts and collagen deposition using histopathology [Figure 2]. Bronchial epithelium might begin inflammatory reflexes by recruitment of inflammatory cells, adhesion molecules, and interaction of epithelial cells with inflammatory cells, and modulation of the activity of parenchymal cells. Studies *in vitro* displayed that bronchial epithelial cells are able to generate a vast range of pre-inflammatory cytokines, which are able to regulate the recruitment and activation of inflammatory cells.^[34]

There are documents that a cyclic cascade of inflammatory cytokines, together with the activation of macrophages, is initiated very early post irradiation.^[2] Attention to the cytokine network in radiation-induced lung damage, TNF- α which is primarily over expressed in pulmonary epithelial cells, likely initiated recruitment macrophages and other inflammatory cells into the locations of inflammation. It is known the TNF- α boosts the production of other cytokines, of which are complicated in recruiting macrophages into the inflammation locations.

GLA has been reported to reduce radiation-induced strand breaks in bone marrow cells and peripheral blood leucocytes by sweeping free radicals,^[20] it might tender preservation from the damage induced in humans by ultraviolet beam radiation,^[35] and also it protects against UVB irradiation-induced photoaging in human dermal fibroblasts. Previous study showed that GLA could added to the list of protective agents since SKMEL-28 cells were really protected from radiation damage.^[36]

The radioprotective effects of GLA on cellular immunocompetence has been reported in the past years.^[12]

In another study, oral administration of SN-GLA, and GLA 1 h before a sublethal dose of radiation exposure reduced the radiation-induced depletion of cellular

antioxidants and lipid peroxidation in various tissues of mice. Administration of GLA, SN-GLA 1 h before gamma radiation exposure also improved the number of visible endogenous spleen colonies per spleen on 12th day post irradiation, which indicates the improved hemopoetic system regeneration following radiation exposure.^[20]

Previous studies have suggested that inflammatory and fibrogenic cytokines may play principal roles in the pathogenesis of radiation-induced lung injury. Among the cytokines studied, TNF- α appears to be a fundamental molecule in the primary stage of inflammation in regions of histopathological lesions.^[37] The prior works on the temporal release of TNF- α in the irradiated lung tissue described that the cellular source of TNF- α could be displayed via TNF- α immunoreactivity as well as histopathologic changes.^[34,38] Immunohistochemistry assessments showed that bronchial epithelium is the great cause of cytokines production, which are consistent with tissue lesions. In our study, we clearly demonstrated the immediate release of the pro-inflammatory cytokine TNF- α , as early as 1 h after thoracic radiation. Although the time course of the mRNA expression of TNF- α in the lung tissue after thoracic irradiation has been depicted earlier,^[39] the results of the PCR analysis in the last reports support our finding of an immediate expression of cytokine after lung irradiation as consistent with the observation of Hong *et al.*,^[40] that TNF- α mRNA level were induced in the lung tissue of C57BL/6 mice within 24 h after irradiation. In addition, our current data are in concordance with the finding of others.^[34,38,41-43] Administration of GLA 1 h prior to thoracic irradiation down regulated the TNF- α mRNA and protein production within the first hours after irradiation. There is major interest in the potential value of GLA as an inhibitor of normal tissue damage caused by radiation. This could be promising primary laboratory researches, as well as the drug's widespread usability and low toxicity rate.

With respect to the mitigation of radiation-induced damage using GLA, several observations need to be done and suggest that GLA might be a useful therapeutic factor.

Conclusion

This study demonstrates a remarkable radiation-induced increase of TNF- α (on mRNA and protein level) in the lung tissue during the first hours after thoracic irradiation. Histopathological findings suggest involvement of this cytokine in the pathogenesis of radiation-induced lung injury. In addition, we observed a pronounced (statistically significant) reduction of TNF- α mRNA and protein production in the animals that received both GLA and radiation (GLA/XRT group) as compared to the radiation-only group (XRT group). Therefore, our results indicate that GLA down-regulates the TNF- α mRNA

and protein production in the lung tissue in response to thoracic irradiation. Suitable inhibition of cytokines at an early stage might provide a modern tool for effective cure of radiation-induced lung injury. Pharmacologic regulation of cytokine production in the first hours post irradiation might provide protection against radiation-induced, cytokine-mediated damage.

Acknowledgments

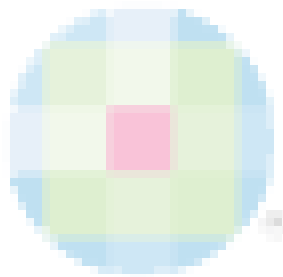
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