

RESEARCH ARTICLE

Preliminary Study of Protective Effects of Flavonoids against Radiation-induced Lung Injury in Mice

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Abstract

Background: Radiation therapy plays an important role in lung carcinoma treatment. However, the incidence of symptomatic radiation-induced lung injury is high. This study aimed to evaluate radioprotective effects of flavonoids extracted from *Astragalus complanatus* and mechanisms of action against radiation damage. **Methods:** Alteration in antioxidant status and levels of several cytokines were investigated in BABL/C mice treated with 4 mg/kg b.wt. flavonoids after exposure to 10Gy thoracic radiation. **Results:** Serum levels of SOD in the flavonoids+radiation group were significantly higher compared to the radiation control group, while TGF- β 1 and IL-6 were lower. Mice in the radiation control group displayed more severe lung damage compared with the flavonoids+radiation group. The expression of TGF- β 1 and TNF- α in the radiation control group was markedly increased in alveolar epithelial cells and macrophages of the alveolar septum. **Conclusions:** From the results of the present study, flavonoids could be excellent candidates as protective agents against radiation-induced lung injury.

Keywords: Radiation-induced lung injury - flavonoids of *Astragalus complanatus* - ELISA - immunohistochemistry

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Introduction

Radiation therapy is an important therapeutic modality in the treatment of several thoracic tumors including lung, breast, esophagus and lymphoma. Although the development of new strategies such as three-dimensional planning and intensity modulation contribute to minimize doses to the normal lung structures, radiation therapy is still sometimes cause radiation-induced pneumonitis especially for patients treated with combined radiation and chemotherapy, higher total dose, larger fraction size and larger volume of irradiated lung (Emami et al., 1991). Accordingly, the incidence of symptomatic radiation-induced lung injury ranges from 5–10% and may be as high as 20% (Vujaskovic et al., 2000).

The lung is the major dose-limiting organ for radiotherapy of cancer in the thoracic region. Damage to endothelial or epithelial cells is assumed to be the initial step leading to radiation pneumonitis and ultimately to pulmonary fibrosis (Rübe et al., 2002). And the cellular damage after irradiation is believed to be a consequence of a cascade of cytokine activity (Rubin et al., 1995), which ultimately begins with oxidative stress from radiolytic hydrolysis and formation of reactive oxygen species (ROS). Hence the radioprotection against the oxidative stress which results in oxidation of proteins, lipids and

nucleotides is drawing increasing attention (Malekirad et al., 2005). Several studies have reported that superoxide dismutase (SOD) played a key role in protecting cells from radiation damage by removing free radicals produced by irradiation. In the presence of SOD, superoxide is dismutated to H₂O₂ and O₂. H₂O₂ is then subsequently eliminated by catalase and glutathione peroxidase via water and oxygen (Tsan et al., 1997).

The flavonoids are naturally occurring diphenylpropanoids that are ubiquitous in the plant kingdom and represent one of the most important classes of biologically active compounds (Hertog et al., 1993). Several studies suggest that flavonoids may act as antioxidants, free radical scavengers or radioprotectors (Martins et al., 1991). Flavonoids possess an interesting anti-inflammatory profile, related to their capability to scavenge various oxidising species i.e. superoxide anion (O²⁻), hydroxyl radical or peroxy radicals (Harborne et al., 2000). Some workers also suggested that synergistic effects of flavonoid mixtures may be responsible for the high activity observed in crude extracts (Terao et al., 1994). Thus administration of flavonoids might be efficacious in the prevention of radiation-induced lung damage. With this aim we have evaluated the protective effect of flavonoids extracted from *Astragalus membranaceus* on radiation-induced lung injury in mice.

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Materials and Methods

Reagents

Flavonoids, in the form of sterile injections of 5ml (equivalent to 20mg) 100% pure flavonoids extracted from *Astragalus membranaceus*, was provided from Changchun University of Chinese Medicine. Flavonoids (4mg/ml) was dissolved in 0.01% dimethyl sulfoxide (DMSO) and was used as the stock solution. The stock solution was then diluted with sterile distilled water to arrive at a final concentration of 0.4mg/ml. 0.2% DMSO was used as a sham control. The kits of SOD, TGF- β 1, TNF- α and IL-6 were purchased from Shanghai Yapei Biological Techniques Institute.

Animals

Female mice (BALB/C, 6weeks old, weighing 20-25g) were purchased from the Shandong Experimental Animal Center and maintained at an animal facility. Five mice were housed per cage containing sterile paddy husk as bedding throughout the experiment and maintained under identical conditions with food and water provided ad libitum. In 1w, 2w, 3w, 4w, 5w, 6w, 8w after irradiation, one or two mice from each group were killed under anesthesia and specimens of blood were centrifuged to obtain serum in 4°C which collected through digging eye method. The lung was then removed and placed in 10% formalin for 48 hours for fixation. The lungs were embedded in paraffin and sections of 5 μ m thick were cut and placed on slides in preparation for histopathology and immunohistochemistry studies. The handling of mice and experimental procedures were conducted in accordance with institutional animal care and use committee guidelines.

Grouping and Treatments for Animals

Forty-eight mice were randomly divided into 4 groups:

Normal control: 0.2% DMSO (0.1ml) twice a week postirradiation.

Flavonoids control: flavonoids solution (0.1ml) twice a week postirradiation.

Radiation control: the bilateral lung of the mice were exposed to a single 10 Gy radiation.

Flavonoids+ radiation: flavonoids solution (0.1ml) twice a week postirradiation+ 10 Gy radiation to the bilateral lung of the mice.

Irradiation

Mice were anesthetized before RT with intraperitoneal injection of pentobarbital (40 mg/kg) and were placed in a specially designed restrainer with adhesive tapes. Twelve-millimeter lead blocks were used to protect the head and the rest of the body. Then mice were exposed to a single dose of 10 Gy irradiation, at a dose rate of 5Gy/min (Varian 600C linear accelerator, USA).

TGF- β 1, IL-6 and SOD assay

SOD, TGF- β 1 and IL-6 levels in blood serum were measured at 1,2,3,4,5,6,8 weeks after irradiation respectively by a sandwich enzyme-linked immunoabsorbent assay (ELISA).

Histology

Paraffin-embedded tissue was sectioned at an average thickness of 5 μ m, stained using haematoxylin and eosin as well as Masson stain for collagen. The sections were H&E stained by dipping the slides in Harris hematoxylin for 1 min and 45 s, rinsing with tap water until clear, dipping in eosin for 1 min and rinsing in H₂O. The slides were allowed to air dry and then dipped twice in 95% ethanol, twice in 100% ethanol, twice in 50:50 ethanol/xylene, and twice in 100% xylene.

Immunohistochemistry

Lungs were assessed for the levels of TGF- β 1 and TNF- α protein expression with Streptavidin-peroxidase methodology. Tissue dewaxing in xylene and rehydration in graded alcohol were performed according to the manufacturer's protocol. Anti-mouse TNF- α and TGF- β 1 antibodies and goat anti-rabbit secondary antibody were purchased from Maixin biotechnology (Fuzhou, P.R. China). Immunohistochemistry (IHC) was carried out according to instructions of the IHC kit. IHC slides were subjected to blinded evaluation by two investigators.

Data analysis

When comparisons were made between the means of two groups, a two-tailed Student's t-test was used, $p < 0.05$. For multiple comparisons an ANOVA was used. Significance was defined as $p < 0.05$. Statistical analyses were performed using SPSS 16.0 software.

Results

TGF- β 1 and IL-6 levels in blood serum

We demonstrated significant differences after thoracic radiation in the extent of elevation in TGF- β 1 and IL-6 levels in the serum of the mice.

In mice killed after 1 week, serum levels of TGF- β 1 were elevated in radiation control group and Flavonoids+radiation group (Figure 1). The peak time of TGF- β 1 are observed at 4th week. The mean level of this cytokine during the experiment were significantly higher in the radiation (55.91 \pm 15.24 and 28.15 \pm 4.94 ng/ml, respectively, $p=0.002$) and Flavonoids+radiation group (39.79 \pm 9.17 and 28.15 \pm 4.94 ng/ml, respectively, $p=0.012$) compared with the normal control group. The difference between the radiation and Flavonoids+radiation group (55.91 \pm 15.24 and 39.79 \pm 9.17 ng/ml, respectively, $p=0.034$) was significant. There was no significant

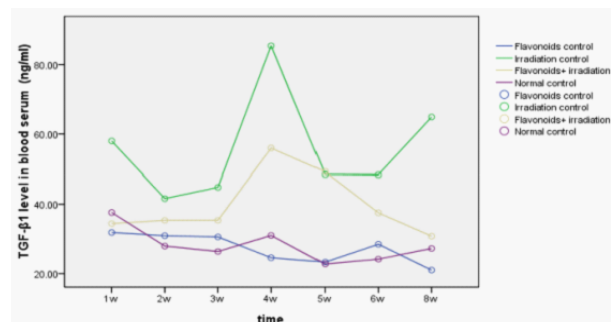


Figure 1. TGF- β 1 Levels in Blood Serum of Mice

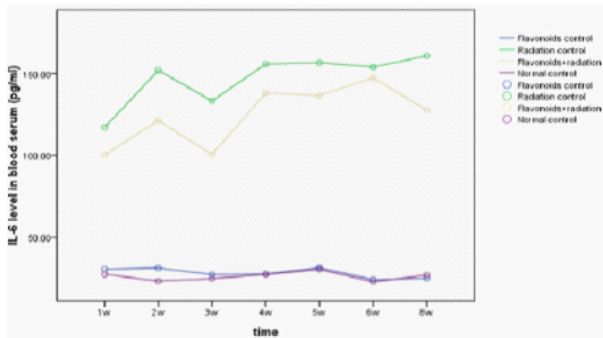


Figure 2. IL-6 Levels in Blood Serum of Mice

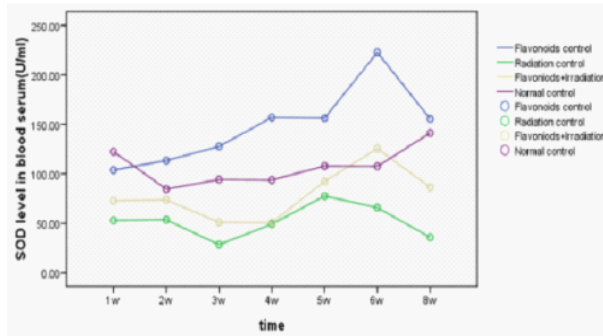


Figure 3. SOD Levels in Blood Serum of Mice

difference in serum TGF- β 1 between the normal and flavonoids control group (28.15 ± 4.94 and 27.24 ± 4.25 ng/ml, respectively, $P = 0.718$) (Figure 1).

After radiation, The levels of IL-6 were significantly higher in the radiation (147.09 ± 15.86 and 26.24 ± 2.70 pg/ml, respectively, $p < 0.001$) and Flavonoids+radiation group (124.59 ± 18.38 and 26.24 ± 2.70 pg/ml, respectively, $p < 0.001$) than the normal control group. There was significantly difference between the radiation control and flavonoids+radiation group (147.09 ± 15.86 and 124.59 ± 18.38 pg/ml, respectively, $P = 0.03$). Meanwhile, results showed no significant difference between the normal and flavonoids control group (26.24 ± 2.70 and 28.28 ± 2.99 pg/ml, respectively, $P = 0.204$) (Figure 2).

SOD levels in blood serum

The mean value for SOD activity in mice during the experiment after flavonoids injection was 148.06 ± 39.48 U/ml. This was significantly higher than the value found in normal control group (107.37 ± 19.38 U/ml) ($p = 0.031$). SOD levels in the flavonoids+radiation group was significantly higher than those in the radiation control group (78.88 ± 25.99 and 51.85 ± 16.70 pg/ml, respectively, $P = 0.039$). The descriptive graph for the trend of SOD level was shown in Figure 3.

Histology

Histological examinations were performed on lungs of the mice. Representative stained sections are shown in Figures 4. Considerable acute lung damage was noted at 4wks following exposure to 10Gy. Mice in the radiation-alone group displayed more severe lung damage, including macrophage infiltration in air spaces; edema in the alveolar wall and/or air spaces; thickening of the alveoli septa; changes in the alveolar and bronchial epithelium and blood vessels compared with the flavonoids plus radiation group.

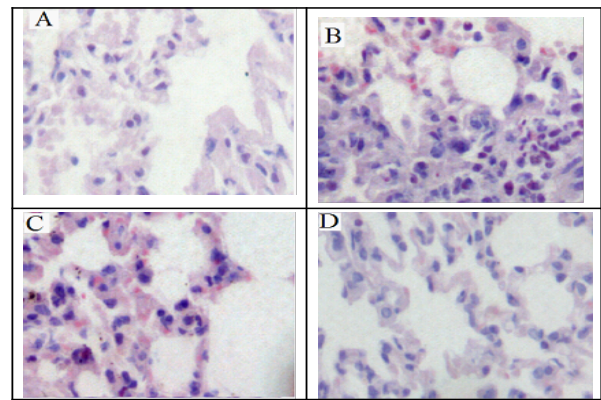


Figure 4. Morphological Changes in the Lung of Mice Injured by Radiation (HE, $\times 200$). The sections were stained with HE. A, Flavonoids control ; B, Radiation control ; C, Flavonoids+Radiation; F, Normal control

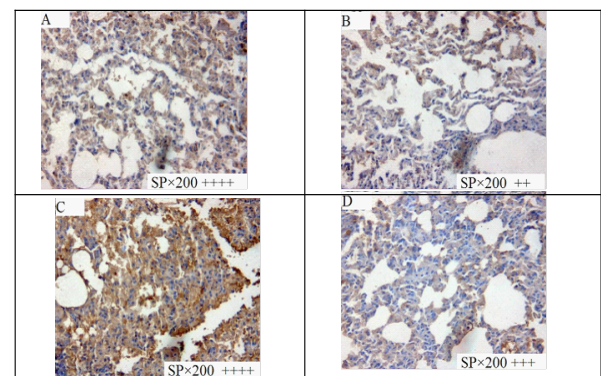


Figure 5. Immunohistochemical Staining of TGF- β 1 and TNF- α in Irradiated Lung Tissue. 4 weeks after irradiation. A, staining of TGF- β 1: positive alveolar macrophages in the lung parenchyma in radiation control group; B, staining of TGF- β 1: positive alveolar macrophages in the lung parenchyma in flavonoids+radiation group; C, staining of TNF- α : positive macrophages in perivascular region in radiation control group; D, staining of TNF- α : positive macrophages in perivascular region in flavonoids+radiation group

Other significant findings included interstitial thickening, vessels wall hyalinization, interstitial thickening and obliteration of the alveoli. Besides, we found severe parenchymal patch consolidation and alveolar epithelial edema in the lungs of the radiation-alone group. In contrast, in mice that also received flavonoids, thickening of the alveolar septum and epithelial cell injury were ameliorated compared with the radiation control group.

Immunohistochemical Analysis of Cytokine Expression in the Irradiated Mice Lung

The expression of TGF- β 1 and TNF- α in the radiation control group was markedly increased in alveolar epithelial cells and macrophages of the alveolar septum compared with the normal group. The combination treatment group that received both radiation and flavonoid injection exhibited a reduced effect on cytokine expression in all experimental tissue specimens. Increased cytokines expression was detected predominantly in regions of histopathological radiation injury; in contrast, no significant immunoreactivity was observed in areas of normal lung tissue.

The most striking increase in TGF- β 1 immunoreactivity was seen at the beginning of the pneumonic phase (2 and 4 weeks after irradiation). At 2 weeks, differential stains are obvious among groups with different treatments. At 4 weeks, it is obvious that cells are differentially stained in the four groups. There are few cells positive for TGF- β 1 (brown-stain) in normal control group mice. Most positive cells are seen in radiation control group mice. Positive cells in Flavonoids plus radiation group mice are obviously fewer than those in radiation alone mice. Between 30 and 90% of epithelial cells were brightly stained with TGF- β 1 antibody. TGF- β 1 antibody intensely stained the endothelial cells for hours to days after irradiation (from 24 h to 1 week) whereas, during the inter-mediate phase (2, 4 and 8 weeks after irradiation), the endothelium did not react with TGF- β 1 (Figure 5).

Discussion

Acute radiation-induced lung injury are serious side effects that are dose limiting in radiotherapy involving significant volumes of the lung. Radiation damage is, to a large extent, caused by the overproduction of superoxide anion(O²⁻), hydroxyl radical (\bullet OH) and hydrogen peroxide(H₂O₂), that overwhelm the levels of antioxidants, resulting in oxidative stress, cellular damage and DNA strand breakage(Singh et al., 2000). To maintain the redox balance and in order to protect them from free radicals action, living cells have evolved an endogenous antioxidant defence mechanism which includes non-enzymatic entities like glutathione, ascorbic acid and also enzymes like superoxide dismutase, catalase, glutathione peroxidase, etc(Mittal et al., 2001). Hence the mechanistic study of cell damage induced by reactive oxygen species in relation to radiation- induce lung injury and their possible prevention by antioxidants constitutes an active area of research in recent years.

Flavonoids are compounds in which the benzenoid substitution is at the 2 position(Di et al., 1999), they are classified into several groups, such as flavones, flavonols and isoflavones, according to their structural differences. The most frequently studied flavonoids are quercetin, luteolin, kaempferol, apigenin and genistein. Some flavonoids were found to possess a good antiinflammatory activity (Król et al., 1995), which this was related mainly to their inhibiting production of inflammatory mediators such as prostaglandins, leukotrienes or nitric oxide. Besides, an investigation showed that flavonoids had antioxidant effects, including increasing superoxide dismutase, glutathione peroxidase and scavenging free radicals and degrading malondialdehyde (Qi et al., 2011); Another possible contributory mechanism to the antioxidant activity of flavonoids is their ability to stabilise membranes by decreasing membrane fluidity (Arora et al., 2000). These suggested protective effects of flavonoids, together with their potent antioxidative and free-radical scavenging activities observed in in-vitro studies have increased the public's interest in the use of flavonoids for protecting the radiation related damage. The results from this study indicated that flavonoids may be an efficient radioprotector through ELISA and immunohistochemical

analysis of several related biological cytokines and histopathology in the blood serum and lung of mice.

Superoxide dismutase, a known scavenging agent of superoxide radicals, is believed to act as a first line of antioxidant defence against oxygen free radicals that mediate cytotoxicity or cell death. SOD plays an important role in providing protection from radiation exposure, a reduction in the activity of it can result in a number of deleterious effects due to the accumulation of superoxide radicals and H₂O₂. Recent investigations using SOD for radiation-induced normal tissue injury have included Mn-SOD plasmid/liposome and Mn-SOD adenovirus gene therapy (Epperly et al., 2000; Epperly et al., 2001). Another series of studies have demonstrated protection of lung and esophagus from radiation injury by an intraperitoneal delivery of a small molecular weight catalytic metalloporphyrin antioxidant (SOD mimetic) (Vujaskovic et al., 2002). Results from this study showed the mean value for SOD activity during the experiment after flavonoids injection was significantly higher than the value found in normal control group. SOD levels in the flavonoids+radiation group was significantly higher compared to the radiation control group. According to this, we hypothesis that the efficacy of flavonoids in preventing radiation damage may depend on the elevation of SOD.

TGF- β is a potent activator of extracellular matrix macromolecule synthesis, in the development of the post-irradiation fibrosis (Martin et al., 2000). Inflammatory cells, especially macrophages infiltrated in the tissue, but also type II pneumocytes and pulmonary fibroblasts, were the main sources of TGF- β in the irradiated lung (Rube et al., 2000). The peak of TGF- β occurred between 3 and 6 weeks post-irradiation, which coincided with the initial influx of inflammatory cells but preceded the development of pulmonary fibrosis (Yi et al., 1996). IL-6, a major mediator of the acute-phase inflammatory response, can be synthesized by a variety of cells in the lung parenchyma such as fibroblasts and alveolar macrophages and has been found to be upregulated within hours following ionizing radiation (Rübe et al., 2004). IL-6 is an acute phase proinflammatory cytokine produced by activated alveolar macrophages, T helper lymphocytes, lung fibroblasts, and Type II pneumocytes (Kotloff et al., 1990). IL-6 was up-regulated and peaked at 6 hrs after radiation in lung tissue, BAL fluid and blood in C57BL/6 mice (Chen et al., 2005; Rübe et al., 2005). TNF- α is produced by activated macrophages during the fibrotic process and has proinflammatory and immunoregulatory effects. TNF- α induces the expression of adhesion molecules which subsequently recruit leukocytes into the sites of inflammation, primes leukocytes to produce oxidants, and promotes the production of prostaglandins and other inflammatory mediators (Piguet et al., 1990).

There have been previous reports suggesting that flavonoids can reduce radiation injuries, but its radioprotective effects are still not well known. This study indicated the effects of administration of flavonoids in mice following irradiation to the lungs. The present results must be taken into consideration in further developments of these promising compounds, which has great potential as a clinical therapeutic agent. Moreover, the flavonoids,

scutellarein, rutin and nepitrin have been reported to increase the survival time of irradiated mice (Agarwal et al., 1981). Some reports also suggested that they had good anti-inflammatory activity without the ulcerogenic side-effects of other anti-inflammatory drugs. Flavonoids were also described as having anti-ulcer effects (Di et al., 1999). Flavonoids presents several advantages in protecting radiation damage, however, it still exists some uncertain factors demanding future study. The flavonoids is composed of several monomers with different structures, such as quercetin, luteolin and genistein, et al. Quercetin is one of the most abundant natural flavonoids. Cai' study demonstrated that quercetin dose-dependently inhibited O₂- produced by xanthine/xanthine oxidase, it can also dose-dependently scavenged H₂O₂ in vitro (Cai et al., 1997). Recently, Wei et al. (1995; 1996) reported that genistein has an inhibitory effect on UV light and Fenton reaction-induced oxidative DNA damage and scavenge free radicals. Therefore, it is necessary to carry out the research for exploring the radioprotective role of the diverse monomers. Furthermore, radiation damage is a complicated procedure which contains many different pathways. This study only focus on the anti-oxidant mechanism of flavonoids for preventing the radiation induced lung damage. There may be other different pathway or regulation mechanisms related with the flavonoids for protecting the radiation damage, which provide new thread for us in the future study.

In conclusion, administration of flavonoids might be efficacious in the prevention of radiation-induced lung damage. In addition, our results showed the changes of several radiation-related cytokines. Further investigation for the pattern of cytokines changes after radiation and the role of diverse monomers is greatly needed. It is our hope that the anti-oxidant function of flavonoids will help to support the radioprotection research.

Acknowledgements

The author(s) declare that they have no competing interests.

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