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Protective effects of berberine on radiation-induced lung injury *via* intercellular adhesion molecular-1 and transforming growth factor-beta-1 in patients with lung cancer

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ABSTRACT

Purpose: To investigate the protective effects of berberine on radiation-induced lung injury (RILI) in non-small cell lung cancer (NSCLC) patients treated with radiotherapy.

Patients and methods: In this randomised, double-blind study, 90 patients with NSCLC were divided into two groups. The trial group received radiation therapy plus berberine, and the control group received radiation therapy plus a placebo for 6 weeks. Soluble intercellular adhesion molecular-1 (sICAM-1) and transforming growth factor-beta-1 (TGF-β1) were measured. RILI and pulmonary function were evaluated at 6 weeks and 6 months after treatment, respectively.

Results: Of the 90 patients enroled, 43 in the control group and 42 in the trial group completed the study. The incidence of RILI was significantly lower in the trial group at 6 weeks and 6 months than that in the control group (45.2% *versus* 72.1% and 35.7% *versus* 65.1%, respectively, both $P < 0.05$). sICAM-1 levels in the trial group were significantly lower at weeks 6 and 12 (373.64 ± 89.33 *versus* 459.53 ± 123.59 and 447.83 ± 111.21 *versus* 513.91 ± 150.46, both $P < 0.01$), and plasma TGF-β1 levels were lower at week 3 and 6 (5.43 ± 1.47 *versus* 6.22 ± 1.78 and 5.93 ± 2.39 *versus* 7.67 ± 2.74, $P < 0.05$ and 0.01, respectively) in comparison with the control group. Significant differences were observed in FEV1 ($P = 0.033$) and DLCO ($P = 0.003$) between patients receiving berberine and those receiving placebo. Independent-samples *T*-test showed reductions from baseline FVC at week 6 ($P < 0.05$), and FEV1 and DLCO at month 6 ($P < 0.05$ and 0.01, respectively) in the trial group were significantly smaller than that in the control group.

Conclusion: Berberine significantly reduced the incidence of RILI, improved PF and decreased the levels of sICAM-1 and TGF-β1. The exact mechanisms remain to be further explored.

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1. Introduction

Radiation-induced lung injury (RILI) following thoracic irradiation is fairly common and it has a poor prognosis. A large proportion of patients with lung cancer receive thoracic irradiation as part of their treatment, and approximately 5–20% develops symptomatic lung injury, 50–100% develop radiological evidence of regional injury, and 50–90% experience declines in pulmonary function.[1] Therefore, active treatment of RILI has important clinical significance for the prognosis of patients treated with radiotherapy.

Generally, the clinical presentation of RILI is extremely variable, usually developing within 6 months following the completion of radiation therapy (RT). The syndrome of RILI is characterised by cough, dyspnoea and fever, and the signs are usually normal, but crackles and/or pleural friction rub may also be heard. The current evidence indicates the pathogenesis of RILI is characterised by a sequence of biological changes, including early radiation pneumonitis and late radiation fibrosis. The phase of pneumonitis is characterised by the loss of type I pneumocytes, increased capillary permeability, interstitial oedema, alveolar capillary congestion and inflammatory cell accumulation in the alveolar space. Pulmonary fibrosis is the repair process that follows the pneumonitis stage, and fibrosis usually develops together with the loss of capillaries, thickened alveolar septa and obliteration of the alveolar space.[2]

Inflammatory cell infiltration of the lung is a predominant histopathological change that occurs during RILI.[3,4] This process is dynamic and involves a number of proinflammatory cytokines, profibrotic cytokines and chemokines produced by a variety of cell types, including macrophages, epithelial cells and fibroblasts. Transforming growth factor-beta-1 (TGF-β1) and intercellular adhesion molecular-1 (ICAM-1) are critical to the pathogenesis of RILI and may be involved in the development of RILI.[3,5] TGF-β1 can stimulate proliferation of fibroblasts and ICAM-1 expressed in the pulmonary capillary endothelium can regulate the emigration of inflammatory cells from circulation into inflamed tissue.[6,7] Several recent studies have proved that radiation induces the expression of ICAM-1 and TGF-β1 on lung cells and they play an important role in the pathogenesis of radiation-induced inflammation.[8–10] It has been suggested that they are the master switch cytokines, which once activated after radiation, promote a train of cellular events that result in RILI. Monitoring the levels of these cytokines may reflect the efficacy of an intervention aimed at preventing RILI.[11,12] Therefore, we regarded ICAM-1 and TGF-β1 as the biomarkers to evaluate the effects of treatment on RILI.

In existing practice, adrenocorticotropic hormone and cortisone are the main drugs for RILI. However, with the exception of these drugs, few therapies have been proven efficacious in a wide range of patients. Moreover, several adverse effects such as an increased risk of osteoporosis caused some patients to withdraw from treatment.[13] Consequently, drugs of herbal origin with low side-effects are of high interest as alternatives and the traditional Chinese herbal medicine may provide a new therapy. Berberine is a benzyl tetra isoquinoline alkaloid which is widely used as an antimicrobial and an antidiarrhoeal drug.[14–16] Moreover, several experimental studies have revealed that berberine was able to attenuate inflammation and liver fibrosis by decreasing the level of TGF-β1 and TNF-α.[17,18] In addition, it was found that it can scavenge superoxide anion radical and attenuate tissue damage.[19] This study was designed to evaluate the therapeutic effects of berberine on RILI in patients with lung cancer as well as to elucidate the possible mechanisms underlying these effects.

2. Patients and methods

2.1. Eligibility criteria

This study was a prospective study of patients with non-small cell lung cancer. Patients were eligible with (1) locally unresectable stage III proven either by histology or cytology; (2) age range 18–75 years; (3) life expectancy $\geqslant$6 months; (4) Karnofsky performance status (KPS) $\geqslant$70; (4) basal pulmonary function (PF) tests (ratio of forced expiratory volume at 1 s on vital capacity $\geqslant$50%, ratio of diffusion capacity for carbon monoxide on alveolar volume $\geqslant$50%) allowing radiotherapy or without severe interstitial lung disease; (5) adequate marrow, renal and hepatic function; and (6) no history of other malignancy. The study protocol was fully explained, and written informed consent was obtained from each participant. This study was approved by the Ethical Committee for Human Research at our institute.

2.2. Thoracic radiation and berberine therapy

Using a prospective, randomised, placebo-controlled, double-blind design, patients in the trial group and the control group received berberine purchased from the Beijing Medicinal Material Company (at a dose of 20 mg kg^{-1} once a day) or a placebo containing starch for 6 weeks during three-dimensional conformal radiation therapy (3D-CRT), respectively. Neither patients nor trials staff can distinguish the drug/placebo by appearance, taste or side-effects. Briefly, radiation therapy consisted of once-daily treatment with 2-Gy to a total 60–70 Gy. Target volumes were defined using the International Commission on Radiation Units and Measurements (ICRU)-50 report.[20] Doses were calculated taking into account the tissue density heterogeneity, and dose volume histograms (DVHs) of the lungs were calculated based on computed tomography-defined lung volumes. Total mean lung dose, the percentage of irradiated lung volume exceeding 20 Gy (V20) and 30 Gy (V30), was calculated from lung DVHs. All patients were treated with similar medications during the observation period. If necessary, patients will receive concurrent or sequential chemotherapy. Chemotherapeutic regimens varied but were all platinum based. Drugs included carboplatin, cisplatin, paclitaxel and irinotecan.

2.3. Evaluation of RILI

The RILI was graded 0–4 according to the system of the Radiation Therapy Oncology Group/European Organisation for the Research and Treatment of Cancer described previously.[21] RILI was scored on clinical symptoms, radiological abnormalities and loss of pulmonary function. This includes three subjec-

tive scales and two objective scales. Subjective scales: cough; dyspnea; and thoracic pain. Objective scales: chest X-ray and thoracic CT read by an independent committee of experts (pneumologists, radiologists and radiation oncologists); PF tests (reduction of vital capacity and/or diffusion capacity for carbon monoxide on alveolar volume). RILT was defined as the grade $\geqslant 1$. The primary end-point was RILI within 6 months after treatment commenced.

2.4. Circulating cytokines analysis

Blood samples were collected at baseline (prior to treatment), and then at 3, 6 and 12 weeks (i.e. 4 blood samples). One set of blood samples were collected in tubes containing K_2EDTA as the anticoagulant. Blood samples were centrifuged under 10,000*g* for 30 min at 4 °C immediately and stored at −80 °C for further analysis. Plasma TGF-β1 levels were measured using a specific Enzyme Linked Immunosorbant Assay (ELISA) (Human TGF-β1 DuoSet kit, R&D Systems Inc., Minneapolis, MN). Another set of blood samples were collected in normal tubes and used for detecting the serum concentrations of sICAM-1 (ZyQuik sICAM-1 ELISA Kit, Invitrogen, USA). All measurements were performed independently by two researchers and the values averaged. For all assays the intra-observer and inter-observer variation coefficient was less than 5%, respectively.

2.5. Evaluation of PF

PF testing was performed using spirometer (Sensor Medics 6200, USA). The measurements including forced expiratory volume at 1 s (FEV1), diffusion capacity for carbon monoxide (DLCO) and forced vital capacity (FVC) were performed at baseline, then at 6 weeks and 6 months after the treatment. The measured values were expressed as percentage of the predicted value.

2.6. The response and survival

Treatment response was assessed according to World Health Organisation criteria based on a CT scan 2 months after the completion of treatment. A complete response was defined as the disappearance of all known disease for at least 4 weeks. A partial response required a reduction of at least 50% in the size of the tumour for at least 4 weeks. Progressive disease was defined as an increase of 25% or more in the size of the tumour, and stable disease was defined as no change or less than 50% reduction or more than 25% increase.

2.7. Follow-up and monitoring of adverse events

All patients underwent follow-up office visits at 6 weeks after completing radiation therapy and monthly for the first half year, then at 4 months intervals. At each follow-up, evaluations included a complete history, physical examination, blood routine, renal and hepatic function and a CT scan of the thorax. The presence of pneumonitis was determined by clinical examination and the thoracic CT scan. Any radiologic changes were taken into account as diffuse haze and a ground-glass opacification indicated the appearance of pneumonitis. The presence of fibrosis was assessed by a thoracic CT scan at 6 months after the end of the radiation treatment. All the evaluations were performed by physicians or nurses who were blinded to the treatment given, using the same set of questionnaires and guidelines.

2.8. Statistical analysis

With the published event rates for our primary end-point, we estimated the number of subjects required for the study to have >80% power ($\alpha = 0.05$) to detect an absolute 30% reduction in the incidence of the end-point. The study with 42 subjects received study drug and 43 subjects as placebo control had a power of 81%. Continuous data were expressed as mean ± SD, and discrete data were given as counts and percentages. The primary end-point was RILI during or after the course of treatment which was assessed by Mann–Whitney *U* test. Pearson Chi-Square test or Fisher's exact test was used to compare categorical variables. Independent-samples *T*-test or one-way ANOVA was used for quantitative variables, as appropriate. Objective tumour response and adverse events were analysed with Mann–Whitney *U* test. Pulmonary function was analysed using repeated-measures analysis of variance, and reduction from baseline pulmonary function was analysed using Independent-samples *T*-test. Kaplan–Meier method was used to estimate the overall survival. A *P*-value of 0.05 or less was considered to indicate the statistical significance. All computations were carried out using an SPSS software package (version 13.0; SPSS Inc.).

3. Results

3.1. Patient characteristics

From July 2004 to July 2006, 90 consecutive patients were enroled in this prospective study from radiation oncology department of Qilu Hospital. Of the 90 patients enroled, three patients in the trial group and two patients in the control group who died within six months after radiation therapy were excluded from this study. Two patients died of complications from metastatic disease, one patient died of complications from metastatic disease with RILI, and two patients died of heart disease. As a result, a total of 85 patients (42 in the trial group and 43 in the control group) completed the study. The main patient characteristics are summarised in Table 1. The arms of the study were well balanced with respect to age, gender, performance status, disease stage, histology, lung function, gross tumour volume, smoke history, chemotherapy and percentage of irradiated lung volume.

3.2. Changes of sICAM-1

The serum sICAM-1 levels at baseline, during and after treatment are shown in Fig. 1. No difference at baseline was found between the two groups, whereas during treatment, the levels of sICAM-1 were increasing in both groups and all reached a peak at 12 weeks. The levels of sICAM-1 at 3, 6 and 12 weeks in the control group were all significantly higher than that at baseline ($P < 0.05$ or 0.01, respectively), whilst only the levels of sICAM-1 at 6 and 12 weeks in the trial group were

Table 1 – Patient characteristics in both groups

Variables	Control group ($n = 43$)	Trial group ($n = 42$)	P-value
Median age (range)	63 (52–74)	65.5 (50–75)	0.65
Gender (male/female)	28/15	32/10	0.26
Stage (IIIA/IIIB)	20/23	23/19	0.45
Histology (SSC/AC)	24/19	30/12	0.14
KPS	83.95 ± 9.29	85.95 ± 9.90	0.34
DLCO (%)	85.20 ± 10.92	84.46 ± 11.64	0.83
FEV1 (%)	84.76 ± 9.52	85.12 ± 12.20	0.88
FVC (%)	89.32 ± 6.76	91.00 ± 7.32	0.27
GTV (cm^3)	249.31 ± 59.88	245.59 ± 70.71	0.79
V20 (%)	31.10 ± 15.32	33.90 ± 17.81	0.44
V30 (%)	27.79 ± 12.51	30.07 ± 14.57	0.44
CHE (yes/no)	28/15	31/11	0.38
Smoke			
Post (yes/no)	35/6	30/8	0.46
Present (yes/no)	29/14	22/20	0.168

KPS = Karnofsky performance status; TLC = Total lung capacity; DLCO = Diffusion capacity for carbon monoxide; FEV1 = Forced expiratory volume at 1 s; FVC = Forced vital capacity; GTV = Gross tumour volume; V20 = Percentage of irradiated lung volume exceeding 20 Gy; V30 = Percentage of irradiated lung volume exceeding 30 Gy; CHE = chemotherapy.

significantly higher (both $P < 0.01$). The sICAM-1 levels in the trial group were significantly lower than that in the control group at week 6 and 12 (373.64 ± 89.33 *versus* 459.53 ± 123.59 and 447.83 ± 111.21 *versus* 513.91 ± 150.46, both $P < 0.01$).

3.3. *Changes of TGF-β1*

Fig. 2 shows the absolute TGF-β1 levels at baseline, during and after the treatment. The plasma TGF-β1 levels in both groups increased during treatment and reached a peak at 6 weeks. The levels of TGF-β1 in the trial group were significantly lower than that in the control group at week 3 and week 6 (5.43 ± 1.47 *versus* 6.22 ± 1.78 and 5.93 ± 2.39 *versus* 7.67 ± 2.74, $P < 0.05$ and 0.01, respectively). Compared with the baseline, TGF-β1 levels in the control group were significantly higher at week 3, 6 and 12 (all $P < 0.01$). While in the trial group, only the levels of TGF-β1 at week 3 and 6 were significantly higher ($P < 0.05$ or 0.01, respectively).

3.4. *Changes of PF*

We initially used the multivariate analysis of variance repeated measure model to compare the treatment effect. We found that the treatment by berberine achieved statistical sig-

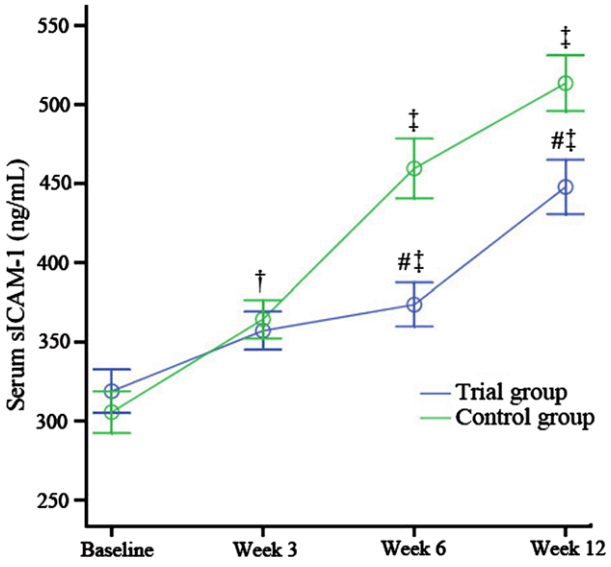

Fig. 1 – Change of sICAM-1 during and after the treatment. Variations of soluble intercellular adhesion molecular-1 (sICAM-1) at baseline, during and after treatment in both groups. Values are presented as mean ± S.E. ‡$P < 0.01$ *versus* the baseline; †$P < 0.05$ *versus* the baseline; #$P < 0.01$ *versus* the control group.

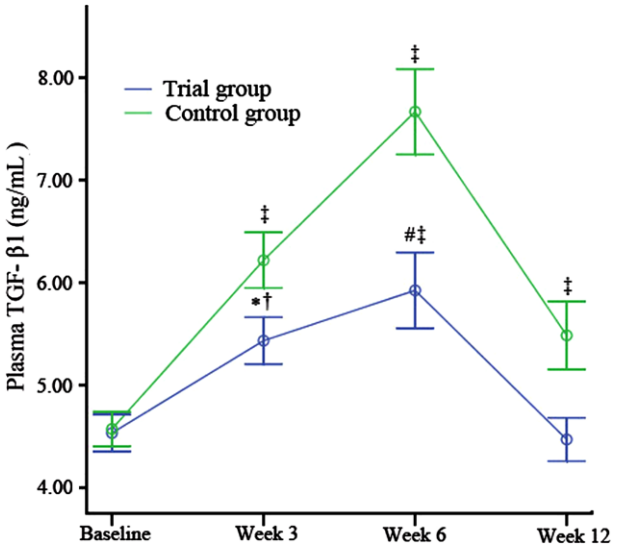

Fig. 2 – Change of TGF-β1 during and after the treatment. Variations of transforming growth factor-beta-1 (TGF-β1) at baseline, during and after treatment in both groups. Values are presented as mean ± S.E. ‡$P < 0.01$ *versus* the baseline; †$P < 0.05$ *versus* the baseline; #$P < 0.01$ *versus* the control group; *$P < 0.05$ *versus* the control group.

nificance in FEV1 (Wilks' Lambda = 0.920, $P = 0.033$) and DLCO (Wilks' Lambda = 0.868, $P = 0.003$), whereas obtained a marginal significance in FVC (Wilks' Lambda = 0.931, $P = 0.053$).

We then further compared the reductions in FVC, FEV1 and DLCO from the baseline values between treatment and placebo group (Table 2). PF variables decreased from baseline in both groups at week 6 and month 6. At week 6, reductions from baseline FVC were smaller in the trial group than that in the control group ($P < 0.05$). At month 6, reductions from baseline FEV1 and DLCO in the trial group were more pronounced than the values recorded at week 6, but were still significantly smaller than that in the control group ($P < 0.05$ and 0.01, respectively), suggesting that berberine protected against the decrease in PF variables. Statistical differences in other comparisons were not observed ($P > 0.05$).

Table 2 – Reduction from baseline pulmonary function (PF) tests at 6 weeks and 6 months after treatment

PF variables	Control group ($n = 43$)	Trial group ($n = 42$)	P-value
FVC			
6 weeks	−3.53 ± 5.47	−1.16 ± 2.98	0.015
6 months	−4.66 ± 6.23	−3.83 ± 4.56	0.486
FEV1			
6 weeks	−3.42 ± 8.13	−0.73 ± 6.14	0.090
6 months	−5.65 ± 7.91	−1.50 ± 6.96	0.012
DLCO			
6 weeks	−5.45 ± 9.55	−2.14 ± 9.80	0.118
6 months	−9.05 ± 9.97	−2.86 ± 8.70	0.003

Values represent mean ± SD.
FVC = Forced vital capacity; FEV1 = Forced expiratory volume at 1 s; DLCO = diffusion capacity for carbon monoxide.

Table 3 – The grades of radiation-induced lung injury in both groups

Variables	Control group ($n = 43$)	Trial group ($n = 42$)	P
6 weeks			<0.05
Grade 1	18 (41.9%)	12 (28.6%)	
Grade 2	9 (20.9%)	6 (14.3%)	
Grade 3	4 (9.3%)	1 (2.4%)	
6 months			<0.05
Grade 1	17 (39.5%)	10 (23.8%)	
Grade 2	8 (18.6%)	5 (11.9%)	
Grade 3	3 (7.0%)	0 (0.0%)	

3.5. *The incidence of RILI*

As shown in Table 3, 19/42 (45.2%) patients in the trial group and 31/43 (72.1%) patients in the control group at 6 weeks occurred grade ⩾1 RILI. At 6 months, 15/42 (35.7%) patients in the trial group and 28/43 (65.1%) patients in the control group experienced grade ⩾1 RILI. No grade 4 RILI was found in both groups. The gross incidence of RILI in the trial group was significant lower than that in the control group at 6 weeks and 6 months (both $P < 0.05$).

3.6. *Objective tumour response and survival*

The median duration of follow-up was 21 months. The median survival was 22.1 (95% CI, 17.4–25.8) months in the trial group and 19.5 (95% CI, 15.3–23.5) months in the control group. The overall response rates (complete response and partial response) were 61.9% (95% CI, 46–76%) in the trial group and 58.2% (95% CI, 42–73%) in the control group, respectively (Table 4). Survival curves of each group according to the Kaplan–Meier method are shown in Fig. 3. No differences of survival and tumour response rate were found between the two groups.

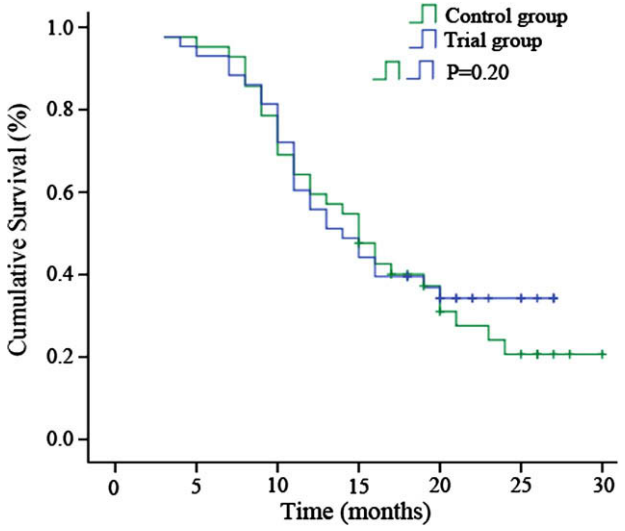

Fig. 3 – Survival analyses. For the trial group and control group patients, 1-year survival rates were 59.5% (95% CI, 43–74%) and 55.8% (95% CI, 40–71%), respectively. No significant difference was found.

Table 4 – Objective tumour response in both groups

Group	n	CR (%)	PR (%)	SD (%)	PG	P
Control group	43	6 (14.0)	19 (44.2)	12 (27.9)	6 (14.0)	0.93
Trial group	42	7 (16.7)	19 (45.2)	12 (28.6)	4 (0.95)	

CR: Complete response; PR: Partial response; SD: Stable disease; PG: Progression.

Table 5 – Adverse events in both groups

Variables	Control group (n = 43)			Trial group (n = 42)		
	Grade 1	Grade 2	Grade 3	Grade 1	Grade 2	Grade 3
RE	18	9	1	17	7	1
LE	12	8	2	10	6	1
N/V	16	9	2	14	5	2
DI	6	4	0	8	3	0

RE = radiation esophagitis; LE = Leukopenia; N/V = Nausea/Vomitting; DI = diarrhoea.

3.7. *Adverse Events*

Adverse events are listed in Table 5. Oesophagitis was the most usual toxicities noted in the study, but no treatment delay or death was observed throughout this trial. Oesophagitis was not statistically different between the two groups. Grade 3 esophagitis occurred in 1 patient (2.4%) in the trial group and in 1 patient (2.3%) in the control group. Other adverse events were similar in both treatment arms.

4. Discussion

Radiation is one of the main therapies for lung cancer and other malignancies, but RILI is a limiting factor for therapy and is sometimes life-threatening. Therefore, finding drugs with the ability to protect normal tissue against radiation with minimal tumour protection is very important. In this study, we tested the radioprotective effect of berberine on RILI in patients with lung cancer and demonstrated that it can reduce the incidence of RILI, improve PF and decrease the level of sICAM-1 and TGF-β1. Berberine had no apparent effect on survival, and no obvious adverse events were found. These results suggested for the first time that berberine may provide a new way for the treatment of RILI.

In relation to the pathogenesis of RILI, previous studies consistently demonstrated a temporal correlation between the increases in extracellular matrix, sICAM-1, TGF-β and the development of RILI.[22] In addition, evidence supports the assumption that the chronic inflammation stimulates fibrosis development through the release of cytokines, growth factors and chemokines and their associated receptors.[4,6] Among these cytokines, sICAM-1 and TGF-β1 are the most valuable factors, because they take part in the pathogenesis of RILI and can be useful in predicting the outcomes of patients with RILI. Plasma TGF-β1 level is the key factor associated with the risk of RILI,[23–27] therefore, it has been used as a sensitive plasma marker of RILI after thoracic irradiation. Animal studies have shown that anti-TGF-β antibodies can attenuate RILI by decreasing the level of TGF-β1 during thoracic irradiation.[28,29] ICAM-1, a member of the immunoglobulin superfamily of adhesion molecules, is expressed on various cell surfaces.[30] ICAM-1 is a ligand for the lymphocyte function-associated antigen-1 (LFA-1), which is found on lymphocytes and other leukocytes.[31] The ICAM-1/LFA-1 interaction is important in T-lymphocyte activation and lymphocyte migration into inflammatory sites.[32] Recently, it has been reported that radiation induces the expression of ICAM-1 on lung cells and that ICAM-1 plays an important role in the pathogenesis of radiation-induced inflammation.[9,10] Measurement of the soluble ICAM-1 (sICAM-1) has been reported to be useful in evaluating and monitoring disease of RILI.

Previous studies have reported that amifostine and intensity-modulated radiotherapy technique can reduce the severity and incidence of acute pulmonary toxicity.[33,34] But there is no report about the traditional herb for RILI. Berberine has been used as a traditional Chinese medicine since ancient times and today it is still present in various herbal preparations. Berberine was mainly used to anti-inflammation in clinic.[35] Recent research demonstrated that berberine can serve to anti-inflammation through suppressing the synthesis of some inflammation cytokines, inhibiting chemotaxis and phagocytic activity of neutrophils and macrophage migration and phagocytosis.[15,18] Experimental evidence showed that berberine not only significantly antagonised the effect of TGF-β1,[17] but also decreased productions of urokinase-plasminogen activator and matrix metalloproteinase-2 which play a central role in the synthesis of extracellular material.[36] Moreover, berberine not only has significant therapeutic effects on liver fibrosis through decreasing regulation of the anti-oxidant system and lipid peroxidation,[17] but also enhanced the tumour-killing effect of radiation.[37] In this study, berberine was administered daily throughout the treatment and the results demonstrated that it could decrease the levels of sICAM-1 and plasma TGF-β1 in patients treated with 3D-CRT. Experimental research also demonstrated that berberine can block the translocation of NF-κB which is a critical signal molecule for the inflammatory process.[38] As for the effects of berberine on the survival and tumour response, there is no difference in tumour response and survival, which suggest that changes in TGF-β1 and sICAM-1 only reflect lung injury. These results suggested that the decrease of TGF-β1 and sICAM-1 probably play an important role in the therapeutic effect of berberine on RILI .

5. Conclusion

RILI is a common complication associated with radiation that can result in significant morbidity. In this study, berberine significantly reduced the incidence of RILI, improved PF and decreased the level of TGF-β1 and sICAM-1. Better results may be obtained with a longer period of treatment. The herbs are available, well tolerated and inexpensive, and we believe that it constitutes an effective treatment. This trial might provide a new way for the drug treatment of RILI. Nevertheless, the precise mechanism of the drug deserves additional atten-

tion. Therefore, further larger randomised trials are therefore necessary to confirm the anti-inflammation and antifibrotic effects of berberine.

Conflict of interest statement

None declared.

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