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Research Paper

Protection afforded by a herbal medicine, Sho-seiryu-to (TJ-19), against oleic acid-induced acute lung injury in guinea-pigs

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Abstract

Objectives The effect of a herbal medicine, Sho-seiryu-to (TJ-19), on oleic acid-induced lung injury, an animal model of acute respiratory distress syndrome or acute lung injury (ARDS/ALI), was examined.

Methods Acute lung injury was induced by an intravenous injection of 15 μl/kg oleic acid to guinea-pigs. TJ-19 was administered by a single oral dose (3 g/kg) or by multiple oral doses (0.75 g/kg).

Key findings The decrease in partial oxygen pressure of arterial blood (Pao_2) and the increase in airway vascular permeability induced by the oleic acid injection were attenuated by a single dose of TJ-19. When TJ-19 was administered orally twice a day for two weeks and then oleic acid was injected, a potent prophylactic effect of the drug was observed. TJ-19 also prevented airway vascular hyperpermeability, lung cell injury, oxidative stress and thromboxane A_2 generation, associated with the oleic acid injection.

Conclusions TJ-19 significantly attenuated the oleic acid-induced lung injury probably through the antioxidative effect and inhibitory effect of thromboxane A_2 generation, although the precise inhibitory mechanisms were not fully elucidated due to the diversity in constituents of the herbal medicine. We suggest that TJ-19 is a promising drug candidate and a medicinal resource for preventing ARDS/ALI.

Keywords ARDS; lung injury; reactive oxygen species; thromboxane A_2; TJ-19

Introduction

Acute respiratory distress syndrome or acute lung injury (ARDS/ALI) is among the most severe forms of lung injury with serious hypoxaemia, which is seen in patients with sepsis, severe trauma, fat embolism and so on.[1,2]

In ARDS/ALI, hypoxaemia and non-cardiogenic pulmonary oedema develop as a result of vascular hyperpermeability induced by inflammatory reactions.[1] Some inflammatory mediators, such as cytokines, reactive oxygen species (ROS) and eicosanoids, released from inflammatory cells such as neutrophils, are known to be involved in the development of ARDS/ALI. Based on the pathophysiological features of ARDS/ALI, some anti-inflammatory drugs, such as antioxidants, inhibitors of neutrophil activation (e.g. ketoconazole) and non-steroidal anti-inflammatory drugs (NSAIDs), have been tested in clinical trials for the treatment of ARDS/ALI.[3] However, these anti-inflammatory drugs, even corticosteroids, are not effective against ARDS/ALI. Despite recent advances in intensive care, the mortality rate of ARDS/ALI is still high (approx. 30–40%),[4–6] because there are few drugs available for its treatment. Therefore, it is critical to develop a new drug effective against ARDS/ALI.

Since the inflammation of ARDS/ALI arises from multiple mediators, a drug that can simultaneously suppress plural inflammatory mediators would be favourable for the treatment or prevention of ARDS/ALI. Herbal medicines (Chinese/Japanese traditional medicines, used in south-east Asia) consist of pharmacologically active diverse ingredients,

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which can cooperatively exhibit favourable actions. As the use of natural products increases in western countries, they attract the attention of clinicians and researchers.

A herbal medicine, Sho-seiryu-to (Chinese name: Xiao-qing-long-tang) has a potential antioxidative effect,[7] and inhibits prostanoid synthesis,[8] neutrophil activation,[9] histamine release[10] and platelet activating factor (PAF) generation.[11] Based on these actions, Sho-seiryu-to has been used in Japan for the treatment of pulmonary diseases, such as asthma, bronchitis and allergic diseases.[12–14] In addition, some basic and clinical studies have been conducted to confirm the usefulness of Sho-seiryu-to.[15–17] However, little has been reported on the effects of Sho-seiryu-to against ARDS/ALI.

In animals, an intravenous injection of oleic acid, an endogenous unsaturated fatty acid, can produce lung injury with hypoxaemia and pulmonary vascular hyperpermeability through an inflammatory reaction. Since this oleic acid-induced acute lung injury shares pathophysiological features with ARDS/ALI, it provides a well-characterized animal model of these diseases.[18,19] We have developed a system of acute lung injury induced by oleic acid in guinea-pigs to screen drugs against ARDS/ALI.[20–22] Using this screening system, we have found some candidate drugs effective against ARDS/ALI and clarified some of the mechanisms of oleic acid-induced lung injury.[23–25]

Building upon our earlier studies, this study was conducted to examine whether Sho-seiryu-to could be a drug candidate against ARDS/ALI. We used TJ-19 (Sho-seiryu-to extract granules for ethical use), which is an extract from a mixture of eight herbal components, Pinellia Tuber, Glycyrrhiza Root, Cinnamon Bark, Schisandra Fruit, Asiasarum Root, Paeony Root, Ephedra Herb and Ginger Rhizome. We examined the effects of TJ-19 on the decrease in partial oxygen pressure of arterial blood (Pao_2) and pulmonary vascular hyperpermeability in oleic acid-induced lung injury in guinea-pigs. Furthermore, we examined the effects of TJ-19 on lung cell damage, oxidative stress and thromboxane A_2 (TXA_2) generation, which were assessed by the analysis of bronchoalveolar lavage fluid (BALF) in guinea-pigs injected with oleic acid.

Materials and Methods

Materials

TJ-19 was purchased from Tsumura & Co. (Tokyo, Japan). The lipid peroxidation test kit was purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). GSH/GSSG assay kit was purchased from Oxis International, Inc. (Portland, USA). Thromboxane B_2 (TXB_2) enzyme immunoassay kit was purchased from Cayman Chemical Co. (Ann Arbor, USA). Pentobarbital sodium (Nembutal injection) was purchased from Dainabott Co. (Osaka, Japan). Evans blue, formamide, ethylene-diaminetetraacetic acid disodium (EDTA), indometacin and procaine were purchased from Sigma Chemical Co. (St Louis, USA). Other reagents and solvents were of reagent grade. De-ionized and distilled water was used throughout the study.

Three-dimensional HPLC analysis

To analyse the chemical constituents of TJ-19, three-dimensional HPLC was performed according to a method reported previously.[15] A granule of TJ-19 (1.0 g) was extracted with methanol (20 ml) under ultrasonication for 30 min, and was centrifuged at 3000 rev/min for 5 min. The supernatant was filtered with a membrane filter (0.45 μm) and then submitted for HPLC analysis (30 μl). HPLC apparatus consisted of an Agilent 1200 Series (Agilent Technologies, Palo Alto, USA) equipped with a multiple wavelength detector (UV 200–400 nm). HPLC conditions were as follows: column, ODS (TSK-GEL 80TS, 250 × 4.6 mm i.d.; Tosoh, Tokyo, Japan); eluant, (A) 0.05 M ammonium acetate (pH 3.6), (B) 100% acetonitrile. A linear gradient of 90% A and 10% B changing over 60 min to 0% A and 100% B was used (and 100% B was continued for 20 min); temperature, 40°C; flow rate, 1.0 ml/min.

Animal care and handling

This study was approved by the Animal Care and Use Committee of Kumamoto University. The care and handling of the animals were performed in accordance with National Institutes of Health guidelines for the care and handling of animals.

Animal operation and oleic acid induced lung injury

Hartley guinea-pigs (male, 561 ± 42 g) were anaesthetized with pentobarbital sodium (25 mg/kg, i.p.) and procaine was used for local anaesthesia. Catheters (1.1 mm o.d.) were inserted into the subclavian vein and artery for the injection of reagents and for blood sampling, respectively. To induce acute lung injury, oleic acid was injected at a bolus dose of 15 μl/kg using a micro syringe (Hamilton Co., Reno, USA) through the catheter inserted into the subclavian vein. Promptly, 1 ml/kg saline was used to push out the oleic acid from the catheter into the vein. All operations and measurements mentioned below were performed as described previously.[20–25]

Measurement of blood gases

To examine the effect of TJ-19 on oleic acid-induced hypoxaemia, we measured arterial blood gas parameters (Pao_2, $Paco_2$ and pH). We chose the doses of TJ-19 on the basis of previous reports[14,15,26,27] and small scale preliminary experiments. Briefly, 24 guinea-pigs were divided into the following four groups ($n = 6$ for each group) for the single oral dose experiment (groups 1 and 2) and the multiple oral dose experiment (groups 3 and 4). In the single oral dose experiment, 3 h before the oleic acid injection, groups 1 and 2 received saline (oral, 10 ml per kg body weight) and TJ-19 (oral, 3 g TJ-19 in 10 ml saline per kg body weight), respectively. In the multiple oral doses experiment, groups 3 and 4 received saline (oral, 2.5 ml per kg body weight) and TJ-19 (oral, 0.75 g TJ-19 in 2.5 ml saline per kg body weight) twice a day (at 8:00 h and 20:00 h) for two weeks, respectively. Oleic acid was injected 3 h after the last dose in groups 3 and 4. Arterial blood (200 μl) was collected 5, 10 and 15 min before, and 6, 10, 15, 35, 55 and 75 min after

oleic acid injection and analysed with a blood gas analyser (ABL 300; Radiometer Ltd, Copenhagen, Denmark). The mean value of the blood gas parameters before the oleic acid injection was defined as the initial value.

Determination of vascular permeability

To examine the effect of TJ-19 on oleic acid-induced pulmonary vascular hyperpermeability, we measured extravasation of Evans blue dye. Oleic acid was injected 3 h after the dose in groups 1 and 2 or 3 h after the last dose in groups 3 and 4, and Evans blue (30 mg/kg) was intravenously administered 1 min before the oleic acid injection. Ninety minutes after the oleic acid injection, the chest cavity was opened. Evans blue in the intravascular space was washed out by perfusing with saline. This procedure was accomplished by inserting a 13-gauge blunt cannula through the right ventricle into the pulmonary artery, and perfusate outflow came out from the dissected left atrium. The lungs were perfused with 100 ml of saline at a rate of 3 ml/min using a pump (EYELA Micro Tube Pump MP-3; Rikakikai Co., Tokyo, Japan). Evans blue could not be detected in any fraction of the perfusate. After the perfusion, the airways and lungs were removed and weighed. The airways were separated into the trachea, main bronchus and intrapulmonary bronchus. The intrapulmonary bronchus was further cut in two in the middle and divided into the proximal bronchus and the distal bronchus. Evans blue dye was extracted from each tissue fraction with 2 ml of formamide at 37°C for 18 h, and the amount of the dye was determined spectrophotometrically at 620 nm with a spectrophotometer (U 3200; Hitachi, Tokyo, Japan). The amount of Evans blue extracted from the tissue was expressed as ng per mg wet weight tissue.

Determination of the markers of lung cell damage, oxidative stress and thromboxane A_2 generation in BALF

The effects of TJ-19 on oleic acid-induced lung cell damage, oxidative stress and TXA_2 generation were examined in the multiple oral dose experiments (groups 3 and 4), assessed by changes in markers observed in BALF. To perform bronchoalveolar lavage, the guinea-pigs received a further 50 mg/kg pentobarbital sodium intraperitoneally 10 min before the lavage. Ninety minutes after the oleic acid injection, 10 ml cold saline cooled by ice was injected and withdrawn slowly through the trachea twice and BALF was recovered (recovery ratio was 90% or greater). For TXA_2 determination, 0.7 mM EDTA in ice-cooled saline and indometacin (final concentration: 10 μg/ml) were added to the BALF to inhibit further metabolism of arachidonic acid to thromboxanes. The BALF was centrifuged at 400*g* for 10 min at 4°C and then the supernatant was divided into portions. These were stored at –80°C until assay. The cell damage in the lungs was assessed by the concentration of total proteins and the activity of lactate dehydrogenase (LDH) leaked into BALF from the cells, analysed by a bioanalyser (Hitachi 7600). Oxidative stress was assessed by the extent of lipid peroxidation, estimated as the concentration of thiobarbituric acid reactive products (TBARS) and glutathione/oxidized glutathione (GSH/SGGS) concentration ratio, using a lipid peroxidation test kit and GSH/GSSG assay kit. The concentration of TXB_2, a stable metabolite of TXA_2, was analysed using the enzyme immunoassay kit.

Statistical analysis

Results were expressed as mean ± SEM. Multiple comparisons were made to examine the statistical significance of the data. When uniform variance of data was identified by Bartlett's analysis ($P < 0.05$), one-way analysis of variance or repeated measures analysis of variance was used to test for statistical differences. When significant differences ($P < 0.05$) were identified, the data were further analysed by Tukey's or Dunnett's multiple range test for significant differences among the values. If uniform variance of data was not identified, non-parametric multiple comparisons were made. After confirming significant differences ($P < 0.05$) by using Kruskal–Wallis analysis, the differences were examined by applying Dunns' test.

Results

Three-dimensional HPLC analysis of TJ-19

The HPLC profile of TJ-19 extract is shown in Figure 1. At least 20 constituents, such as liquiritin and glycyrrhizin (originating from Glycyrrhizae Radix), paeoniflorin and albiflorin (Paeoniae Radix), schizandrin (Schizandrae Fructus), cinnamic aldehyde and cinnamic acid (Cinnamomi Cortex) and 6-gingerol (Ginger Rhizome) were identified by this analysis.

Changes in blood gases

An intravenous injection of oleic acid at a dose of 15 μl/kg to guinea-pigs caused a significant decrease in Pao_2. The maximum decrease in Pao_2, approximately 50% of the initial value, was observed 10 min after the oleic acid injection. Seventy-five minutes after the oleic acid injection, Pao_2 recovered to near 80–90% of the initial value (Figure 2a, b).

When 3 g/kg TJ-19 was administered orally 3 h before the oleic acid injection, it prevented the decrease in Pao_2 induced by oleic acid (Figure 2a). When 0.75 g/kg TJ-19 was administered orally twice a day for two weeks and then oleic acid was injected intravenously 3 h after the last oral dose of TJ-19, a potent prophylactic effect of the drug was observed (Figure 2b). No significant change in $Paco_2$ and pH was observed between the control and TJ-19 treated groups (Figure 2a, b).

Changes in airway vascular permeability

As indicated by the extravasation of Evans blue given intravenously, the oleic acid-induced increase in vascular permeability in the trachea, proximal bronchus and distal bronchus of the guinea-pigs was significantly attenuated by multiple oral doses of TJ-19 (Figure 3b). In addition, distinctive patches of intense haemorrhage with Evans blue on the surface of the lungs were observed after oleic acid was injected intravenously. On the other hand, there were few patches of haemorrhage in the group administered oleic acid with multiple oral doses of TJ-19 (data not shown). Although the single oral administration of TJ-19 tended to prevent the

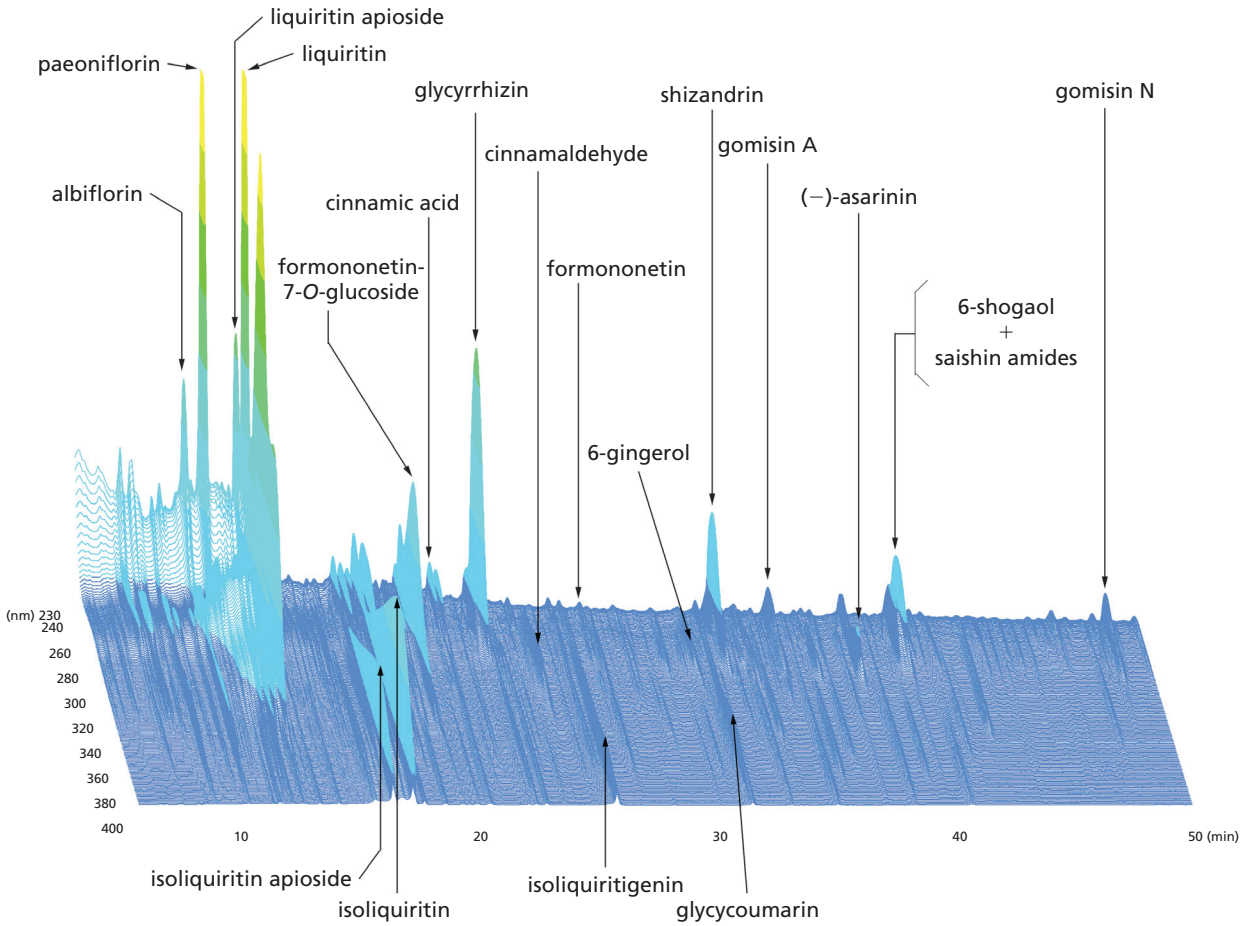

Figure 1 Chemical profile of TJ-19 analysed by three-dimensional HPLC

extravasation of the dye, statistical significance was not observed (Figure 3a).

Changes in parameters of lung cell damage, oxidative stresses and thromboxane A_2 generation

As indicated by the increase in LDH activity and total protein concentration in BALF, the oleic acid injection induced cell damage in guinea-pig lungs. The multiple oral doses of TJ-19 significantly inhibited the oleic acid-induced increase in LDH activity in BALF (Figure 4a), and tended to ameliorate the increase in total protein concentration in BALF (Figure 4b). The multiple oral doses of TJ-19 markedly suppressed the oleic acid-induced oxidative stress, as indicated by changes in TBARS concentration (Figure 5a) and GSH/GSSG ratio in BALF (Figure 5b). In addition, the concentration of TXB_2 in BALF was increased by the oleic acid injection and such an increase was significantly diminished by TJ-19 (Figure 6).

Discussion

Hypoxaemia is a critical state in patients with ARDS/ALI, the state of which eventually results in brain damage and death.[28] In this study, we demonstrated that a single oral administration of TJ-19 markedly ameliorated the decrease in Pao_2 induced with oleic acid in guinea-pigs. In comparison with the results in which TJ-19 intended for prophylactic use was administered orally twice a day for 2 weeks, the efficacy of multiple oral doses of TJ-19 was much greater than that of the single oral dose.

The multiple oral administration of TJ-19 suppressed the pulmonary vascular hyperpermeability and cell damage induced by oleic acid, which were measured by increases in the extravasation of Evans blue and the LDH activity and total protein concentration in BALF. Our previous studies have demonstrated that the decrease in Pao_2 was closely associated with pulmonary vascular hyperpermeability and lung cell injury.[20,21] Therefore, the prominent effect of TJ-19 against hypoxaemia, at least in part, may be through the inhibition of vascular hyperpermeability in airways and lung cell injury, events of which would be associated interdependently.

In this study, the multiple oral administration of TJ-19 prevented the decrease in the GSH/ GSSG ratio in BALF and inhibited the increase in TBARS in BALF induced by oleic acid. This indicates that TJ-19 inhibits oxidative stress in oleic acid-induced lung injury. Some constitutive herbs of TJ-19, such as Glycyrrhiza and Schisandra fruit, have been reported to possess superoxide scavenging activity.[7] Moreover, 3D-HPLC data indicate that TJ-19 contains the radical

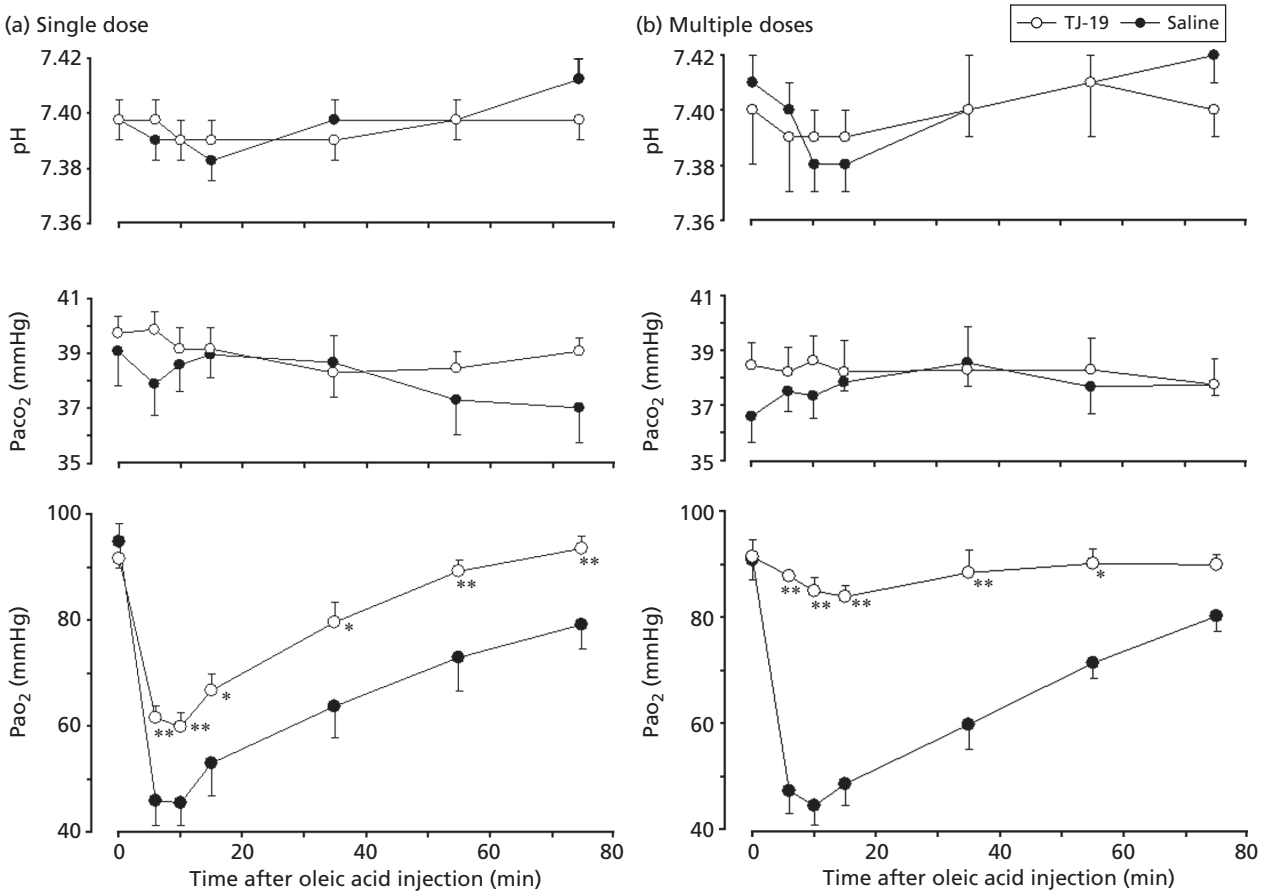

Figure 2 Preventive effects of orally administered TJ-19 on the decrease in Pao_2 induced by oleic acid. Each point represents the mean ± SEM of data from 6 guinea-pigs. (a) Single oral dose experiment: TJ-19 (3 g TJ-19 in 10 ml saline per kg body weight) or saline (10 ml/kg) was orally administered to guinea-pigs 3 h before the oleic acid injection. (b) Multiple oral dose experiment: TJ-19 (0.75 g TJ-19 in 2.5 ml saline per kg body weight) or saline (2.5 ml/kg) was orally administered to guinea-pigs twice a day for 14 consecutive days before the oleic acid injection. TJ-19 significantly reduced the decrease in Pao_2 induced by oleic acid, and the effect of the multiple oral doses of TJ-19 was much greater than that of the single oral dose. In all groups, compared with the value at 0 min, no significant changes in pH and $Paco_2$ were observed. $^*P < 0.05$, $^{**}P < 0.01$ compared with the saline group.

scavenger, 6-gingerol.[29,30] This scavenging action of TJ-19 may partially contribute to the inhibition of oleic acid-induced pulmonary vascular hyperpermeability.

Schuster[31] reported that TXA_2 plays an important role in oleic acid-induced lung injury through platelet aggregation, increasing both airway and pulmonary vascular resistance, inducing pulmonary hypertension and enhancing oedema formation. Our recent studies have shown that TXA_2 participated in oleic acid-induced lung injury as an early phase mediator, and rapidly-acting TXA_2 synthase inhibitors were effective in the prevention of acute lung injury.[24] In this study, we showed that TJ-19 inhibited the production of TXA_2 induced by oleic acid. It is likely that glycyrrhizin, a main active ingredient of TJ-19, inhibits the production of TXA_2 probably through inhibiting the activity of phospholipase A_2.[32]

Other inflammatory mediators may have relevance to the preventive effect of TJ-19. Histamine[33] and PAF[34,35] are likely to play an important part in the development of oleic acid-induced lung injury as well as ARDS/ALI. Previous studies have concluded that TJ-19 had the potential to inhibit the release or production of histamine[10] and PAF.[11,32] In addition, a recent in-vitro study[36] indicated that glycyrrhizin, a constituent of TJ-19, suppresses interleukin-8 production and nuclear factor-kappa B activity, important factors in the development of ARDS/ALI.[1] Liquiritin derivatives, paeoniflorin and gomisins, which have been detected in TJ-19 by 3D-HPLC, have anti-inflammatory effects, such as inhibition of cytokine release, anti-apoptotic effect and so on.[37–40] These effects of TJ-19 may also be involved in the attenuation of the lung injury induced by oleic acid, although further studies are needed to clarify the precise mechanism of action of TJ-19.

TJ-19 seems to have potential as a preventive drug if the drug is administered when patients develop the diseases that precede ARDS/ALI, such as fat emboli and trauma. Although the efficacy and safety of TJ-19 for intended use has been established in clinical practice, further clinical experiments

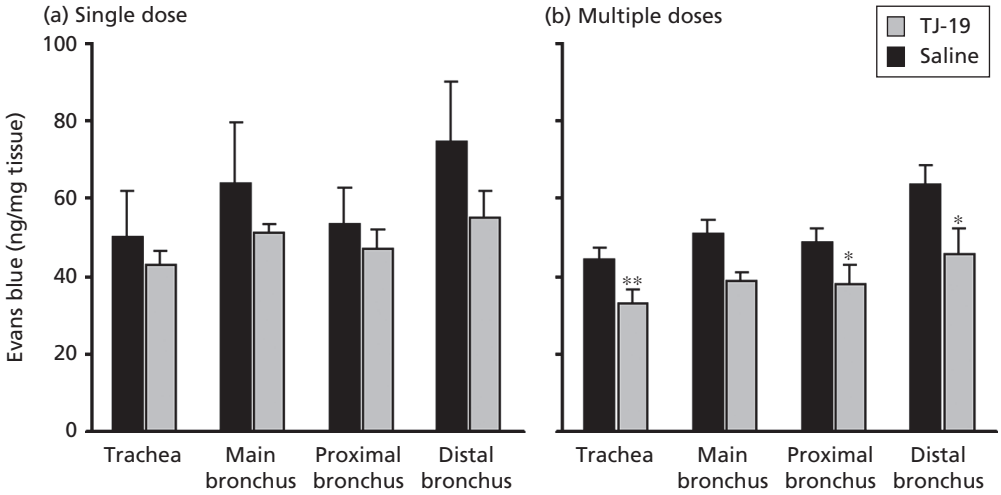

Figure 3 Effect of single or multiple administration of TJ-19 on the increase in airway vascular permeability induced by oleic acid in guinea-pigs. Each bar represents the mean ± SEM of data from 9 or 10 guinea-pigs. (a) Single dose experiment: TJ-19 (3 g TJ-19 in 10 ml saline per kg body weight) or saline (10 ml/kg) was orally administered 3 h before oleic acid injection. (b) Multiple dose experiment: TJ-19 (0.75 g TJ-19 in 2.5 ml saline per kg body weight) or saline (2.5 ml/kg) was orally administered twice a day for 14 consecutive days before the oleic acid injection. The effect of the multiple oral doses of TJ-19 was much greater than that of the single oral dose. $*P < 0.05$, $**P < 0.01$ compared with the saline group.

are needed to establish the efficacy and safety of TJ-19 for critically ill and injured patients.

These limited results indicate that TJ-19 attenuates oleic acid-induced lung injury, probably through the interdependent pharmacological effects of its diverse active ingredients against several inflammatory mediators. One of the limitations of this study is the lack of mechanistic investigation regarding the active components within TJ-19. One issue is the difficulty in the pharmacokinetic/pharmacodynamic assessment of the effects of TJ-19, which is composed of many ingredients. Future studies to examine the effect of each component of TJ-19 in the lung injury model and to measure the concentration of the representative components in the plasma of animals treated with TJ-19 are needed. In addition, our lung injury model was developed with a low dose of oleic acid and, thus, showed relatively mild hypoxaemia and vascular permeability, to avoid overlooking the effectiveness of the candidates resulting from the use of an incurable severe model. Therefore, the system reflects, at least in part, an aspect of clinical ARDS/ALI but the model does not fully emulate clinical ARDS/ALI.

Conclusions

TJ-19 drastically attenuated oleic acid-induced lung injury in guinea-pigs, at least in part through the inhibition of ROS

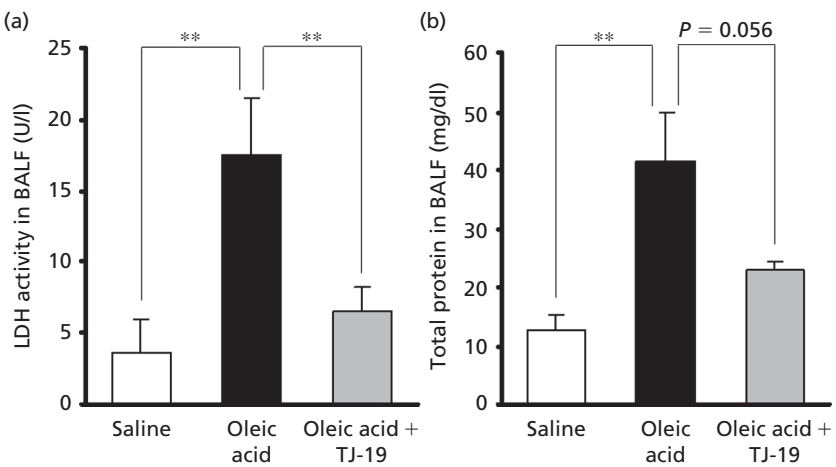

Figure 4 Effect of multiple oral administration of TJ-19 on lactate dehydrogenase activity and total protein concentration in bronchoalveolar lavage fluid in guinea-pigs. TJ-19 (0.75 g TJ-19 in 2.5 ml saline per kg body weight) was administered orally twice a day for 14 days to guinea-pigs. Each bar represents the mean ± SEM of data from 10 guinea-pigs. There was a significant increase in lactate dehydrogenase (LDH) activity (a) and total protein concentration (b) in bronchoalveolar lavage fluid (BALF) in the oleic acid group compared with the saline group. The increase in LDH activity and total protein concentration induced by oleic acid was significantly reduced by multiple administration of TJ-19. $**P < 0.01$.

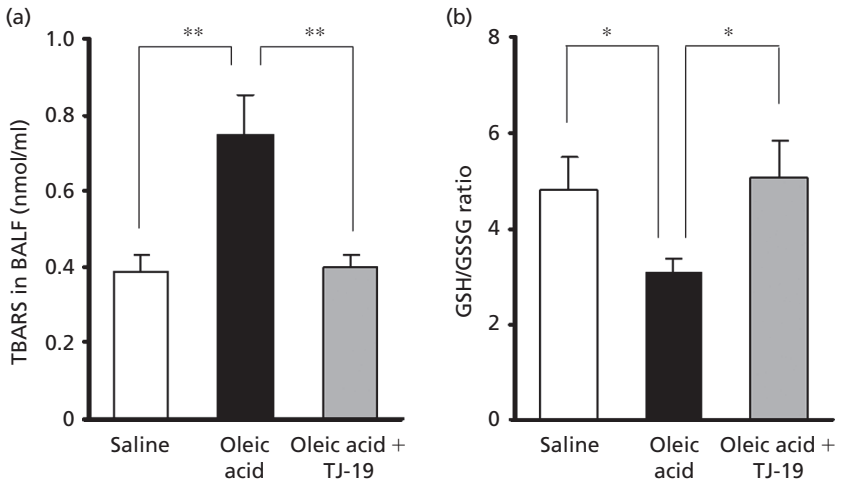

Figure 5 Effect of multiple administration of TJ-19 on the changes in thiobarbituric acid reactive substances and glutathione/oxidized glutathione ratio in bronchoalveolar lavage fluid of guinea-pigs. TJ-19 (0.75 g TJ-19 in 2.5 ml saline per kg body weight) was administered orally twice a day for 14 days to guinea-pigs and the effect on the changes in thiobarbituric acid reactive substances (TBARS) (a) and glutathione/oxidized glutathione (GSH/GSSG) ratio in bronchoalveolar lavage fluid (BALF) was determined. Each bar represents the mean ± SEM of data from 10 guinea-pigs. There was a significant increase in TBARS and decrease in GSH/GSSG ratio in the oleic acid group compared with the saline group. The changes in TBARS and GSH/ GSSG induced by oleic acid were corrected by multiple administration of TJ-19 to near control level. $*P < 0.05$, $**P < 0.01$.

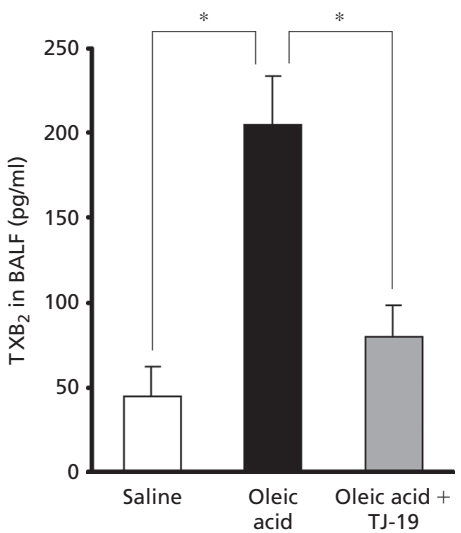

Figure 6 Effect of multiple administration of TJ-19 on the level of thromboxane B_2 in oleic acid-induced lung injury in guinea-pigs. TJ-19 (0.75 g TJ-19 in 2.5 ml saline per kg body weight) was administered orally twice a day for 14 days to guinea-pigs and the effect on the level of thromboxane B_2 (TXB_2) in the oleic acid-induced lung injury was determined. Each bar represents the mean ± SEM of data from 10 guinea-pigs. There was a significant increase of TXB_2 in bronchoalveolar lavage fluid (BALF) in the oleic acid group compared with the saline group. The increase in TXB_2 in BALF induced by oleic acid was significantly reduced by multiple administration of TJ-19. $*P < 0.05$.

and TXA_2, although the precise mechanisms or inhibitory constituents of TJ-19 against the lung injury were not identified in this study. We suggest that the multiple component drugs, such as herbal medicines, can pave the way to 'rounding up' of key mediators involved in the pathogenesis of ARDS/ALI. Our results suggest that a herbal medicine, TJ-19, is a promising candidate and a source for the treatment of hypoxaemia and vascular hyperpermeability in lung injuries that share common mechanisms with oleic acid-induced lung injury (e.g. ARDS/ALI).

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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