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A Chinese Herb Prescription "*Fang-gan* Decoction" Protects Against Damage to Lung and Colon Epithelial Cells Caused by the SARS-CoV-2 Spike Protein by Regulating the TGF-β/Smad2/3 and NF-κB Pathways

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ABSTRACT

Objective To explore the effects and mechanisms of a traditional Chinese medicine (TCM) prescription, "*Fang-gan* Decoction" (FGD), in protecting against SARS-CoV-2 spike protein-induced lung and intestinal injuries *in vitro* and *in vivo*.

Methods Female BALB/c mice and three cell lines pretreated with FGD were stimulated with recombinant SARS-CoV-2 spike protein (spike protein). Hematoxylin-eosin (HE) staining and pathologic scoring of tissues, cell permeability and viability, and angiotensin-converting enzyme 2 (ACE2) expression in the lung and colon were detected. Enzyme-linked immunosorbent assay (ELISA) was performed to detect the levels of inflammatory factors in serum and cell supernatant. The expression of NF-κB p65, p-NF-κB p65, p-IκBα, p-Smad2/3, TGF-β1, Caspase3, and Bcl-2 was evaluated by Western blotting.

Results FGD protected against the damage to the lung and colon caused by the spike protein *in vivo* and *in vitro* according to the pathologic score and cell permeability and viability ($P<0.05$). FGD up-regulated ACE2 expression, which was reduced by the spike protein in the lung and colon, significantly improved the deregulation of inflammatory markers caused by the spike protein, and regulated the activity of TGF-β/Smads and NF-κB signaling.

Conclusion Traditional Chinese medicine has a protective effect on lung and intestinal tissue injury stimulated by the spike protein through possible regulatory functions of the NF-κB and TGF-β1/Smad pathways with tissue type specificity.

Key words: COVID-19; SARS-CoV-2; traditional chinese medicine; prevention; transforming growth factor-β

INTRODUCTION

Coronavirus disease 2019 (COVID-2019), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is featured by rapid spread[1] and high fatality rate[2,3]. Traditional Chinese medicine (TCM) prescriptions have been confirmed to be remarkably effective for treating COVID-19 patients since they were first applied in Wuhan, China[4]. A variety of TCM treatments preventing SARS-CoV-2 have been produced[5,6]. According to reports from China, 90% of Chinese patients with COVID-19 used TCM, and the effectiveness rate was up to 80%[7-9].

A meta-analysis suggests that the improvement in clinical symptoms with the combination of Qingfei Paidu decoction and conventional Western medicine (CWM) was remarkable compared to CWM alone, along with fewer adverse events in patients taking

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the TCM prescription[10]. Among the six TCM recipes for the treatment of viral infections, Jinhua Qinggan granules and Lianhua Qingwen capsules are recommended. Lung Cleansing and Detoxifying Decoction is recommended for treating both severe and non-severe patients, together with Xuanfeibaidu granules, Huashibaidu and Xuebijing. Molecular biological studies have demonstrated that the active ingredients of TCM prescriptions can target angiotensin converting enzyme 2 (ACE2), 3C-like protease ($3CL^{pro}$) and interleukin-6 (IL-6)[11]. However, many mechanisms remain unclear, and further high-quality studies are needed to clarify these findings.

A prescription, named *Fang-gan* Decoction (FGD), consisting of several Chinese herbs was specially created by the Second Affiliated Hospital of Shenzhen University for the unique geographical and climatic environment of Shenzhen, China. More than 20,000 doses of this prescription have been sent to various districts in Shenzhen during the COVID-19 pandemic since January 2020 and are highly appreciated by local residents. However, the specific anti-SARS-CoV-2 effects and mechanisms have not yet been studied. This study aimed to investigate the protective effect of FGD on injury to target tissues, including the lung and intestine, induced by the SARS-CoV-2 spike protein and the related mechanisms.

MATERIALS AND METHODS

Primary materials

Forty female BALB/c mice (6-8 weeks) were obtained from Guangdong Yaokang Biotechnology Co., Ltd. (No. 448247400011736). Eight SPF male SD rats were purchased from Zhuhai Baichaitong Biological Technology Co., Ltd. (No. 44822700004214).

Human type Ⅱ alveolar epithelial cells (hTⅡAECs) were purchased from Guangzhou Taylor Biological Technology Co., Ltd. (HUM-iCell-a002, China). Primary human small intestinal mucosa epithelial cells (HsIMECs) were obtained from Guangzhou Taylor Biological Technology Co., Ltd. (HUM-iCell-d007). The normal colon epithelial cell line HCoEpiC was obtained from Guangzhou Genio Biological Technology Co., Ltd. (JNO-1787). Recombinant SARS-CoV-2 (2019-nCOV) spike RBD-His protein (spike protein) was purchased from Sino Biological, Inc. (0.25 mg/ml, 40592-V08H70-100). Freund's incomplete adjuvant (P2031) was purchased from Guangzhou Jiayan Biotechnology Co., Ltd.. The human interleukin 6 (IL-6) ELISA kit (MM-0049H1), human interleukin 10 (IL-10) ELISA kit (MM-0066H1), human interleukin 13 (IL-13) ELISA kit (MM-0062H1), and human tumour necrosis factor α (TNF-α) ELISA kit (MM-0122H1) were purchased from Wuhan Meimian Biotechnology Co., Ltd., and the human transforming growth factor-β1 (TGF-β1) ELISA kit (ml026482) was purchased from Enzyme-linked Biotechnology Co., Ltd. (Shanghai). The human nuclear factor-κB (NF-κB) ELISA kit was purchased from Cayman (Beijing, 10006912).

The following antibodies were used in these experiments: rabbit anti-extracellular signal-regulated kinase (ERK1/ERK2) (Ab184699), anti-NF-κB p65 (Ab32536), anti-p38 MAPK (Ab170099), anti-p-Smad2/3 (Ab272332), anti-TGF-β1 (Ab215715), anti-p-NF-κB p65 (Ab76302), and anti-p-IκBα (Ab133462), which were purchased from Abcam; and rabbit anti-ACE2, which was purchased from Gene Tex (GTX101395).

The TCM prescription FGD was from the Second Affiliated Hospital of Shenzhen University and consisted of the following selected 12 Chinese herbs: *Astragalus membranaceus* 20 g, fried *Rhizoma atractylodis* 10 g, *Radices sileris* 10 g, *Radix pseudostellariae* 30 g, *Glycyrrhiza* 10 g, *Folia perillae acutae* 10 g, *Agastache rugosus* 10 g, *Artemisia apiacea* 10 g, *Platycodon grandiflorum* 10 g, red dates 20 g, *Bupleurum falcatum* 10 g, and medicated leaven 10 g.

In vivo stimulation of the spike protein and treatment with the TCM prescription

Forty BALB/C mice were fed adaptively for 5 days and randomly divided into a normal group, model group, normal saline (NS) group, and TCM group (10 in each group). The above 12 Chinese herbs were prepared into a decoction and concentrated to 100 ml. The spike protein (3 mg) was emulsified with Freund's incomplete adjuvant. Mice in the NS and TCM groups were pretreated with the above prepared Chinese herb decoction by gavage for 5 days and then stimulated with 0.2 ml of emulsified spike protein (50 μg) through intraperitoneal injection for 3 and 5 days. The model group was stimulated with the same dose of spike protein for 3 and 5 days. The NS group was given the same volume of normal saline as the Chinese herbal decoction for 5 days before recombinant spike protein stimulation. Serum was collected after 3 days of stimulation. After 5 days of stimulation, the mice were sac-

rificed by cervical vertebrae dissection to obtain specimens under anesthesia with 10% chloral hydrate, and serum and tissue specimens, including lung and colon specimens, were also obtained.

In vitro induction of lung and intestinal epithelial cells by the spike protein

hTⅡAEC, HsIMEC and HCoEpiC cells were plated in six-well plates and incubated overnight. S protein (200 ng/ml) was added to the wells, and the cells were cultured for another 48 hours.

Preparation of serum containing Chinese herbs and treatment

The above 12 Chinese herbs were boiled in water and then evaporated and concentrated to approximately 100 ml (TCM solution). Eight male SD rats (weighting 100 g–150 g) were fed adaptively for 5 days and transferred to an SPF feeding room. The rats were grouped into control and TCM groups. The control group was given an equal volume of normal saline. The TCM group was given FGD solution by gavage (2.5 ml/rat) every day for 5 days. After the rats were treated with an intraperitoneal injection of 10% chloral hydrate solution (5 ml/kg), blood was collected to obtain serum through the abdominal aorta, which was named TCM serum. TCM serum was diluted in medium by 10%, 25%, and 50%. All experiments were conducted in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC). The experimental protocol was reviewed and approved by the Laboratory Animal Management and Use Committee of our institution before implementation.

Hematoxylin and eosin (H&E) staining and pathologic score

Lung and colon tissues were fixed with 4% paraformaldehyde. The samples were trimmed, dehydrated, embedded, sliced, and stained, and microscopic examination was performed in strict accordance with the procedures of the pathology experiments. Pathologic changes in the sections were observed under a microscope at different magnifications. Basic pathological features included hyperaemia, congestion, haemorrhage, oedema, degeneration, necrosis, hyperplasia, fibrosis, granulation tissue, and inflammatory changes. Histopathological injury was scored according to the following criteria: 0, normal; 1, very slight, changes are just above the normal range; 2, slight, lesions can be observed, but they are not severe; 3, moderate, the lesion is obvious and probably more severe; and 4, serious, the lesion is very severe (the lesion has taken over the whole tissue or organ).

Enzyme-linked immunosorbent assay (ELISA) of inflammatory markers in mouse sera and cell supernatants

Mouse sera and cell supernatants were added to a 96-well plate. The detection procedure was performed according to the protocol of kits including those for IL-6, IL-10, IL-13, TNF-α, C-reactive protein (CRP), NF-κB, and TGF-β1. Fifty microlitres of sample diluent were added to each well and incubated at 37°C for 30 min. Then, 50 μl of horseradish peroxidase (HRP)-conjugated reagent was added to each well, and 50 μl of chromogenic reagent A and 50 μl of chromogenic reagent B were added to each well for 10 min. The optical density (OD) was measured at 450 nm.

Fluorescence detection of ACE2 expression in the lung and colon tissues

The lung and colon specimens of mice fixed with 10% formaldehyde were used to generate paraffin sections, followed by dewaxing with xylene, dehydration with absolute ethyl alcohol, and proteinase K repair. After incubation with buffer at room temperature, the slices were treated with fluorescein isothiocyanate (FITC)-labelled anti-ACE2 antibody (1:50) at 37 °C for 2 hours. Diamidinophenyl indole (DAPI) was used to counterstain the nuclei. The images were examined under a fluorescence microscope (FITC excitation wavelength: 465 nm–495 nm).

Detection of cell activity by enhanced cell counting kit-8 (CCK-8)

The influence of the spike protein and serum containing Chinese herbs on cell viability was detected by an enhanced CCK-8 according to the instructions. In brief, the CCK-8 mixture was diluted 10 times with serum-free medium in advance, and then hTⅡAECs, HsIMECs, and HCoEpiCs pretreated with the spike protein and TCM serum were added to the CCK-8 dilution (100 μl/well) and incubated at 37°C in 5% CO_2 for 3 hours. The OD value was measured at 450 nm.

Detection of *in vitro* cell permeability by HRP test

The inoculation of hTⅡAECs, HsIMECs, and HCoEpiCs

was performed in a double permeable transwell chamber (0.4 μm) at a density of 3×10^5/well and treated with spike protein and TCM serum. After the upper and lower chamber media were removed, 0.5 μmol/L of HRP buffer was added to the upper chamber of the transwell and allowed to react for 1 hour, followed by collection of the bottom liquid. Then, 100 μl of TMB substrate was mixed to detect the HRP permeability using an OD value of 450 nm.

Western blotting

The relative expression of proteins in tissues and cells was detected by Western blotting (WB) in strict accordance with procedures. In brief, tissues were ground into powder in liquid nitrogen. The powder and cells were treated with ristocetin-induced platelet agglutination (RIPA) [plus 1% phenylmethanesulfonyl fluoride (PMSF)] lysis solution to extract total protein, followed by centrifugation at 12,000 r/min at 4°C for 20 min. After the protein concentration was measured using a Bradford kit or bicinchoninic acid (BCA) kit, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), transfer membranes, and blocking were also carried out. The membranes were immunologically reacted with antibodies against ERK, p38-MAPK, Bcl-2, Caspase-3, GAPDH, NF-κB p65, p-NF-κB p65, p-IκBα, p-Smad2/3, and TGF-β1 (1:1,000) and HRP-labelled secondary antibody (1:10,000) for 1 hour, followed by treatment with ECL exposure solution. The protein bands were observed, and grey values were also measured.

Statistical analysis

All data were analyzed using the Statistical Package for Social Sciences (SPSS 23.0) software package. Comparative sampling of the mean between multiple groups was performed by one-way ANOVA. The post-hoc multiple comparison between groups was performed by the LSD method. $P<0.05$ was considered statistically significant.

RESULTS

FGD relieved the damage to the lung and colon caused by spike protein *in vivo*

Alveolar epithelial cells may be the first target tissue attacked by the S protein. As shown in **Fig. 1A**, large areas of alveolar wall thickening and stenosis were seen in the lung tissue stimulated by the spike protein, with numerous inflammatory cells infiltrating into the alveolar walls. In addition, dilated alveoli and broken alveolar walls were observed. There was local alveolar and bronchial haemorrhage. After treatment with FGD, small areas of mild alveolar wall thickening were observed in the lung tissue despite extensive narrowing of the alveolar space, and a few inflammatory cells infiltrated the alveolar walls with a small amount of intravascular congestion. In addition, no obvious abnormalities were observed at any level of the bronchi, which was consistent with the pathological injury score of the lung (**Fig. 1B**).

Stimulation of the spike protein also resulted in extensive damage to the intestine. In the colon tissues of the model group, a large number of necrotic epithelial cells and pyknosis of necrotic cells were observed in the mucosal layer, the structure of the lamina propria was loose and disorganized, and extensive necrotic cell debris was present in the intestinal lumen. The necrotic, erosive intestinal glands were replaced by proliferative connective tissue, accompanied by numerous inflammatory cell infiltrates. In the colon tissues of mice treated with FGD (the TCM group), a small number of necrotic epithelial cells were seen in the mucosal layer, a small amount of necrotic cell debris were visible in the intestinal lumen, and occasional small focal erosion was accompanied by a small amount of inflammatory cell infiltration, which was demonstrated by the pathological score of the colon (**Fig. 1C**).

FGD upregulated the expression of ACE2 reduced by the spike protein in the lung and colon tissues

ACE2 is the receptor of SARS-CoV-2 in the colon and lung epithelium. We found that stimulation with the spike protein resulted in a significant downregulation of the ACE2 receptor compared with the normal group (**Fig. 2**). However, this phenomenon was reversed by treatment with FGD, in which ACE2 expression in the colon and lung tissues was higher than that in the model group.

FGD significantly improved the dysregulation of inflammatory markers caused by the spike protein

The relative levels of several inflammatory cytokines in mouse serum, including IL-6, IL-10, IL-13, TNF-α, and CRP, are shown in **Table 1**. We observed that the spike protein caused higher levels of cytokines compared to the normal control ($P<0.05$). The intervention with FGD had different effects on the reduction of

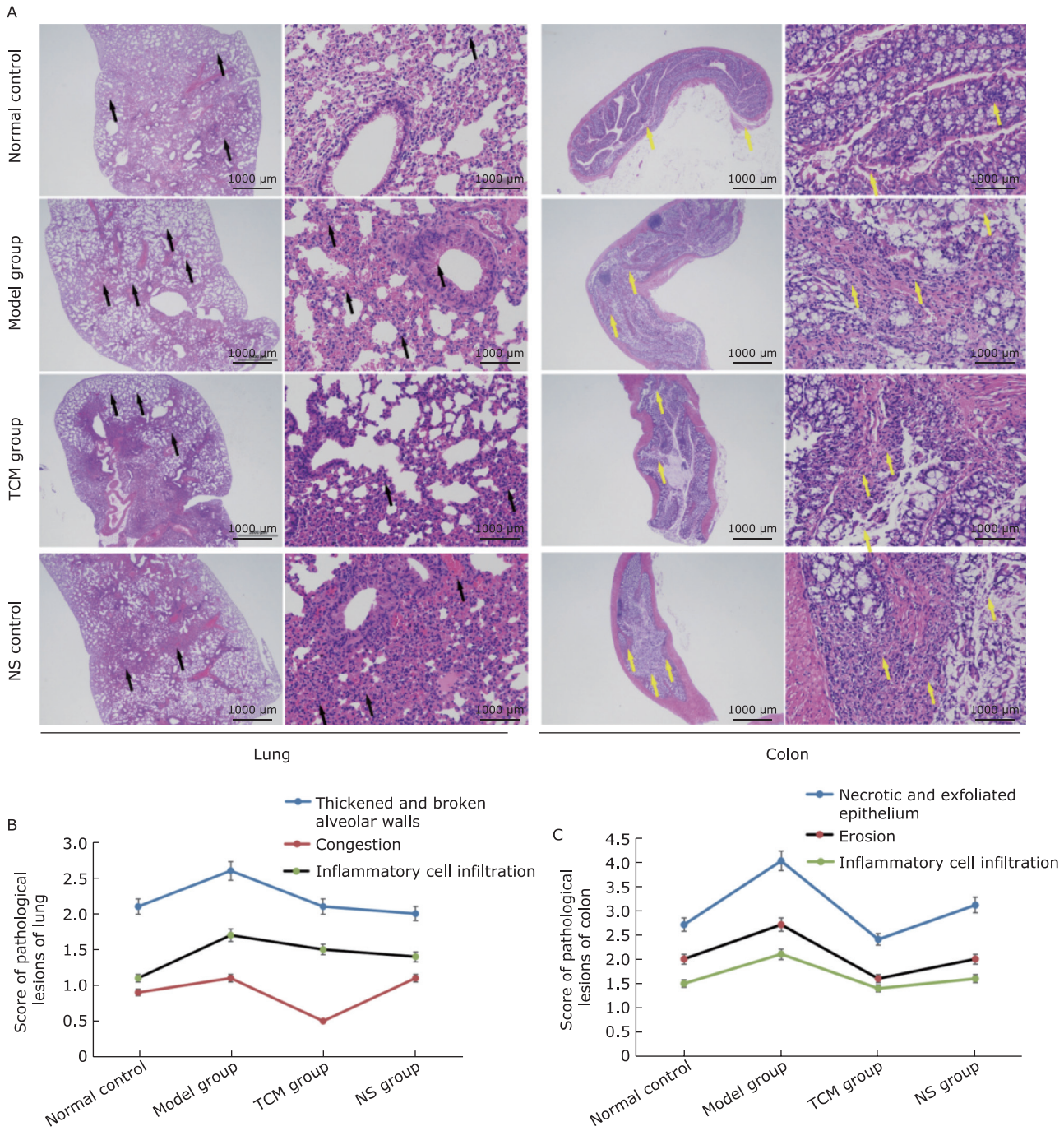

Figure 1. Pathological observation of lung and colon tissues using HE staining. (A) In the lung tissue, there was thickening and narrowing of the alveolar wall, infiltration of inflammatory cells on the alveolar wall, expansion of the alveolar wall, breakage of the alveolar wall, fusion with each other, and haemorrhage or stasis in the blood vessels (black arrows). In the colon tissue, necrosis and detachment of mucosal epithelial cells and pyknosis of necrotic cells were observed in the mucosal layer. Inflammatory cell infiltration in the lamina propria, loose arrangement of muscle fibres in the muscularis, erosion of mucosal tissue, disappearance of necrotic intestinal glands, and replacement by proliferative connective tissue were observed (yellow arrows). (B) Pathological injury scores of lungs. (C) Pathological injury scores of colon tissues.

cytokines. In general, FGD intervention significantly reduced the levels of IL-6, IL-10, TNF-α, and CRP at 5 d compared with the model group (P<0.05). Obvious changes in IL-13 levels were not found.

FGD might regulate the activity of TGF-β/Smads and NF-κB signaling

The inflammatory response triggered by spike protein stimulation cannot be separated from the involvement

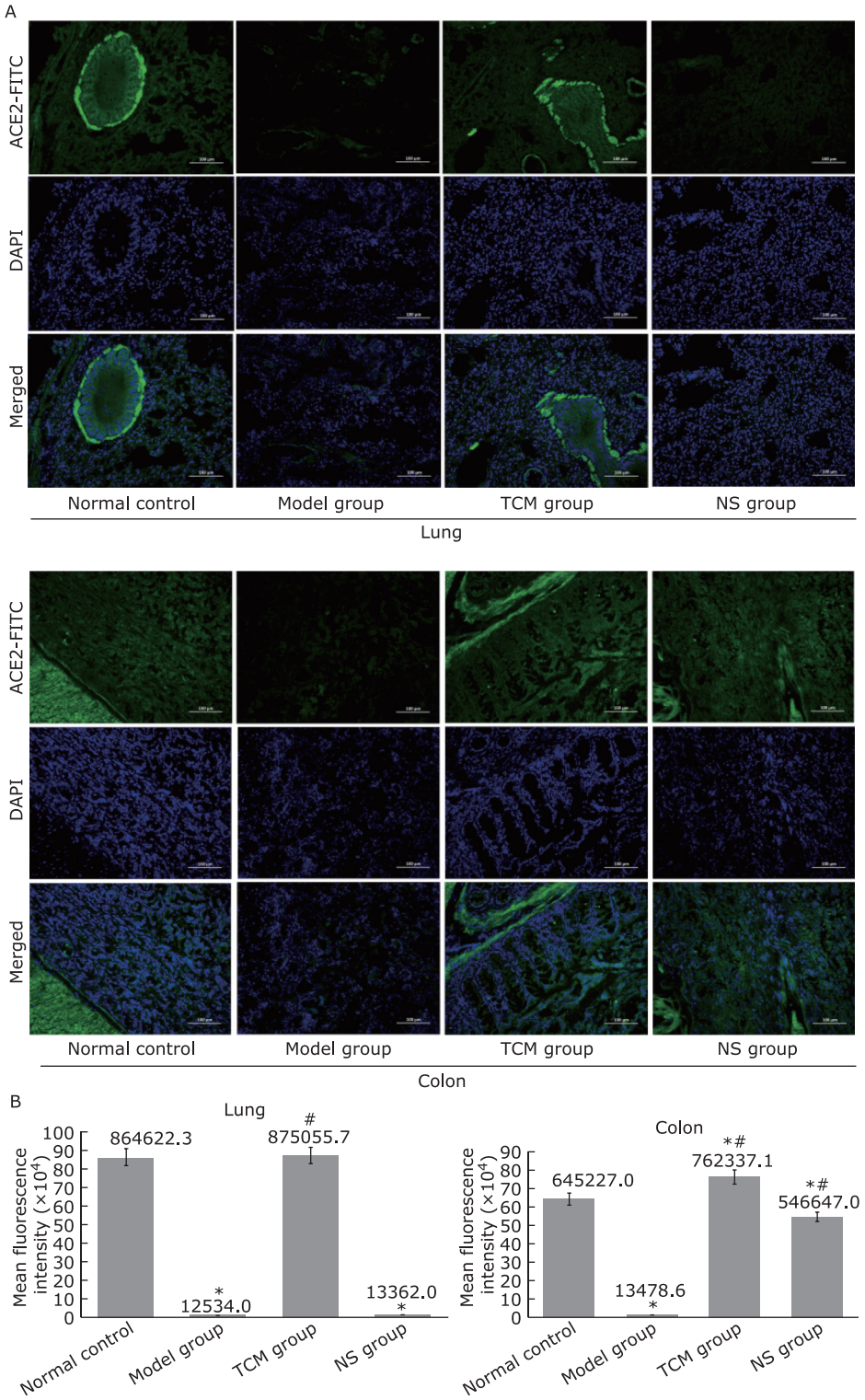

Figure 2. Immunofluorescence and relative intensity of ACE2 expression. (A) Immunofluorescence detection (200×) of ACE2 receptor expression in lung and colon tissues. (B) Histogram of relative fluorescence intensity. * $P<0.05$ *vs.* the normal control. # $P<0.05$ *vs.* the model group.

of signaling pathways. We observed that the influence of the spike protein on ERK1/2 and p38 MAPK expression was not obvious compared with that of the normal control (**Fig. 3**). Spike protein upregulated the expression of NF-κB p65 but not that of p-IκBα or p-NF-κB p65 in the lungs (**Fig. 3**), which was different from what was observed in the colon, where the spike protein increased p-IκBα expression but decreased NF-κB

Table 1. Inflammatory cytokine expressions in the serum of mice (by ELISA)

Markers	Time	Normal group	Model Gorup	NS group	TCM group	*P* value
IL-6	3d	644.61	717.36*	696.89*	702.85*	0.009
	5d	268.02	342.09*	344.88*	298.89#‡	<0.001
IL-10	3d	25.14	82.12*	74.86*	44.7#	0.009
	5d	87.61	145.79*	123.84*	121.52	0.019
IL-13	3d	352.69	344.69	362.46	351.09	0.998
	5d	91.9	102.87	92.42	100.23	0.931
TNF-α	3d	566.19	853.87	840.04	633.38	0.141
	5d	575.77	1042.36*	1236.73*#	864.43*#‡	<0.001
CRP	3d	18175.8	21129.27*	21550.72*	19786.15	0.042
	5d	5933.09	7413.08*	6567.13#	6093.33#	0.001

* *P*<0.05 *vs.* the normal control; # *P*<0.05 *vs.* the model group; ‡ *P*<0.05 *vs.* the NS group.

p65 expression (both *P*<0.05). TCM treatment dramatically increased the levels of NF-κB p65, TGF-β1, and p-Smad2/3 expression in the lungs compared with the model group (all *P*<0.05), while it decreased NF-κB p65 and p-IκBα expressions and increased p-Smad2/3 expression in the colon (all *P*<0.05) (**Fig. 3**).

FGD relieved the damage to lung and colon epithelial cells and mediated the dysfunction of inflammatory cytokines caused by the spike protein *in vitro*

As revealed in **Fig. 4A**, the stimulation of the spike protein caused decreased activity and increased permeability of hTⅡAEC cells *in vitro* compared to the control (*P*<0.05), with the promotion of the apoptotic marker caspase 3 (**Fig. 4B**). The serum containing Chinese herbs (spike protein + TCM groups) significantly improved the viability of hTⅡAECs induced by S protein in a concentration-dependent manner compared to that of spike protein treatment (spike model, *P*<0.05), with decreased cell permeability and caspase 3 expression in contrast to the S model (**Fig. 4A,** *P*<0.05). In addition, the spike protein promoted high levels of inflammatory

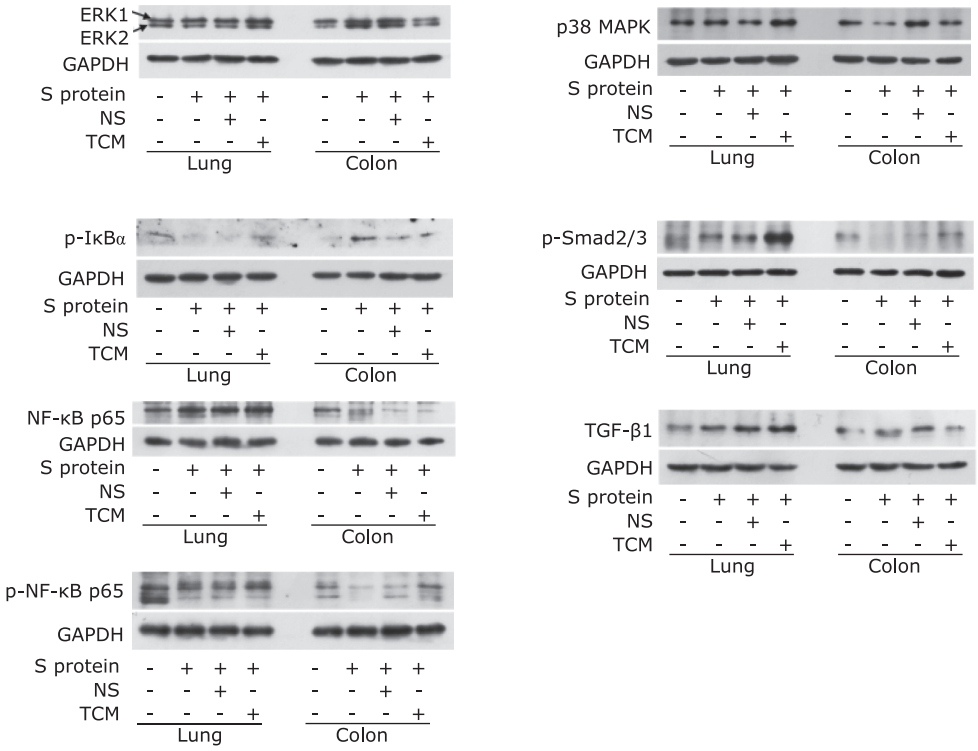

Figure 3. Changes in the expression of related signaling pathway proteins in lung and colon tissues by Western blotting, including ERK, p38 MAPK, p-IκBα, NF-κB p65, p-NF-κB p65, p-Smad2/3, and TGF-β1.

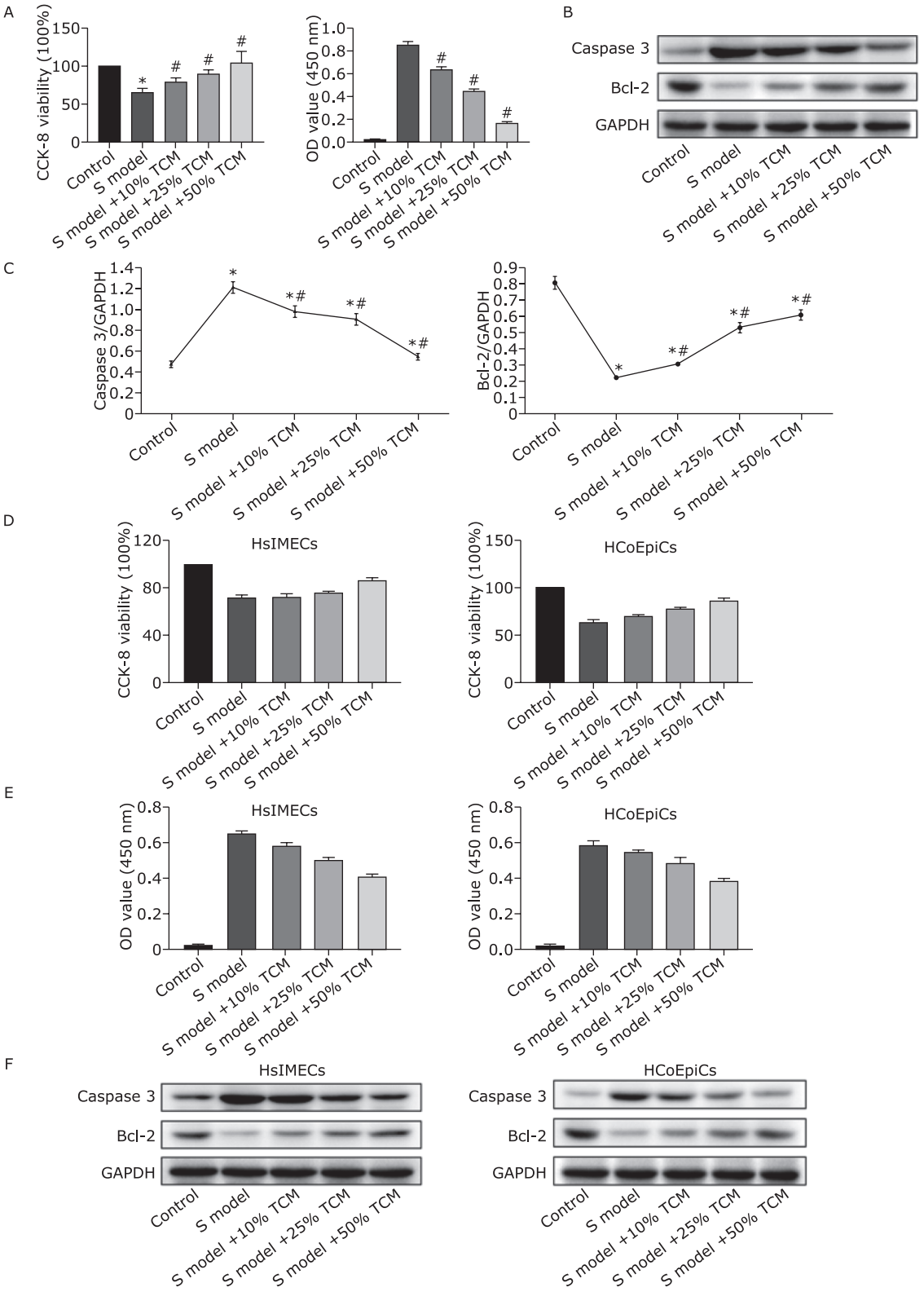

Figure 4. Detection of relative proteins, cell viability and permeability. (A) The influence of serum on the viability (Left) and cell permeability (Right) of spike protein-treated hTIIAEC cells. (B) The influence of serum on the S protein-induced expression of caspase 3 and Bcl-2 (protein bands). (C) Comparison of the grey value of caspase 3/GAPDH and Bcl-2/GAPDH. (D) The influence of the serum at different concentrations on the viability of HsIMECs and HCoEpiCs. (E) The effect of serum on the cell permeability of HsIMECs and HCoEpiCs. (F) The influence of serum on the expression of caspase 3 and Bcl-2 in HsIMECs and HCoEpiCs. * $P<0.05$ *vs.* the normal control. # $P<0.05$ *vs.* the model group.

cytokines, including IL-6 and TNF-α, with decreased IL-13 and TGF-β1 levels (P<0.05). However, the changes in IL-6 and TNF-α but not IL-13 and TGF-β1 levels were reversed by the serum containing Chinese herbs (**Table 2**). The serum promoted the levels of IL-13 and TGF-β1 in TCM group compared with those in the S model (P<0.05).

The effects of induction of spike protein and intervention with serum containing Chinese herbs on the small intestine and colon were similar to those on alveolar epithelial cells (**Fig. 4D-F**). However, the effects of the spike protein and Chinese herbs on inflammatory cytokines were different. As shown in **Table 3**, the spike protein increased the levels of IL-6 and TNF-α in HsIMECs but decreased the levels of IL-10 in HsIMECs and IL-13 in HCoEpiCs (P<0.05). Serum containing Chinese herbs reversed the levels of IL-6 and TNF-α in HsIMECs and HCoEpiCs and increased the levels of IL-10 in HsIMECs and IL-13 in HCoEpiCs (P<0.05).

DISCUSSION

In early May 2023, the World Health Organization (WHO) declared that COVID-19 no longer constitutes a "public health emergency of international concern". However, COVID-19 still presents a low level of epidemic in China. Unfortunately, the mechanism of the disease is not fully understood, and there is a lack of highly effective treatment. Luckily, TCM prescriptions have played an important role in the fight against COVID-19, and the underlying mechanisms need to be further clarified.

From the TCM perspective, COVID-19 belongs to the *Doctrine of Wen Disease category*, a theoretical system of exogenous heat-evils. Our present study initially investigated the protective effects of Chinese herbs on lung and intestine injury stimulated by the spike protein and the related mechanisms *in vitro* and *in vivo*. The vast majority of COVID-19 patients present with respiratory and gastrointestinal symptoms, in-

Table 2. The effect of serum on spike protein-induced inflammatory cytokines in hTⅡAECs

Markers	Control group	S model group	S model+10% TCM group	S model+25% TCM group	S model+50% TCM group	*P* value
IL-6 (ng/L)	20.24	33.01*	29.81*#	27.95*#	20.01#	<0.001
IL-10 (ng/L)	428.32	435.40	435.54	435.38	418.36	0.762
IL-13 (ng/L)	41.33	27.17*	30.75*#	33.67*#	35.04*#	<0.001
TNF-α (ng/L)	212.14	309.29*	277.51*	263.84	222.83	<0.001
NF-κB (ng/L)	215.69	187.85	183.57	206.98	213.29	0.154
TGF-β1 (ng/L)	285.49	156.97*	185.03*#	196.94*#	272.26#	<0.001

hTⅡAECs: human type Ⅱ alveolar epithelial cells. * P<0.05 *vs.* the normal control; # P<0.05 *vs.* the model group.

Table 3. The relative levels of inflammatory cytokines in the supernatants of HsIMECs and HCoEpiCs by ELISA

Cells	Markers	Control group	S model group	S model+10% TCM group	S model+25% TCM group	S model+50% TCM group	*P* value
HsIMECs	IL-6 (ng/L)	22.40	30.49*	27.62*	25.39#	22.11#	<0.001
	IL-10 (ng/L)	348.14	307.22*	318.12*	327.84*#	339.72#	0.001
	IL-13 (ng/L)	30.47	30.59	28.49	31.09	29.16	0.757
	TNF-α (ng/L)	121.90	273.29*	271.30*	234.02*	202.70*#	<0.001
	NF-κB (ng/L)	178.52	177.08	175.88	178.74	180.86	0.859
	TGF-β1 (ng/L)	149.71	146.78	143.00	141.65	145.37	0.986
HCoEpiCs	IL-6 (ng/L)	24.13	28.28*	25.26#	23.78#	21.95#	0.001
	IL-10 (ng/L)	372.10	384.02	378.86	388.56	388.92	0.910
	IL-13 (ng/L)	23.69	15.99*	20.01	22.77#	21.53#	0.021
	TNF-α (ng/L)	168.63	286.16*	281.62*	230.50*#	221.83*#	<0.001
	NF-κB (ng/L)	133.02	131.88	134.09	126.03	134.28	0.201
	TGF-β1 (ng/L)	177.31	173.80	176.35	177.77	171.37	0.452

HsIMECs: human small intestinal mucosa epithelial cells; HCoEpiCs: human colonic epithelial cells. *P<0.05 *vs.* the normal control. #P<0.05 *vs.* the model group.

cluding cough, abdominal pain, nausea, vomiting, and diarrhoea[12-16], in addition to systemic symptoms such as fever and muscular soreness. Indeed, SARS-CoV-2 not only acts on the respiratory system through its binding to the angiotensin converting enzyme 2 (ACE2) receptor on the lung cell surface, but the intestine and other tissues are also considered alternative targets[17]. Our present results demonstrated that the spike protein induced a reduction in ACE2 expression in lung and colon tissues, which was reversed to normal levels by pretreatment with FGD. Although SARS-CoV-2 entry into cells requires the binding of a receptor binding domain (RBD) on the spike protein to its receptor ACE2[18], the disruption in ACE2 expression can cause altered tissue functions[19]. Therefore, spike protein-induced histiocytic damage may result in a reduction in ACE2 receptors on the cell surface, and these ACE2 receptors expressed on the cell surface are increased with the repair of cell damage by treatment with FGD. It has been shown that certain compounds in TCM herbs can bind to ACE2 in a special way, similar to the pattern SARS-CoV-2 bound with ACE2, and these compounds can prevent SARS-CoV-2 from entering cells through ACE2[20].

Consistent with the results of other studies[21-23], the spike protein of SARS-CoV-2 in our present study also induced pathologic injury of the lung and colon *in vivo* and *in vitro* in alveolar and intestinal epithelial cells by enhancing cell permeability and reducing cell viability. Pretreatment with FGD significantly reversed cell permeability and apoptosis and improved cell viability in a concentration-dependent manner, with *in vivo* amelioration of pathological injury. These results suggest that FGD can protect against spike protein-triggered damage to target tissues (including the lung and intestine).

In addition, an improvement in the dysregulation of inflammatory cytokines was found in mouse serum and the supernatant of *in vitro* cultured alveolar and intestinal epithelial cells treated with FGD. Generally, on the 5th day after intervention, the downregulatory effect of FGD on inflammatory factors in mice was higher than that on the 3rd day, suggesting that the protective effect of FGD on the inflammatory response triggered by the spike protein may have a cumulative effect over time.

On the other hand, the spike protein increased the levels of IL-6 and TNF-α but decreased the levels of IL-13 and TGF-β1 in hTⅡAECs. Serum containing Chinese herbs reversed the expressions of IL-6 and TNF-α and increased the levels of IL-13 and TGF-β1 in hTⅡAECs. Thus, IL-13 and TGF-β1 may play a positive role in the protection against cell injury by Chinese herbs, which is consistent with the results of the mouse experiments. Although several studies have revealed that SARS-CoV-2 infection may lead to the activation of inflammatory pathways (including TGF-β signaling)[24,25], TGF-β was not highly expressed in the injured lung tissues of necropsy patients who died from COVID-19[26], highlighting the complex mechanism of TGF-β in SARS-CoV-2-induced lung injury. A recent study demonstrated the amelioration of lung injuries by tocotrienol through the induction of TGF-β1/Smad[27]. TGF-β/Smad can induce epithelial cell damage through cell cycle arrest. TGF-β is also one of the multiple inflammatory pathways triggered by SARS-CoV-2[28]. Pulmonary fibrosis is a key feature of COVID-19. TGF-β/Smad can induce pulmonary fibrosis by activating phenotypic changes of alveolar epithelial cells and extracellular matrix remodeling[29]. Thus, the mediation of TGF-β1 expression may also be a potential mechanism by which Chinese herbs prevent lung injury.

The S protein can promote the production of various inflammatory cytokines[30-33]. Similarly, we found that the spike protein promoted the levels of IL-6 and TNF-α in intestinal epithelial cells but reduced the levels of IL-10 in the small intestine cell line HsIMEC and IL-13 in human colonic epithelial cells (HCoEpiCs), which were reversed by herbal medicine-containing serum. However, FGD had no effect on the levels of NF-κB and TGF-β1 in intestinal epithelial cells, suggesting that there are different effects and mechanisms among different cell types.

Indeed, the SARS-CoV-2 protein affects the activity of NF-κB and ERK signaling[34]. NF-κB, an essential mediator of the inflammatory response, regulates the expression of various pro-inflammatory cytokines involved in acute lung injury. Activation of NF-κB signaling in epithelial cells can cause cytokine storm[35]. ERK activation is required for the transcription of viruses including polyomavirus, Ebola virus, and influenza A virus[36], suggesting that activated ERK1/2 can strengthen the infectivity of relative viruses, such as SARS-CoV-2[37]. A study revealed that the SARS-CoV-2 S protein could induce the upstream activation of the ERK pathway. ERK participates in spike protein-induced transcription of the cyclooxygenase-2 (COX-2) gene and its protein production in HEK293T cells. In our

present study, the expression of ERK1/2 increased after stimulation with S protein in colon and lung tissues, although the change was not statistically significant, suggesting the potential effects of the S protein on ERK in the colon and lung. After TCM intervention, although the expression of ERK1/2 in lung tissue was not decreased, the expression of ERK1/2 in colon tissue was significantly lower than that in the model group, indicating that TCM herbs may act as a possible ERK inhibitor in colon tissue; however, such an effect was not significant in lung tissue and may be related to tissue specificity. Recent studies have also demonstrated that ERK1/2 inhibition can alleviate proinflammatory cytokines induced by SARS-CoV-2[38-40].

Increasing studies have revealed the specific activation of NF-κB in SARS-CoV-2-infected cells[41-43]. Similarly, we found that the spike protein stimulated the expression of NF-κB p65 in lung tissue and p-IκBα in colon tissue but decreased their expressions in colon tissue, suggesting that the spike protein may promote the activation of NF-κB in lung tissue injury, which is consistent with the results of other previous studies[44,45]; however, this mechanism may be tissue type-dependent. Treatment with FGD significantly increased the expression of NF-κB p65, TGF-β1, and p-Smad2/3 in the lungs of mice; however, compared with the model group, the herbs decreased the expression of NF-κB p65 and p-IκBα in the colons of mice and increased p-Smad2/3 expression in the colon, indicating that Chinese medicine inhibits spike protein-induced lung and intestinal injuries by supressing NF-kB p65 and promoting the TGF-β1/Smad pathway.

In conclusion, the spike protein induces lung and intestinal tissue injuries and may lead to a significant decrease in the expression of its receptor ACE2 and the dysregulation of inflammatory factors *in vitro* and *in vivo*. TCM has a protective effect on lung and intestinal tissue injuries induced by the spike protein, but the mechanisms are extremely complex and may be tissue type-specific. Both NF-κB and TGF-β1/Smad pathways may be involved in the protective effect of TCM herbs on tissue injuries caused by the spike protein.

Conflicts of interest

All authors declare no conflicts of interest.

Authors' contributions

Huang C wrote and corrected the draft. Shen WZ supervised the experiments. Liu HS, Liang BJ, Liao SR, and Huang C performed the experiments. All authors agreed to be accountable for all aspects of work ensuring integrity and accuracy.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding authors upon reasonable request.

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论著

中药复方防感汤对 SARS-CoV-2 刺突蛋白引起的肺和结肠上皮细胞损伤的保护作用与 TGF-β/Smad2/3 和 NF-κB 通路的调节有关

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摘要

背景与目的 探讨中药复方防感汤对 SARS-CoV-2 刺突蛋白诱导的肺、肠损伤的体外和体内保护作用及其机制。

方法 用重组 SARS-CoV-2 刺突蛋白刺激 BALB/c 雌性小鼠和 3 株经防感汤预处理的细胞系。检测各组组织苏木精 - 伊红（HE）染色、病理评分、细胞通透性和活力以及肺、结肠 ACE2 表达。酶联免疫吸附测定法（ELISA）检测小鼠血清和细胞上清中炎症因子水平。蛋白质印迹法（Western blotting）检测 NF-κB p65、p-NF-κB p65、p-IκBα、p-Smad2/3、TGF-β1、Caspase3、Bcl-2 的表达。

结果 从病理评分以及细胞通透性和活力来看，中草药在体内和体外均对刺突蛋白诱发的肺和结肠损伤均有保护作用（P<0.05）。中草药上调肺和结肠中被刺突蛋白降低的 ACE2 表达，显著改善刺突蛋白引起的炎症标志物的分泌紊乱，同时调节 TGF-β/Smads 和 NF-κB 通路的活性。

结论 中药复方防感汤对 SARS-CoV-2 刺突蛋白刺激的肺和肠组织损伤具有一定的保护作用，其机制基于对可能具有组织类型特异的 NF-κB 和 TGF-β1/Smad 通路的调控作用。

关键词：COVID-19；SARS-CoV-2；中草药；预防；转化生长因子 -β

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