Article

J. Biol. Regul. Homeost. Agents. 2022; 36(4): 1125–1136
https://doi.org/10.23812/j.biol.regul.homeost.agents.20223604.123

Electroacupuncture Relieves Postoperative Chronic Cough in Lung by Regulating TRPV1 Pathway and Neurogenic Factors

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Published: 20 August 2022

Background: Chronic cough is common in postoperative patients with non-small cell lung cancer (NSCLC). But the mechanism of chronic cough is unclear.

Methods: Forty NSCLC patients with postoperative chronic cough (CC) after surgery were randomly divided into electroacupuncture (EA) and no therapy groups, each treated for 28 days. The quality of life (QOL) module in the European Organization for Research and Treatment of Cancer Treatment of Cancer Quality of Life Questionnaire-Core 30 (EORTC QLQ-C30), the Dyspnea module in the EORTC QLQ-Lung Cancer Module 13 (EORTC QLQ-LC13) and the Mandarin Chinese version of the Leicester Cough Questionnaire (LCQ-MC) were completed before and after treatment. *In vitro*, thirty guinea pigs were randomly divided into sham operation group (sham group), simple modeling group (model group), electroacupuncture+model group (EA+M group) and treated for one week. After detecting cough frequency, samples were taken and the contents of markers in bronchoalveolar lavage fluid, blood and lung tissue were detected. Finally, immunofluorescence was used for verification.

Results: Compared to those before treatment, the scores of EORTC QLQ-C30 QOL model, the Dyspnea module in EORTC QLQ-LC13 and LCQ-MC physical dimension were significantly better ($p < 0.01$); The bradykinin (BK), Prostaglandin E2 (PGE-2), substance P (SP), calcitonin gene related peptide (CGRP) and transient receptor potential vanilloid 1 (TRPV1) levels were significantly lower ($p < 0.01$) significantly after treatment. *In-vitro* experiment revealed that compared to the Model group, the cough frequency of guinea pigs in either the EA+M group or the sham group was significantly lower ($p < 0.01$). Compared to those in the model group, the BK, PGE-2, SP and CGRP levels in bronchoalveolar lavage fluid, blood or lung tissues were also lower in the EA+M group ($p < 0.01$); Their levels were positively correlated with the cough frequency ($p < 0.01$). Finally, compared to the model group, p-TRPV1 immunofluorescence showed that it's level in the EA+M and sham groups were significantly lower ($p < 0.01$).

Conclusions: The study suggests that EA could physiologically alleviate the symptoms of cough and dyspnea in patients with CC and improve their QOL. The mechanism might be linked to the decreased expression of TRPV1 provide in full on first mention in the abstract and neurogenic factors after electroacupuncture treatment.

Keywords: electroacupuncture; chronic cough; non-small cell lung cancer; transient receptor potential vanilloid 1; neurogenic factors; post-operative

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Introduction

Lung cancer is one of the main causes of cancer-related death worldwide [1]. For resectable non-small cell lung cancer (NSCLC), approximately 25–50% had cough after surgery [2,3]. Some patients may experience this cough for a long time, leading to a reduction in their quality of life (QOL) [4–6] in physical, psychological and social aspects [7]. However, effective therapies are lacking, mainly due to the limited understanding of the pathogenesis of this cough, which reflects the lack of well-designed experiments [8]. At present, chronic cough (CC) after surgery for non-small cell lung cancer is mainly treated by opioids, which might result in drug dependence, respiratory depression, and gastrointestinal symptoms [9,10].

Existing studies suggest that increased sensitivity to cough is related to airway neurogenic inflammation [11,12]. There are two kinds of cough afferent nerve receptors: Myelinated Aδ fibers and unmyelinated diameter C fibers [13]. A large number of transient receptor potential (TRP) pathways are distributed in afferent nerve fibers [14], of which TRPV1 provide in full on first mention in the main text is present in the peripheral nerve of lung C fiber, and could be activated by various neuroinflammatory mediators [14,15], further promoting cough reflex [16]. Neuropeptides, such as SP provide in full on first mention in the main text and CGRP provide in full on first mention in the main text, could cause neurogenic inflammation and increase the sensitivity to cough [17]. In a study [18], the level of TRPV1 after surgery for non-small cell lung cancer was significantly higher than the baseline, especially in those with acute or CC. Therefore, it could be inferred that TRPV1 is linked with the occurrence of CC after surgery for non-small cell lung cancer.

Acupuncture, which is part of Chinese medicine can reduce post-operative inflammatory response [19,20]. Liu *et al.* [21] revealed that lung-related complications in patients after surgery in general or for non-small cell lung cancer were significantly reduced after electroacupuncture. Besides, Tu *et al.* [22] reported that percutaneous acupoint electrical stimulation could accelerate postoperative recovery and improve the prognosis (which aspect of it, need to be clear, as this could be understood to mean prognosis from cancer point of view) of patients. Meanwhile, the authors found that acupuncture could improve the symptoms of cough in patients with non-small cell lung cancer after surgery [23], suggesting that acupuncture might have a potential in treating CC.

The aim of this study therefore was to assess the value of electroacupuncture in treating CC for after surgery for NSCLC (Fig. 1).

Methods

Clinical Study

Patient Selection

Forty patients undergoing radical resection of lung cancer from October 2020 to December 2020 were selected. The inclusion criteria were: (1) Aged 19–75 years; (2) NSCLC diagnosed by histopathology; (3) Lobectomy and systemic lymph node dissection; (4) R0 resection; (5) CC lasting more than 8 weeks; The exclusion criteria were: (1) Patients who started anti-cancer treatment during the study, within 6 weeks after the beginning of chemotherapy, within 8 weeks after the beginning of tyrosine kinase inhibitor or within 12 weeks after receiving/completing chest radiotherapy. (2) Patients taking other treatments that may regulate cough, such as steroids, opioids, pregabalin or gabapentin. (3) Patients with history of allergy, asthma or tuberculosis; (4) Patients receiving antibiotic treatment.

The degree of cough was assessed by visual analogue scale (VAS) [15]. Cough was diagnosed when VAS $\geq$60 mm (range 0–100 mm). Based on the standard of the American College of Chest Physicians, CC is a cough lasting more than 8 weeks [24].

Grouping and Treatment Methods

Estimated sample size included 33 patients with 90% power and 5% alpha to detect refractory CC [25] assuming that the smallest clinical significant difference of cough frequency was 20–30% [26] and the data was in normal distribution (including allowance for 10% loss to follow-up). This is an unusual way of power calculation. Power calculation should be based on a specified change in the primary outcome measure, which was not mentioned.

Patients were randomly divided into two groups by random number table method: The electroacupuncture group (EA) and the control group. The electroacupuncture group was treated with acupuncture for 28 days. The standardized acupoints used in this study were; (1) LU7 (Lieque); (2) Lu9 (Taiyuan) of the lung meridian [21,22]. After local disinfection, two needles of Φ0.30 $\times$ 25 mm were inserted into SDZ-IV electronic acupuncture apparatus, and were inserted to a depth of 25 to 40 mm vertically. The EA stimulation parameters were; Current (IP-P) of 1 mA, frequency of 2 Hz and duration of 30 min.

Evaluation Method

Data on demographics, cancer characteristics, anti-cancer treatment and comorbidities were collected during screening. The cough status of patients was assessed before and after treatment. The assessment was carried out using the Global Health Status (QOL) module in the European Organization for Research and Treatment of Cancer Treatment of Cancer Quality of Life Questionnaire-Core 30 (EORTC QLQ-C30) [27], the Dyspnea module in the EORTC QLQ-Lung Cancer Module 13 (EORTC QLQ-LC13). EORTC

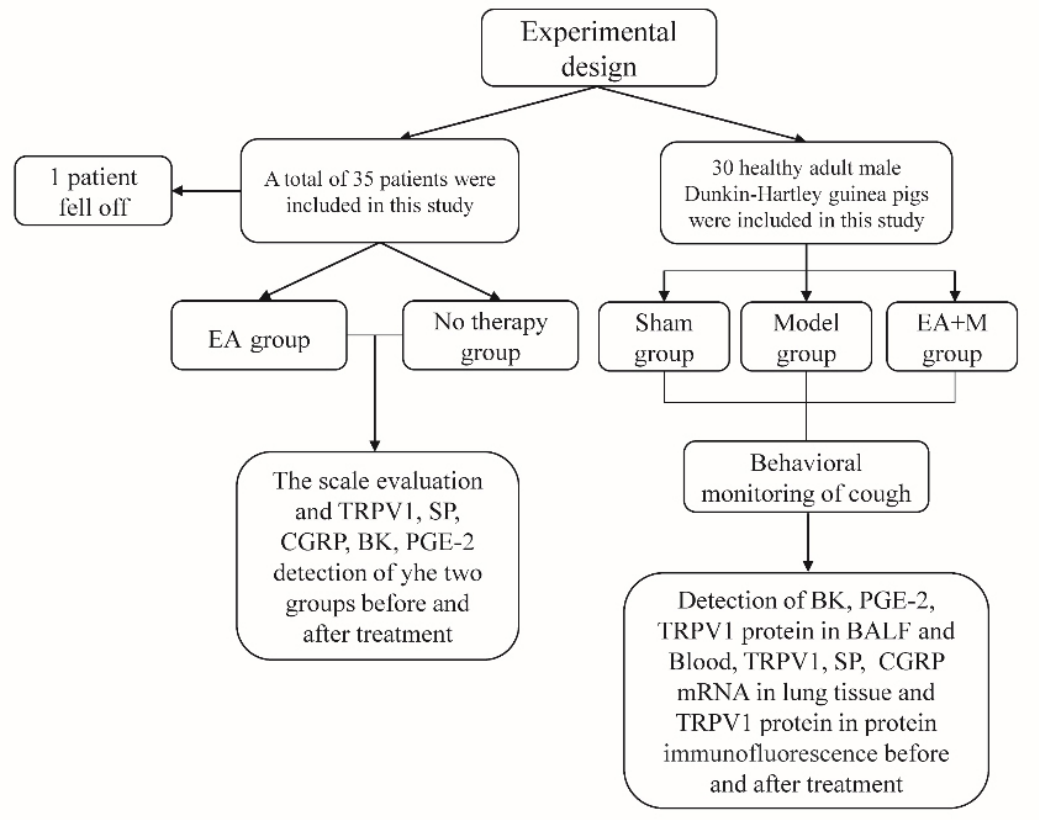

Fig. 1. Technology roadmap.

QLQ-LC13 evaluates lung cancer-related symptoms from multiple dimensions including cough, hemoptysis, dyspnea, and pain. The questionnaire has a 1-week time frame and uses a four-point response format, the higher the score, the more severe the symptoms. While the EORTC QLQ-C30 QoL model has a seven-point response format, the scores are linearly transformed to a score between 0 and 100, with a higher score indicating better health [28].

The Mandarin Chinese version of the Leicester Cough Questionnaire (LCQ-MC) was used to assess cough severity from physiological, psychological and social perspectives. There are 19 multiple choice questions, including eight physiological items, seven psychological items and four social items. There are seven options for each question, the score of each dimension is 1–7, and the total score range is 3–21. The higher the score, the lighter the cough. The common terminology standard of adverse events (CTCAE 4th edition) was adopted to score the adverse conditions.

TRPV1, SP, CGRP, BK, PGE-2 by Enzyme Linked Immunosorbent Assay (ELISA)

Samples of whole blood were stored at room temperature for 120 min, followed by centrifugation (1000 rpm, 20 min). ELISA provide in full on first mention was conducted complying with the instructions provided by the manufacture.

In vitro Experiment

Animals and Groups

Thirty healthy adult male Dunkin-Hartley guinea pigs (5–7 weeks old, 300–350 g) were provided by Experimental Animal Center of Anhui University of Traditional Chinese Medicine (License No: Anhui SCXK2017-001). The guinea pigs used in the experiment were raised under constant temperature. After adaptive feeding for 1 week, animals were equally and randomly allocated into three groups, including: (1) Sham operation group (Sham Group): Only thoracotomy was performed without lung resection, (2) Simple modeling group (Model Group): Preparation of CC model after lung surgery, (3) EA+M Group: CC model after lung surgery+EA at bilateral lung acupoints (Taiyuan, Lieque).

CC Model after Pulmonary Surgery in Guinea Pigs

The Model group were established as follows: (1) Based a complete water-only fasting for 8 h before operation, the guinea pigs were intraperitoneally anesthetized (30 g/L pentobarbital sodium, 30 mg/kg) and maintained in the left lateral position. (2) The right lung was pulled out of the chest with non-invasive tweezers through the right fifth intercostal incision. The right lung with the best degree of freedom of the lobe was selected, and 5-0 non-invasive suture was used to continuously suture to remove the root lobe in advance. After the resection of the lobe, the root of the

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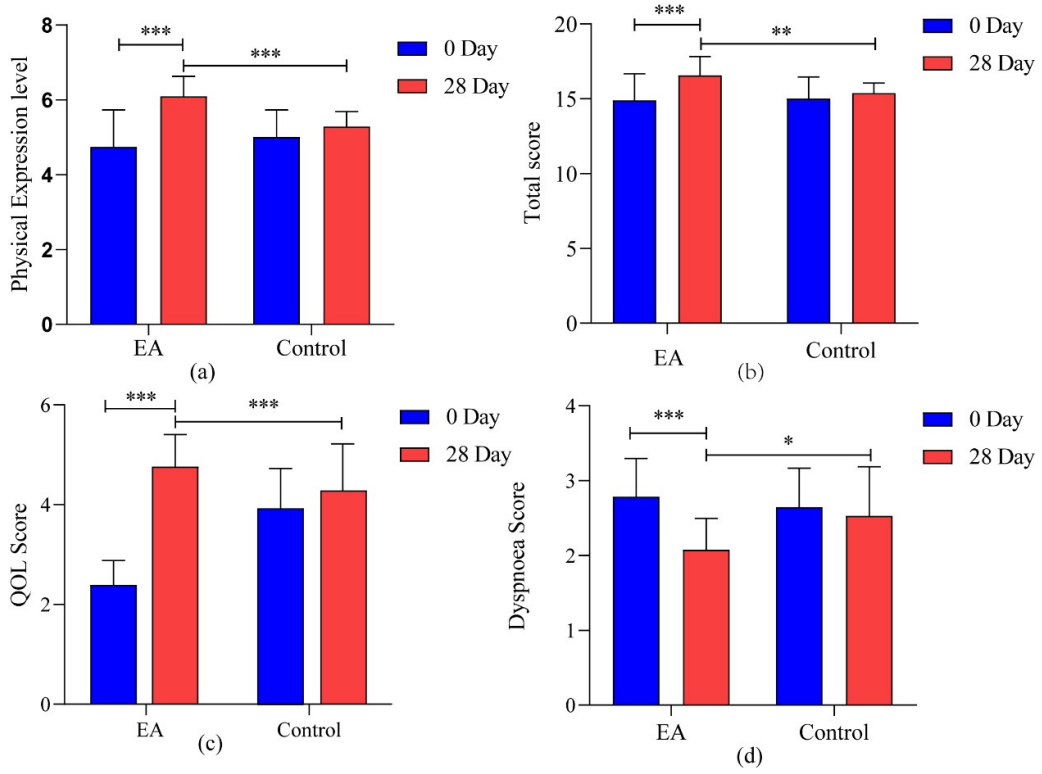

Fig. 2. Differences in LCQ-MC, EORTC-C30 QOL score and EORTC-LC13 dyspnea score between the EA group and control group. (a) The physical score of the EA group on the 28th day was much higher compared to that before treatment or that of the control group on the 28th day. (b) The total score of the EA group on the 28th day was much higher compared to that before treatment ($p < 0.001$) or that of the control group on the 28th day. (c) In the EA group, the QOL score was significant different before and after treatment. Besides, the difference of the QOL score in the EA and control groups was not significant before treatment ($p > 0.05$) but significant after treatment. (d) In contrast to that before treatment, the dyspnea score in the EA group after treatment was better. The difference of the dyspnea score of the EA and control groups was not significant before treatment ($p > 0.05$) but significant after treatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

lobe was sutured again. (3) Careful hemostasis, chest closure after placement of thoracic drainage tube, and removal of thoracic drainage tube after recovery of guinea pigs [4]. The whole process was operated under binocular magnifying glasses. In the Sham group, only thoracotomy was performed.

EA Intervention Treatment

Referring to the route of human meridians, and the 'Chinese Veterinary Acupuncture' about the positioning standard of acupuncture points in guinea pigs, the acupuncture lung meridian group selected Taiyuan (LU9) and Lieque (LU7) of hand Taiyin lung meridian [29]. Two needles of Φ0.30 × 25 mm were inserted into SDZ-IV electronic acupuncture apparatus. The EA stimulation parameters were current (IP-P) of 1 mA, frequency of 2 Hz and duration of 30 min. Acupuncture was applied for 1 week.

Behavioral Monitoring of Cough

Before behavioral experiments, guinea pigs needed to adapt to the environment for at least 10 min. Cough was recognized according to the characteristic postures of guinea pigs, such as the mouth opening, the front foot extending, or the neck extending forward. The WBP provide in full on first mention whole body volume description system (Shanghai Tawang Intelligent Technology Co, Ltd., WBP-8R, Tarzen-tech, Shanghai, China) was utilized to count the number of coughs. The guinea pigs were settled in a transparent room and moved freely. Capsaicin was used as the cough inducer. After atomization for 3 min, the number of coughs within 6 min was recorded using the WBP whole body volume recorder.

PGE2, BK and TRPV1 Protein Expression in Bronchoalveolar Lavage Fluid (BALF) and Blood of Guinea Pigs by ELISA

After anesthesia, the guinea pigs were placed on the operation table, and the trachea, together with the heart and lung, was freed and placed on the table. Was ligated and fixed. The left lung was collected. Recovery liquid (2 mL each time for three times) sputum or mouth was placed in sterile centrifuge tube on the ice block.

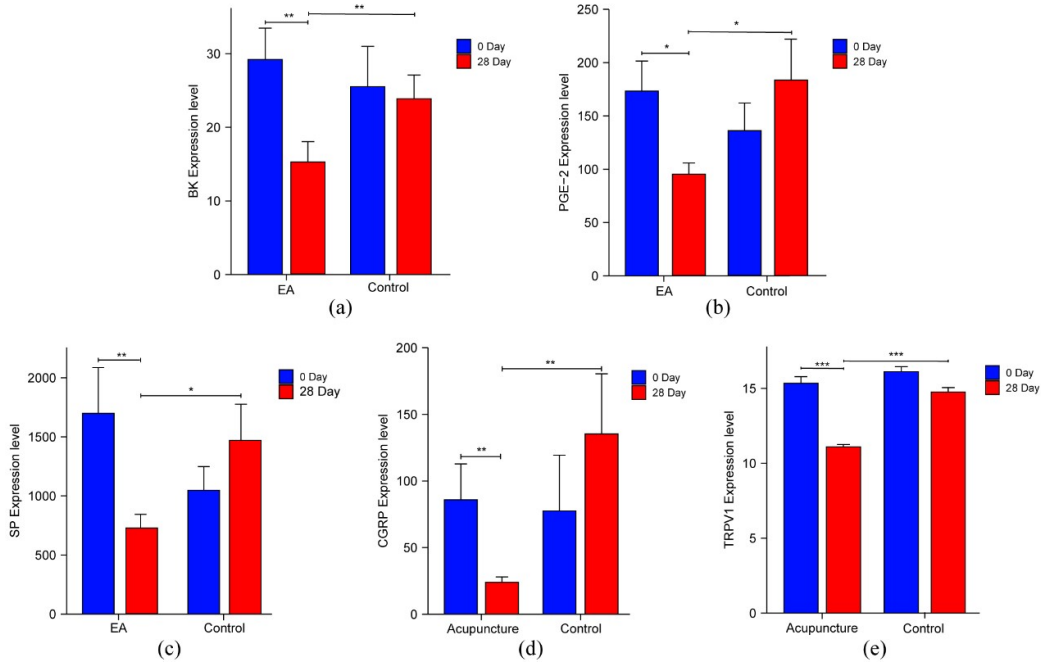

Fig. 3. Expression of TRPV1, SP, CGRP, BK and PGE-2 before and after treatment in two groups were detected by Elisa. The expression of TRPV1 after treatment was significantly different from that before treatment, and the expression in the EA group was significantly better than that in the control group. The expression level of SP, CGRP, BK, PGE-2 in the EA group was significantly lower in contrast to that in the control group after treatment, but there was no significant difference in the control group before and after treatment ($p > 0.05$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

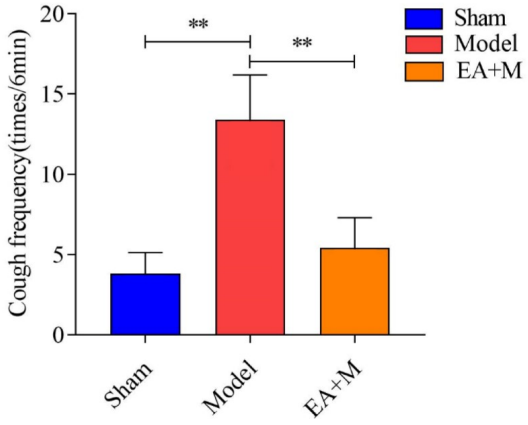

Fig. 4. In contrast to that in the Model group, the number of coughs were lower in either the Sham group or the EA+M group. $^{**}p < 0.01$.

After filtration through two layers of sterile gauze, the recovery rate was measured (about 75%). After centrifugation at a rate of 1500 r/min for 10 min, the supernatant was discarded and cell precipitation was dissolved with PBS, of which 30 μL was put in blood cell counter to calculate the total number of white blood cells; And another 30 μL was used to make cell smears, followed by conventional HE staining and cell morphology classification count by optical microscope (at least 200 cells), with the percentage of various cells calculated. According to the instructions of PGE2, the bradykinin (BK) (E-EL-GP0144c, Elabscience, Wuhan, China) and TRPV1 (SEF839Ra, Uscnk, Wuhan, China) kits, the serum was centrifuged at 3000 g for 10 min, the supernatant was taken, and 100 μL of standard and sample diluent was added to the blank well, and 100 μL of standard or sample to be tested was added to the remaining wells.

After incubation at 37 °C for 1 h, the plate was washed, and then 90 μL of TMB was added. The enzyme-labeled plate was incubated with the film at 37 °C for 10–20 min. The OD value of each empty hole read at 450 nm by a microplate reader, and the OD value of each hole was subtracted from the OD value of the blank control hole for zero adjustment. The standard curve was made according to the standard sample, and then the antigen content of each sample was calculated separately.

TRPV1, SP and CGRP mRNA Expression in Lung Tissue of Guinea Pig by Real-Time Fluorescence Quantitative PCR (RTFQ PCR)

A small amount of liquid nitrogen-frozen lung tissue was taken and suspended in 1.5 mL Eppendorf tube. One mL Trizol (15596018, Ambion, Texas, USA) reagent was added to extract the RNA. The purity of total RNA concentration meter in each group was determined, and then reverse transcription PCR was performed. Finally, the spe-

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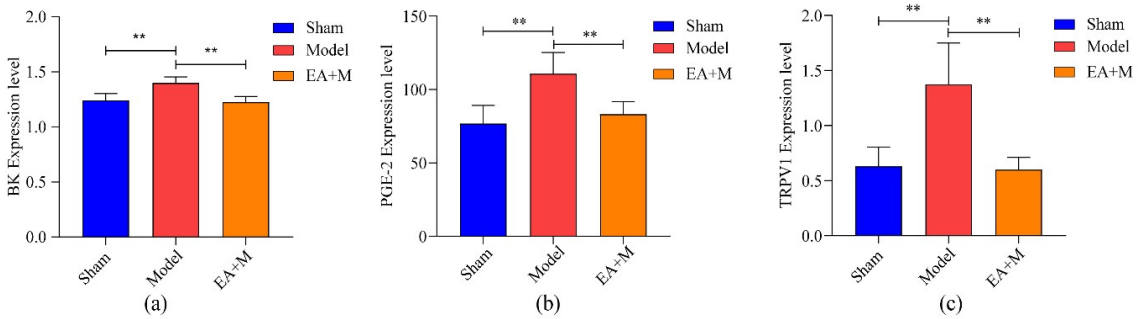

Fig. 5. The expression levels of PGE2, BK as well as TRPV1 in BALF. (a) In contrast to the Model group, the expression level of BK was lower in either the Sham group or the EA+M group. (b) In contrast to the Model group, the expression level of PGE-2 was lower in either the Sham group or the EA+M group. (c) In contrast to the Model group, the expression level of TRPV1 was lower in either the Sham group or the EA+M group. $^{**}p < 0.01$.

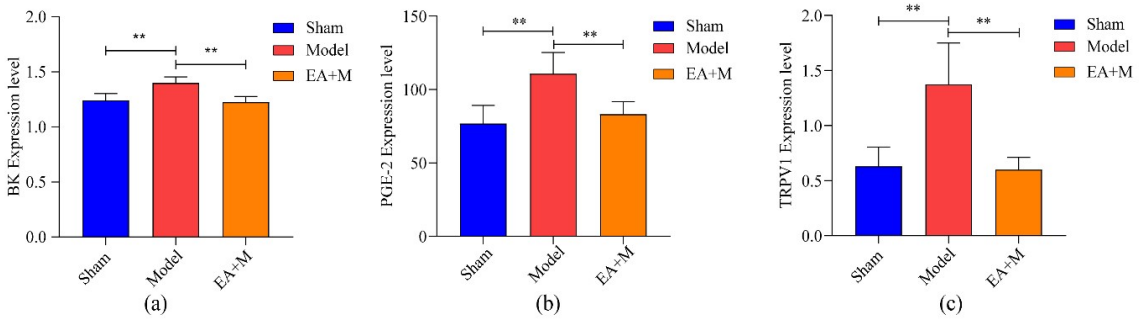

Fig. 6. Changes of BK, PGE-2 and TRPV1 protein expression in guinea pig blood. (a) In contrast to the Model group, the BK level was decreased in either the Sham group or the EA+M group. (b) In contrast to the Model group, the PGE-2 level was decreased in either the Sham group or the EA+M group. (c) In contrast to the Model group, the TRPV1 level was decreased in EA+M group, and Sham group was lower than Model group. $^{**}p < 0.01$.

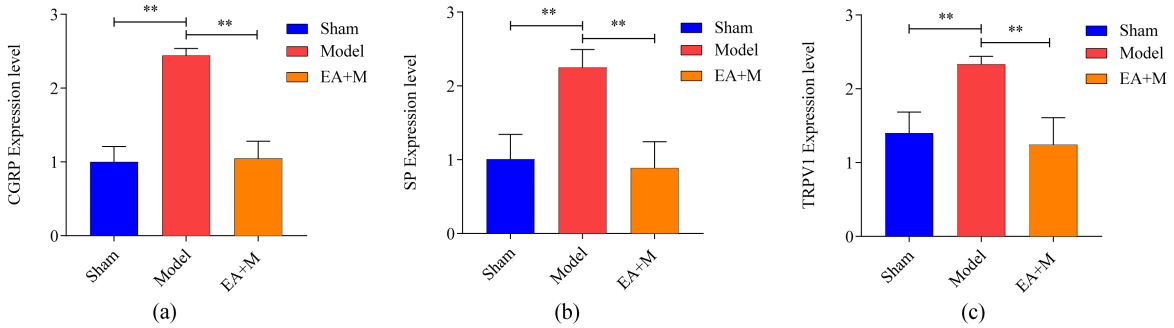

Fig. 7. The differences of CGRP, SP as well as TRPV1 mRNA expression. (a) In contrast to the Model group, the CGRP level were decreased in either the Sham group or the EA+M group. (b) In contrast to the Model group, the SP level was decreased in the Sham group and the EA+M group. (c) In contrast to the Model group, the TRPV1 level was decreased in either the Sham group or the EA+M group. $^{**}p < 0.01$.

cific primers of the genes to be tested were used for quantitative PCR to observe the change of gene mRNA expression (Vazyme, Nanjing, China). Relative levels were calculated by relative quantitative $2^{-\Delta\Delta Ct}$ method. The average value of three complex holes was calculated to determine the final differential expression. The primer sequences are shown in Table 1.

p-TRPV1 Protein Expression in Lung Tissue by Immunofluorescence

The guinea pigs in each group were sacrificed, by cervical dislocation. Their thoracic cavities were cut off on the ice surface and theor tracheae was ligated to prevent alveolar collapse. Complete trachea, bronchus and lung tissues were isolated and prepared for tissue homogenate.

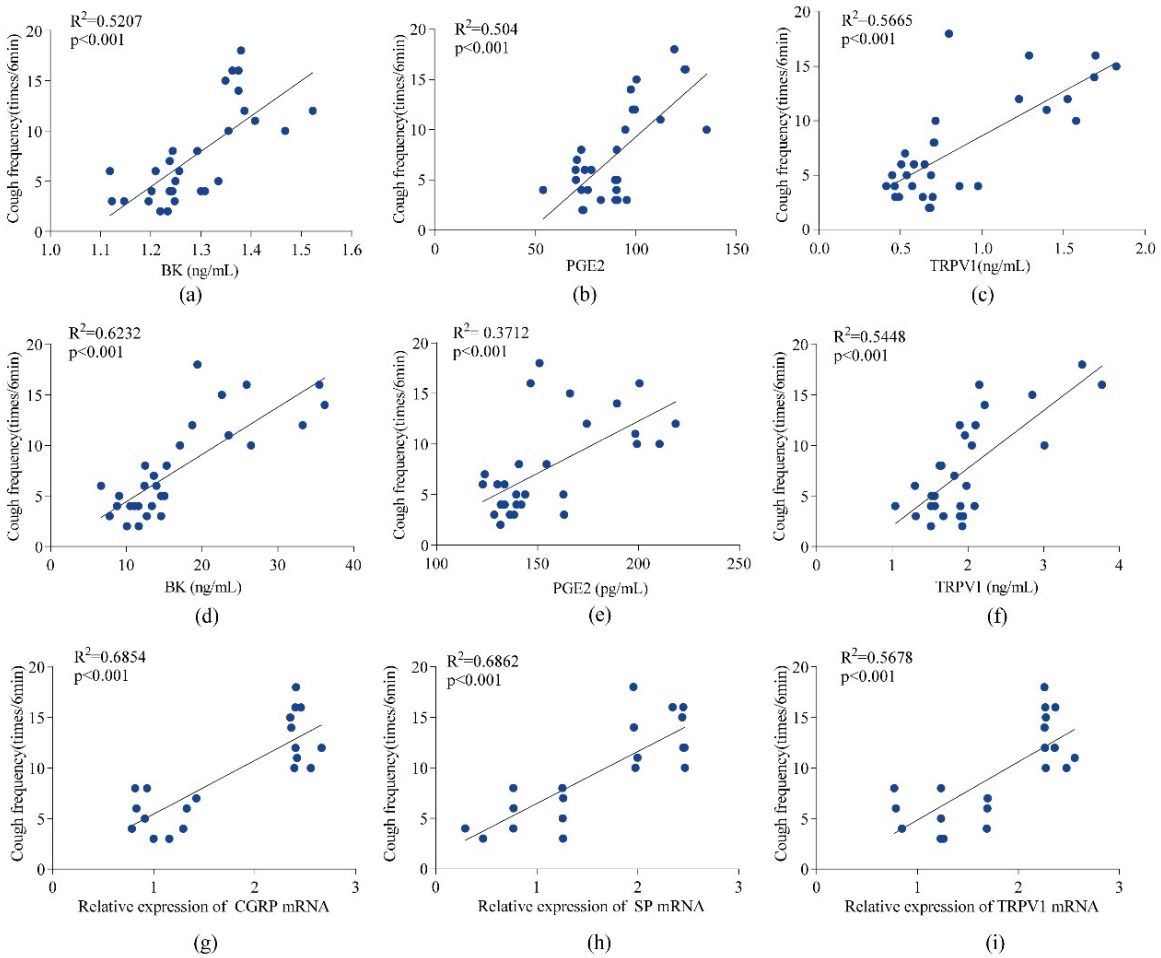

Fig. 8. Correlation analysis. (a) The level of BK was positively related to cough frequency ($p < 0.001$, $r^2 = 0.5207$). (b) The level of PGE-2 was positively related to cough frequency ($p < 0.001$, $r^2 = 0.504$). (c) The level of TRPV1 was positively related to cough frequency ($p < 0.001$, $r^2 = 0.504$). (d) The level of BK in blood was positively related with cough ($p < 0.001$, $r = 0.6232$). (e) The level of PGE-2 was positively related with cough ($p < 0.001$, $r^2 = 0.3712$). (f) The level of TRPV1 in blood was positively related with cough frequency ($p < 0.001$, $r^2 = 0.5448$). (g) CGRP expression was positively related with cough frequency ($p < 0.001$, $r^2 = 0.6854$). (h) SP expression was positively related with cough frequency ($p < 0.001$, $r^2 = 0.6862$). (i) TRPV1 also promotes cough in lung tissues ($p < 0.001$, $r^2 = 0.5678$).

Besides, 0.01 mol/L TBS solution (pH 6.0) was used for antigen repair by microwave heating and 3% H_2O_2 solution (10 min) was used to inactivate endogenous peroxidase. Normal goat serum blocking solution was added. Primary antibody (1:100) (AF8396, Affinity, Jiangsu, China) was added to cell lysates and incubated at 4 °C overnight. After rewarmed at room temperature for 10 min, secondary antibody (1:100) (AF8396, Affinity, Jiangsu, China) solution was added and incubated at 37 °C water bath for 30 min. Subsequently, horseradish enzyme-labeled streptomyces ovalbumin solution was added. After DAB staining, it was washed with distilled water, followed by staining with hematoxylin, dehydrating, transparenting, and fixing. Finally, photos were taken. Immunofluorescence was used to detect the expression of p-TRPV1 proteins in lung.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software (v8.0, GraphPad Software Inc., San Diego, CA, USA). Continuous data were checked for normality of distribution, using Shapiro-Wilk test. Normally distributed continuous data were described as mean ± standard deviation (SD) and compared using student's *t* test, when comparing two groups, resorting to Analysis of the Variance (ANOVA) when comparing more than two groups. Whenever a statistically significant difference was found between more than two groups, each pair were compared. Bonferroni correction is used to correct the problems of repeat comparisons, rather than compare variables. Correlation analysis of two or more sample rates (constituent ratio) and two categorical variables using the chi-square test. Correlation between variables was checked for using Spear-

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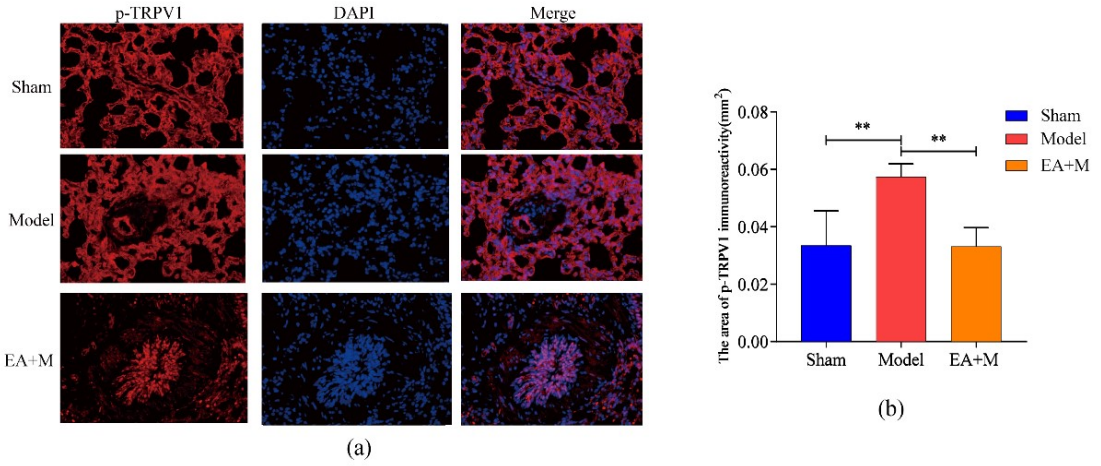

Fig. 9. Immunofluorescence assays of p-TRPV1 protein expression. (a) p-TRPV1 expression in guinea pigs' lung tissues. (b) In contrast to the Sham group, the positive area of p-TRPV1 immune response in the Model group was larger. In contrast to the Model group, the positive area of p-TRPV1 immune response in the EA+M lung tissue was decreased with statistical significance. $^{**}p < 0.01$.

Table 1. Primer sequences.

Gene	Primer	Sequence (5′-3′)	PCR Products
b-actin	Forward	CACGATGGAGGGGCCGGACTCATC	240 bp
	Reverse	TAAAGACCTCTATGCCAACACAGT	
TRPV1	Forward	CAGTGGGAAGATTGGGGTCT	207 bp
	Reverse	TCATGGCGATTAGGGGTCTC	
SP	Forward	CGGAGGAAAACACAGCCATT	240 bp
	Reverse	ACTCACAGATGGTCAGTCGG	
CGRP	Forward	CGAAGGACTCTAGCTCACCG	193 bp
	Reverse	CCAGGTCTAGGCTGTTGTCT	

man's correlation analysis. A p value of <0.05 was considered statistically significant.

Results

Patient Group and Disease Composition

A total of 40 patients were recruited in this experiment. Their baseline features are in Table 2 In the experiment, 4 patients dropped out due to disease progression, and 2 patients withdrew, and total of 34 patients completed treatment.

LCQ-MC Score, EORTC-C30 QOL Score and EORTC-LC13 Dyspnea Score before and after Treatment

In LCQ-MC physical dimension, the score in the EA group was 4.74 ± 0.99 before treatment and 6.10 ± 0.53 after treatment ($p < 0.001$). However, in other dimensions, the scores in the EA and control groups were not statistically significant before and after treatment (Fig. 2a). And for the EA group, the total LCQ-MC score was 14.90 ± 1.77 before treatment and 15.01 ± 1.44 after treatment ($p < 0.001$). In the control group, the total LCQ-MC score before and after treatment was not significantly different ($p > 0.05$). (Fig. 2b) After 28 days of treatment, the EORTC-C30 QOL score in the EA group was 4.76 ± 0.64, which was higher than that before treatment 2.38 ± 0.49 ($p < 0.001$) as well as the control group (3.94 ± 0.83) ($p < 0.001$) with statistical significance (Fig. 2c). Besides, EORTC-LC13 dyspnea score in the EA group after treatment (2.07 ± 0.41) was less than that in the EA group before treatment (2.78 ± 0.51) ($p < 0.001$) and that in the control group after treatment (2.53 ± 0.66) ($p < 0.05$) with statistical significance (Fig. 2d).

SP, CGRP, BK, PGE-2 and TRPV1 Expressions before and after Treatment

After treatment, the expression levels of BK, ProstaglandinE2 (PGE-2), SP, CGRP, TRPV1 in the EA group were all significantly lower than that in the control group ($p < 0.05$), and were significantly lower than that before treatment ($p < 0.05$). In contrast, there was no statistical significance in the expression of each group before and after treatment in the control group ($p > 0.05$) (Fig. 3).

Table 2. Baseline clinical and demographic characteristics.

Characteristic		EA group	No therapy group	*p*
N		20	20	
T stage, n (%)				0.433
	T1	12 (30%)	8 (20%)	
	T2	5 (12.5%)	9 (22.5%)	
	T3	3 (7.5%)	3 (7.5%)	
N stage, n (%)				1.000
	N0	8 (20%)	8 (20%)	
	N1	7 (17.5%)	6 (15%)	
	N2	5 (12.5%)	6 (15%)	
M stage, n (%)				0.231
	M0	17 (42.5%)	20 (50%)	
	M1	3 (7.5%)	0 (0%)	
Stage, n (%)				0.357
	I	8 (20%)	4 (10%)	
	II	4 (10%)	9 (22.5%)	
	III	4 (10%)	4 (10%)	
	IV	4 (10%)	3 (7.5%)	
Age, mean ± SD (years)		56.25 ± 11.93	58.75 ± 11.31	0.501
Gender, n (%)				0.748
	Female	9 (22.5%)	7 (17.5%)	
	Male	11 (27.5%)	13 (32.5%)	
Smoke, n (%)				0.751
	NO	12 (30%)	10 (25%)	
	Yes	8 (20%)	10 (25%)	
cardiovascular disease, n (%)				1.000
	NO	10 (25%)	9 (22.5%)	
	Yes	10 (25%)	11 (27.5%)	

In vitro Experiment

Cough Frequency in Guinea Pigs

In contrast to the Model group, the numbers of coughs in the Sham group (3.8 ± 1.32 times/6 min) and the EA+M group (5.4 ± 1.90 times/6 min) were significantly fewer ($p < 0.01$) (Fig. 4).

PGE2, BK and TRPV1 Protein Expression in BALF of Guinea Pigs by ELISA

The expression of BK, PGE-2, and TRPV1 proteins in BALF of guinea pigs in the Model group were different with either the Sham group ($p < 0.01$) or the EA+M group ($p < 0.01$) with statistical significance. But the expressions of BK, PGE-2, and TRPV1 proteins in the Sham group and the EA+M group were not significantly different ($p > 0.05$) (Fig. 5).

Expression of PGE-2, BK and TRPV1 in the Blood of Guinea Pigs by ELISA

After treatment, the expression levels of BK, PGE-2 and TRPV1 in the EA+M group were significantly lower than those in the model group ($p < 0.01$), which proved that electroacupuncture could significantly reduce the release of TRPV1 and its related inflammatory factors, which was consistent with our hypothesis. At the same time, the expression levels of BK, PGE-2 and TRPV1 in the model group were significantly higher than those in the sham model group, which proved that TRPV1 and related inflammatory factors in guinea pigs increased significantly after surgery ($p > 0.05$) (Fig. 6).

TRPV1, SP as well as CGRP mRNA Levels in Lung Tissues of Guinea Pig by Quantitative Real-Time PCR (qRT-PCR)

The mRNA levels of CGRP, SP and TRPV1 in the lung tissues of guinea pigs in each group were detected by qRT-PCR. The expression trends of CGRP, SP and TRPV1 in the three groups were consistent. Compared with the Model group, the mRNA expression of CGRP, SP and TRPV1 in the EA+M group was significantly decreased, and the difference was statistically significant ($p < 0.01$). At the same time, the expression of the Sham group was also significantly lower than that of the model group, which was consistent with our hypothesis ($p < 0.01$) (Fig. 7).

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Correlation Analysis

The correlation between cough frequency and other observed factors in guinea pigs was analyzed. After pneumonectomy, the expressions of BK, PGE-2 and TRPV1 levels in BALF were all positively correlated with cough frequency ($p < 0.001$) (Fig. 8a–c). At the same time, the levels of BK, PGE-2 and TRPV1 in blood were all positively correlated with the frequency of cough ($p < 0.001$) (Fig. 8d–f), which was consistent with the results in BALF. Finally, the expressions levels of CGRP, SP, and TRPV1 were all positively correlated with cough frequency ($p < 0.001$) (Fig. 8g–i).

Immunofluorescence Assays of p-TRPV1 Protein Expression in Lung Tissue

Based on immunofluorescence labeling, the expression of p-TRPV1 was detected in the guinea pigs' lung tissues (Fig. 9a). On the 15th day of CC modeling after pneumonectomy, the positive area of p-TRPV1 immune response in the guinea pigs' lung tissues in the CC model was larger than that in the Sham group ($p < 0.01$) and that in the EA+M group ($p < 0.01$) significantly (Fig. 9b). Thus, acupuncture might inhibit the expression of TRPV1 in lung tissue through TRPV1 receptor-dependent manner.

Discussion

As a pivotal element of traditional Chinese medicine (TCM), acupuncture has been applied in various lung diseases including cough. Studies have demonstrated that TRPV1 channel is a critical factor in acupuncture treatment [30,31]. In previous studies [23], we have proved that acupuncture could significantly alleviate the symptoms of patients with CC after LC surgery. However, its mechanism is still unclear and rarely studied. This is also the focus of this study.

Continuous cough after pneumonectomy is a common complication after pneumonectomy. Keller *et al.* [32] confirmed that compared with non-mediastinal lymph node dissection, mediastinal lymph node dissection increased the probability of postoperative refractory and intractable cough. Our previous studies have shown that right LC, respiratory difficulties, and acute cough were independent factors predicting postoperative CC [23]. In addition, the occurrence of cough after LC surgery was also related to the drugs, the method of anesthesia and the time of anesthesia [6]. Most of the existing studies believed that postoperative LC could stimulate the respiratory system by various physical and chemical factors, such as postoperative airway physical changes.

C fibers are one of the cough receptors and highly sensitive to chemical stimuli in the airway, such as different inflammatory mediators [33,34]. As a ligand-gated non-selective cation channel protein, TRPV1 is located at the end of C fiber and closely related to the occurrence of cough [35]. TRPV1 was found to be involved in the mechanism of CC [23,36]. Clarke *et al.* [37] have shown that TRPV1 expression in respiratory nerve fibers among CC patients was 4 times higher than that among healthy people. Guan *et al.* [38] discovered that the expression of TRPV1/TRPA1 was up-regulated in the guinea pig model of CC. After activated, C fibers could release neuropeptides, including SP as well as CGRP, thereby inducing airway neurogenic inflammation in the and increasing the sensitivity to cough. The use of trpv1-/-mice showed that TRPV1 is required for capsaicin and anandamide to activate C fibers, and this channel plays a regulatory role in bradykinin and acid-induced effects. Xu *et al.* [39] and found that C fibers activated by TRPV1 released SP and CGRP and led to subsequent airway neurogenic inflammation. Subsequently, neuropeptides might exert an effect on C fibers in the airway, resulting in increased expression of TRPV1 and changed plasticity of airway, establishing a positive feedback loop in the formation of cough hypersensitivity. Grace *et al.* [40]. found that the expression of PGE2 and BK protein were associated with CC after lung surgery, which was consistent with our previous results. Therefore, TRPV1 plays an important role in cough reflex. However, the above studies were all about internal diseases, and the related studies on CC and TRPV1 activation after lung surgery have not been reported yet.

In this study, we found that the BK, PGE-2, SP, CGRP, TRPV level of patients with CC treated with electroacupuncture was significantly lower than that of the control group, and the QOL and cough symptoms of patients were significantly improved, which proved the potential of electroacupuncture treatment for postoperative CC in patients with LC. In animal experiments, we found that the inflammatory factor BK, PGE-2 was positively correlated with cough. As upstream inflammatory factors of TRPV1, BK and PGE-2 can activate neurogenic inflammatory response as well as increase the expression of TRPV1, which was also confirmed in our experiments. In the downstream pathway of TRPV1, SP and CGRP were also highly expressed, which was in accordance with the results of previous studies. Besides, compared to the Model group and the Sham group, the cough frequency along with SP and CGRP expression of guinea pigs in the EA treatment group were much lower, indicating that electroacupuncture may relieve cough by blocking the expression of SP and CGRP. Our study proved that electroacupuncture might improve CC after LC surgery.

However, our study also had several limitations. Firstly, the sample size was relatively small, which limited the universality of acupuncture and moxibustion in the treatment of CC after LC operation. In addition, we have not studied how TRPV1 reduced the expression of SP and CGRP, which needs to be further explored in our follow-up research.

Conclusions

Our results showed that TRPV1 was closely related to CC and exerted a pivotal effect on the anti-myocardial deficiency-mediated CC of TRPV1. This mechanism may be linked with the decreased expression of TRPV1 after electroacupuncture treatment. The effect of electroacupuncture on CC was found to be closely related to the expression of TRPV1 and downstream SP and CGRP, providing an experimental basis for the clinical treatment of CC.

Author Contributions

YFZ—is responsible for design research and paper revision; PCZ—is responsible for animal experiments and paper writing; JZ and LW—are responsible for clinical experiments and data processing; YMC, YHS, JM—is responsible for data collection and preliminary analysis; GXW—contributed to the method and provided software; MRX and MQZ—are responsible for editing manuscripts and contributing papers. All the authors made contributions and agreed to submit the materials.

Ethics Approval and Consent to Participate

The study followed the Helsinki Declaration, and was approved by the Ethics Committee of Anhui University of Traditional Chinese Medicine (Ethical number: 2022MCZQ21). The animal experiment was approved by the Experimental Animal Ethics Committee of Anhui University of Chinese Medicine (Ethical number: AHUCM-mouse-2022059).

Acknowledgment

Not applicable.

Funding

This work was supported by the grants from the Opening Project of Zhejiang Provincial Preponderant and Characteristic Subject of Key University (Chinese Traditional Medicine), Zhejiang Chinese Medical University, the National Natural Science Foundation of China, the Key Projects of Natural Science Research in Anhui Universities in 2021, The Fundamental Research Funds for the Central Universities and Key research and development projects in Anhui Province (No. ZYXZD2019004, 81973643, KJ2021A0557, WK9110000021, and 202004j07020017), Zhejiang Chinese Medicine Science and Technology Project (No. 2023ZL467).

Conflict of Interest

The authors declare no conflict of interest.

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