Effects of *Gubenkeli* capsule on matrix metallopeptidase 9 and tissue inhibitor of metalloproteinase1 levels in lung tissue of a rat model of chronic obstructive pulmonary disease*★

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Abstract

BACKGROUND: The effectiveness and safety of traditional Chinese medicine treatment for chronic obstructive pulmonary disease (COPD) have been preliminarily approved by clinical practices.

OBJECTIVE: To investigate the effects of *Gubenkeli* capsule on the protein expression of matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of metalloproteinase1 (TIMP-1) in lung tissue of COPD model rats.

METHODS: A total of 50 Wistar rats were randomly divided into five groups with 10 rats in each group: normal control, model, prednisone, *Gubenkeli* capsule-low dose, and *Gubenkeli* capsule-high dose. COPD rat models were established in all rats with the exception of the normal control rats by smoking and intratracheal instillation of LPS. At 29 days after COPD induction, rats from the prednisone, *Gubenkeli* capsule-low dose, *Gubenkeli* capsule-high dose groups were intragastrically administered prednisone (1.04 mg/kg per day), *Gubenkeli* capsule (0.4, 0.94 g/kg per day), once a day, to observer rat general conditions. Protein expression of MMP-9 and TIMP-1 in the lung tissue was detected by immunohistochemical methods.

RESULTS AND CONCLUSION: Protein expression of MMP-9 and TIMP-1 in the lung tissue of COPD rats was significantly increased ($P < 0.05$). After drug intervention, the general conditions of COPD rats were greatly improved, and protein expression of MMP-9 and TIMP-1 in the lung tissue was decreased. Prednisone yields the strongest effects, followed by high-dose *Gubenkeli* capsule and low-dose *Gubenkeli* capsule. These findings demonstrate that *Gubenkeli* capsule alleviates the clinical manifestations of COPD model rats, improve airway remodeling, and correct the imbalance between prolease and antiprotease in a dose-response manner.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a disease characterized by airflow limitation which cannot be completely reversed with progressive degeneration. Its morbidity is positively correlated with smoking index (smoking packs/day × years as a smoker) [1-2]. Most current therapies for COPD are symptomatic treatment and chronic airway inflammation treatment[3-8]. However, these therapies are not able to reverse airway remodeling or prevent progressive decline in lung function.

Traditional Chinese medicine treatment for COPD has advantages of good efficacy without side-effect and low recurrence and it is becoming a hot research in recent years. Therefore, it has important implications in the clinical and mechanismic research on the traditional Chinese medical treatment for COPD. This study investigated the effects of *Gubenkeli* capsule on the matrix metallopeptidase 9 (MMP-9) and tissue inhibitor of metalloproteinase1 (TIMP-1) levels in lung tissue of a rat model of COPD.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Tine and setting

All experiments were performed at Qingdao Hiser Medical Group between January and May 2009.

Materials

A total of 50 male Wistar rats, aged 2–3 months old, weighing (200±20) g, of specific pathogen-free grade, were purchased from Shandong LuKang Medical Group Co., Ltd., China (license No. SCXK (lu) 20050017) and were raised for 1 week at 18–22 ℃ and relatively humidity 28%.

Gubenkeli capsule was produced by Department of Pharmaceutics of Qingdao Hiser Medical Group. It comprises processed *radix aconiti lateralis*, *rhizoma zingiberis*, *schisandra chinensis*, *cortex moutan, earthworm*, *dodder seed*, *rehmanniae vaporata* and *raidx astragali* with the ratio of 0:10:10:10:10:10:15:15. Each capsule contains 0.5 g crude drug. 0.05 g/mL and 0.1 g/mL suspension was prepared before use.

Main reagents and instruments

Reagent and instrument	Source
Lipopolysaccharide (LPS)	Sigma, USA
Prednisone (0.066 mg/mL)	Tianjin Tianyao Pharmaceutical Co., Ltd., China
Daqianmen cigarettes	Shanghai Cigarette Factory, China
Rabbit anti-mouse TIMP-1 antibody	Wuhan Boster Bioengineering Co.,Ltd., China
Rabbit anti-mouse MMP-9 antibody, diaminobenzidine (DAB) color kit	Beijing Zhongshan Biological Reagents Co., Ltd., China
OLYPUS-CH microscope and micrographic device	Olympus, Shinjuku-ku, Tokyo, Japan

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Methods

Experimental grouping

A total of 50 male Wistar Rats were randomly divided into five groups with 10 rats in each group: normal control, model, prednisone, *Gubenkeli* capsule-low dose, and *Gubenkeli* capsule-high dose. They were fed with regular fodder, 5 rats per cage.

Model preparation and intervention

Rat models of COPD were established by adopting smoking and intratracheal instillation of LPS[9-12], which are currently used in domestic and international research. Except for the normal control group, the rats together with the cages were placed in the homemade smoking plexiglass box (60 cm × 120 cm × 80 cm) which was connected with a negative pressure pump on one side and a cigarette socket on the other side. The smoke was pumped into the box as the electric current of the pump was set up after lighting the cigarettes. Only 12 cigarettes were lighted for 30 minutes at one time and then fresh air ventilation was provided afterwards in order to prevent the rats from hypoxic heaths. The smoking process was conducted twice a day and lasted for 8 weeks. On the 1st, 14th, 21st, 28th days, the rats are anaesthetized through intraperitoneal injection of 10% chloral hydrate (10 mL/kg). The tongues of the rats were pulled out and the LPS physiological saline (200 μL) was injected into their lungs through bronchus with elbow gavage needles. The solution had already been injected into their lungs judging from big bubbles could be clearly heard. For the normal control group, the rats were not smoked but injected with equivalent physiological saline using the same method. Each group was administered once per day continuously from the 29th day, till the end of the experiment. Dosage: 15.8 mL/kg per day for prednisone, 9.4 mL/kg per day for low dose *Gubenkeli* capsule, 18.8 mL/kg per day for high dose *Gubenkeli* capsule and 2 mL/d for physiological saline in the normal control and model groups. Intragastric administration was used in all groups.

Preparation of tissue samples

The rats were anaesthetized by intraperitoneal injection of 10% chloral hydrate (10 mL/kg) 24 hours after the last smoking, and then fixed. The trachea was isolated from the neck and the thoracic cavity was opened. The right lung tissue was taken out from the hilus pulmonis, fixed with 40 g/L paraformaldehyde, dehydrated with a gradient ethanol series, embedded with paraffin, and sliced for immunohistological staining.

Immunohistological staining

MMP-9 and TIMP-1 levels in the lung tissue were determined by immunohistochemical method. Specific instructions were followed by the kit instructions. The positive reaction is that the cytoplasm shows brown color under optical microscope. PBS was used as primary antibody for negative control. With regard to the protein expressions of MMP-9 and TIMP-1, the semi-quantitative scoring system proposed by Bresalier *et al*[13] was adopted to mark immunohistochemical staining level: 10 high power fields (400 ×) were randomly selected for each slice. Each field was divided into negative staining (0 point), yellow staining (1 point), claybank staining (2 points), and brown staining (3 points) according to staining levels. For a respective field, the product of each staining intensity score and its percentage as well as the sum of four products were calculated. The mean value of ten fields was the result of each slice. The formula is as follows, Is = (0×F0) + (1×F1) + (2×F2) + (3×F3), where Is is the score of each field and Fi (I = 0, 1, 2, 3) is the percentage that each level accounts for.

Main outcome measures

MMP-9 and TIMP-1 protein expression.

Statistical analysis

Statistical software (SPSS 11. 0) was employed to perform statistical analysis. The results were expressed by Mean ± SD. Normal test and analysis of variance were performed for data in each group. If the data meet the normal distribution and homogeneity of variance, one-way analysis of variance was adopted. For comparison among groups, the Least Significant Difference method was employed. If the data meet heterogeneity of variance, multi-rank test was conducted. The significance level was chosen as $P < 0.05$.

RESULTS

Quantitative analysis of experimental animals

All 50 rats were included in the final analysis.

General appearances

The COPD model rats presented the symptoms of grasping, nodding breathing, deep and quick breath, and abdominal muscle spasm. However, symptoms vary from each other in each group. The rats in the model group breathed more difficultly as the progression of experimentation. The most severe rats breathed slowly or arhythmically, and were of slight cyanosis, limbs limp, delayed action or motionless proneness. Their body weight increased slowly and their hair color lost luster. In each treatment group, the symptoms like dyspnea gradually alleviated with drug therapy proceeding. Improvements in the symptoms were more significant in the prednisone group and *Gubenkeli* capsule-high dose group.

MMP-9 and TIMP-1 protein expression in the lung tissue

Compared with the normal control group, MMP-9 and TIMP-1 protein expression in the model group was significantly increased ($P < 0.05$). Compared with the

model group,MMP-9 and TIMP-1 expression was significantly decreased in the prednisone, *Gubenkeli* capsule-low dose and *Gubenkeli* capsule-high dose groups ($P < 0.05$), especially in the prednisone and *Gubenkeli* capsule-low dose groups (Table 1).

Table 1 Matrix metallopeptidase 9 (MMP-9) and tissue inhibitor of metalloproteinase1 (TIMP-1) protein expression in the lung tissue ($\bar{x}\pm s$, n=10)

Group	MMP-9	TIMP-1
Normal control	0.40±0.08	0.41±0.07
Model	0.87±0.05[a]	1.04±0.06[a]
Prednisone	0.55±0.08[ab]	0.62±0.05[ab]
Gubenkeli capsule-low dose	0.71±0.10[abc]	0.86±0.10[abc]
Gubenkeli capsule-high dose	0.63±0.06[abcd]	0.55±0.09[abd]

[a]$P < 0.05$, *vs.* normal control group, [b]$P < 0.05$, *vs.* model group, [c]$P < 0.05$, *vs.* prednisone group, [d]$P < 0.05$, *vs. Gubenkeli* capsule-low dose group.

DISCUSSION

Yang deficiency of lung and kidney, deficiency of both *Qi* and blood as well as stagnation of *Qi* and blood stasis are the basic pathogenesis of COPD in remission. So its treatment should be based on warming and nourishing lungs and kidneys, replenishing *Qi* and consolidating body surface as well as nourishing blood and activating blood circulation. *Gubenkeli* capsule is manufactured in the form of granules according to the above pathogenesis and therapeutic methods. In this prescription: processed *radix aconiti lateralis*, *rhizoma zingiberis* and *dodder seed* are able to warm and nourish lungs and kidneys in addition to dispelling cold. *Raidx astragali* will replenish *Qi* and consolidate body surface. Schisandra chinensis can converge lung *Qi* and nourish kidney due to its acid and warm nature, and its effect of closing together with opening effect of *rhizoma zingiberis* will complement each other. The combined effect of *cortex moutan* and earthworm is able to activate blood circulation and remove obstruction in channels to drive and regulate *Qi*. *Rehmanniae vaporata* nourishes blood. All of them will make an impact on warming and nourishing lungs and kidneys, replenishing *Qi* and consolidating body surface as well as nourishing blood and activating blood circulation. Patients will recover from COPD due to the recovery of *Yang Qi*, consolidation of body surface and regulation of *Qi* and blood.

It is shown in the modern research that COPD is a kind of chronic inflammatory disease that can be prevented and treated, whose pathological changes mainly behave airway remodeling. It is regarded that the imbalance between prolease and antiprolease is one of the main mechanisms of airway remodeling[14]. In this study, the effect of *Gubenkeli* capsule on prolease and antiprolease in COPD model rats is evaluated in quantification along with the comparison of efficacy advantage to investigate the onset mechanisms of *Gubenkeli* capsule on prolease and antiprolease.

The imbalance between the degradation and aggradation of extracellular matrix (ECM) is the main cause for airway remodeling and lung parenchyma damage and interstitial proliferation. MMPs are the main rate-limiting enzyme in regulating the metabolism of ECM, whose leading role is to degrade ECM and basement membrane. MMP-9 is one member of the MMPs family, and it plays an important role in airway damage of COPD due to smoking as well as pulmonary vascular damage[15-17]. It can not only degrade elastin and collagen to induce irreversible damage of alveolar surface and elastin, alveolar wall damage and rebuilding of lung structure, but also regulate the activities of other prolease and cell factors. In addition, it can degrade 1-antitrypsin to protect the activity of elastase in neutrophil and strengthen the activities of collagen in collagen gel and procollagen in MMP-13, and it can participate in the angiogenesis through releasing vascular endothelial growth factor[18]. Overall, MMP-9 plays an important part in COPD development.

TIMPs are a multi-factor family which endogenously inhibits the activities of MMPs. TIMP-1 is a specific inhibitor for MMP-9, which can be combined with active MMP-9 as well as zymogen with a ratio of 1:1. It inhibits the biological effect of MMP-9 and protects the substrate from degradation in two ways[19-20]: to prevent activation of MMP-9 zymogen and to inhibit the enzymatic activity of MMP-9 which have been activated. It is also testified that TIMP-1 has biological characteristics in promoting fibroblast hyperplasia and improving collagen synthesis[21]. Therefore, the increase of TIMP-1 is a sign of airway fibrosis[22], reflecting the process of airway remodeling. It is demonstrated that MMP-9 and TIMP-1 protein expression levels increase significantly in the model control group, particularly the TIMP-1, which is coincided with the study of Li *et al*[23], indicating that it is very active in degradation and aggradation of ECM, implying the rebuilding and remodeling exist as the histology is being damaged. *Gubenkeli* capsule is capable of helping relieve the clinical features and airway inflammation of the COPD model rats. *Gubenkeli* capsule in the high dose and low dose groups can inhibit the overexpression of MMP-9 and TIMP-1 protein, regulate the dynamic balance of MMP-9/TIMP-1, intervene in the process of airway remodeling, and protect the airway structure. The mentioned activities have a dose-response relationship, namely, the activities increase with increasing dose. Hereby, medical workers should control the dose appropriately according to the patients' conditions in order to guarantee the survival quality.

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慢性阻塞性肺疾病模型大鼠肺组织基质金属蛋白酶 9 及组织金属蛋白酶抑制剂 1 与固本颗粒胶囊*★

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摘要

背景：中医药防治慢性阻塞性肺疾病(chronic obstructive pulmonary disease, COPD)的有效性和安全性已初步得到临床认证。

目的：观察 COPD 模型大鼠肺组织中基质金属蛋白酶 9 及其特异性抑制物组织金属蛋白酶抑制剂 1 表达与固本颗粒胶囊干预的影响。

方法：将 50 只 Wistar 大鼠随机等分为 5 组，除正常组外，其余大鼠均以烟熏及气管内滴注脂多糖的方式建立 COPD 模型。造模 29 d，泼尼松组、固本颗粒胶囊低、高剂量组分别灌胃给予醋酸泼尼松 1.04 mg/(kg•d)，固本颗粒胶囊 0.47，0.94 g/(kg•d)，1 次/d，观察记录大鼠的一般状况。免疫组织化学方法检测大鼠肺组织中基质金属蛋白酶 9 及组织金属蛋白酶抑制剂 1 的表达。

结果与结论：COPD 大鼠肺组织中基质金属蛋白酶 9 及组织金属蛋白酶抑制剂 1 的表达显著增强($P < 0.05$)。药物干预后，COPD 大鼠的一般状况明显改善，肺组织中基质金属蛋白酶 9 及组织金属蛋白酶抑制剂 1 的表达有所降低；其中，醋酸泼尼松的作用最为显著，固本颗粒高剂量次之，低剂量最弱。说明固本颗粒胶囊能以剂量依赖的方式缓解 COPD 大鼠的临床表现，改善气道重塑，纠正 COPD 大鼠体内蛋白酶和抗蛋白酶失衡。

关键词：固本颗粒胶囊；慢性阻塞性肺疾病；动物模型；基质金属蛋白酶 9；组织金属蛋白酶抑制剂 1；肺组织；大鼠

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提供证据：检索维普、万方及 Pubmed 数据库，检索时间为建库至 2009-05，检索关键词为：“COPD, MMP-9, TIMP-1”，共检索到中文文献 18 篇，英文文献 30 篇，未见与本文研究内容密切相关的文献。

创新点说明：课题在遵循中医传统理论的前提下，汲取现代科技，制定可操作性强、可量化和标准化的辨证分型方法，建立新的富有现代科学气息的慢性阻塞性肺疾病证候学。并研制开发出能有效防治慢性阻塞性肺疾病的新药固本颗粒。