Original Article

Effects of *Angelica Gigantis* Pharmacopuncture Extracts on the Elastase Activity and DPPH and NO Scavenging Activities

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국문초록

당귀약침액의 Elastase 효소 활성과 DPPH, NO 소거능에 미치는 영향

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목적 : Elastin 섬유는 피부, 폐, 동맥, 정맥 등 조직에서 발견된다. Elastin 섬유의 손실은 대동맥, 동맥, 세동맥의 탄력 등과 연관되어 폐기종, 혈액순환 부전 및 폐동맥 고혈압을 악화시킬 수 있다. 본 연구는 당귀약침액의 elastase 효소활성에 대한 효과와 항산화 효과에 대하여 연구하고자 계획되었다.

방법 : 당귀약침액의 elastase 억제효과와 DPPH, 및 NO 소거능을 측정하여 항산화 효과를 측정하였다.

결과 : 당귀약침액에서 통계적으로 유의한 elastase 활성 억제효과를 관찰할 수 있었다. 또한 통계적으로 강한 DPPH free radical 소거 효과가 관찰되었다. 그리고 NO 소거효과를 관찰할 수 있었으며, 그 효능은 pH 6.0에서 가장 강한 효능을 보였다.

결론 : 본 연구 결과에 의하면, 당귀약침액에는 유효한 elastase 활성 억제효과와 강한 항산화 효과가 있어, 폐기종 및 폐동맥 고혈압 치료에 유효한 작용이 있을 것으로 사료된다.

핵심 단어 : 당귀약침액, elastase, DPPH, NO, 항산화 효과, 폐기종, 폐동맥고혈압

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Ⅰ. Introduction

Acute lung injury is a kind of syndrome, caused by many different factors. It manifests itself when clinical, radiological and biological disorders arise from inflammation of the lungs and increase of permeability[1]. According to the recent studies, the core reason for actue lung injury is that elastase secreted by activated neutrophils damages vascular endothelial cell and alveolar epithelial cell. For this reason, administering a neutrophil elastase suppressant is thought to be effective in helping prevent and cure acute lung injury[2-6].

Elastase is an enzyme from the class of proteases (peptidases) that breaks down a kind of proteins. It breaks down elastin, an elastic fiber that, together with collagen, determines the mechanical properties of connective tissue[7]. This neutrophil elastase is a potent non specific serine protease which plays a role as bactericidal agent and in the degradation of immune complexes by intra-hagosomal processes. It promotes inflammation, pulmonary emphysema, and chronic obstructive pulnary disease. Clinical studies for human pulmonary hypertension and systolic left ventricular failure are now in progress as well[8-11].

The production of reactive oxygen species (ROS) was induced by neutrophil elastase[12,13]. They reported that neutrophil elastase enhancement of Mucin 5, subtypes A and C (MUC5AC) messenger Ribonucleic acid (RNA) levels was dependent on the production of intracellular oxidants or an alteration in the redox state of the cell. It means that ROS may play a role in elastase mediated inflammation. Nitrite plays an pivotal role in elastase mediated diseases as well[14,15].

Angelicae Gigantis Radix is the root of *Angelica gigas* Nakai[16]. It's effects are to tonify blood, invigorate blood circulation, relieve pain, moisten intestines, and unblock the bowels[16,17].

In the present study, I investigated the effects of Angelicae Gigantis Pharmacopuncture Extracts (AGHAE) on elastase activity. Anti-oxdative activities of AGHAE were also examined via measuring the 1,1-iphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and nitrite scavenging activities.

Ⅱ. Materials and methods

A. Sample preparation

Angelica Gigantis Radix was purchased from Omniherb (Korea). AGHAE was prepared as follow. 100g of Angelica Gigantis Radix in 2,000ml distilled water was heated in a heating extractor for 3hours. The extract was filtered and concentrated by using the rotary evaporator. The extracts were lyophilized by using freeze dryer (12.2g). The extract was dissolved in water and filtered three times through micro-filter paper and syringe filter (Whatman #2, 0.45μm to 0.2μm). Filtered material was placed in the disinfected vial.

B. Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

C. Elastase activity inhibition

The elastase activity was evaluated by using a modification of a previously reported method of Kraunsoe et al[18]. In order to evaluate the inhibition of elastase activity, the amount of released p-nitroaniline, which was hydrolyzed from the substrate, N-succinyl-Ala-Ala-Ala-p-nitroanilide, by elastase, was read with a maximum absorbance at 410nm[19]. In brief, 2mM N-succinyl-Ala-Ala-Ala-p-nitroanilide was prepared in a 0.1M Tris - Cl buffer (pH 8.0), and this solution was added to the stock sample. Each sample solution was diluted to final concentrations of 0.1, 1, and 10mg/ml. The solutions were mixed thoroughly by tapping before an elastase (0.1360unit/ml) stock solution was added. Solution was incubated for 10min at 37°C, and the

absorbance was measured at 410nm. The percent scavenging activity was calculated according to the following equation:

Elastase inhibitory activity(%) = [(OD410ofcontrol) - (OD410ofsample)/(OD410ofcontrol)] × 100

D. DPPH free radical scavenging activity

The scavenging effect of sample on DPPH radicals was assayed according to the procedure described by Shimada et al[20]. The DPPH radical shows a deep violet color due to its unpaired electron, and radical scavenging capacity can be followed spectrophotometrically by the loss of absorbance at 540nm[19]. In brief, sample was added to 1ml of freshly prepared ethanolic solution containing a final DPPH radical concentration of 0.2mM. After it stood for 30min in the dark, the absorbance of the mixture was measured at 540nm against an ethanol control with a spectrophotometer. The percent scavenging activity was calculated according to the following equation:

DPPH free radical scavenging activity (%) = [(OD540ofcontrol) - (OD540ofsample)/(OD540ofcontrol)] × 100

E. Nitrite scavenging activity

Nitrite scavenging activity of sample was determined by using Griess reagent[21]. Firstly, 1ml of pharmacopuncture extract was mixed with 1ml of 1mM sodium nitrite. The mixture was added to 8ml of 0.2M citrate buffer (pH 1.2, 3.0, and 6.0) and incubated for 1h at 37°C. Then, 1ml aliquot was removed and added to 2ml of 2% acetic acid and 0.4ml of Griess reagent (1% sulfanilic acid and 1% naphthylamine in a methanol solution containing 30% acetic acid). After vigorous vortex mixing, the mixture was placed at room temperature for 15min and the absorbance was measured at 520nm. The nitrite scavenging activity (%) was calculated by the following equation:

Nitrite scavenging activity (%) = [1 - (A - C) / B] × 100

Where, A is the absorbance of treated sample, C is the absorbance of pharmacopuncture, and B is the absorbance of 1mM $NaNO_2$.

F. Statistical analysis

The results were expressed as means±standard error of the mean (SEM). Significances of changes were evaluated using the Students't-test. Values of $p<0.05$ were considered significant.

Ⅲ. Results

A. Inhibition of the elastase activity

The inhibitory effect of AGHAE on elastase activity was determined according to the method described previously. AGHAE showed the elastase inhibitory effect in dose dependent manner. AGHAE was found to inhibit elastase activity highly at a concentration of 10mg/ml (52.5±2.1%). AGHAE 0.1mg/ml and AGHAE 1mg/ml treated groups showed 82.0±3.1% and 71.1±3.5% of elastase

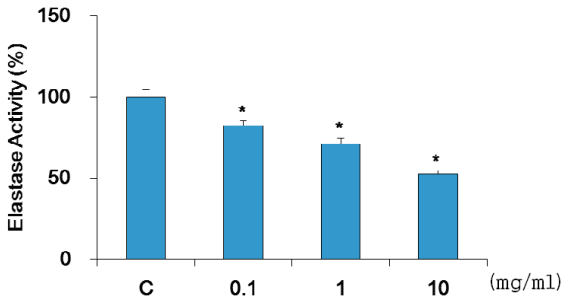

Fig. 1. Effect of AGHAE on inhibition of elastase activity

C : control, distilled water treated group.
0.1, 1, and 10 : AGHAE treated group (0.1, 1, and 10mg/ml).
Data are expressed as the mean±SEM of three experiments.
* : significantly different from control, $p<0.05$.

activities, respectively (Fig. 1).

B. DPPH free radical scavenging capability

Assays of the free radical scavenging capacity were carried out by the DPPH method. The free radical scavenging capacity of sample was measured at each concentration (0, 4, 20, 100, and 500mg/ml). A dose dependent free radical scavenging capability was observed in sample treated groups. AGHAE 100mg/ml treated groups had the highest scavenging capability, of 63.0±2.1%, while 500, 20, and 4mg/ml treated groups had 52.1±23.3%, 28.0±1.7%, and 12.9±1.7%, respectively (Fig. 2).

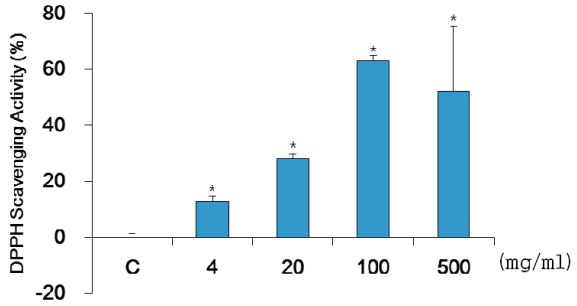

Fig. 2. DPPH free radical scavenging capability

C : control, distilled water treated group.
4, 20, 100, and 500 : AGHAE treated group (4, 20, 100, and 500mg/ml).
Data are expressed as the mean±SEM of three experiments.
* : significantly different from control, $p<0.05$.

C. Nitrite scavenging activity at pH 1.2

Nitrite scavenging activity changes at various pH environments. Accordingly, nitrite scavenging activities at pH 1.2, 3.0, and 6.0 were measured in this study.

The nitrite scavenging activity of sample was measured at each concentration (0, 0.4, 2, 10, and 50mg/ml). A dose dependent nitrite scavenging activity was observed in sample treated groups. AGHAE 50mg/ml treated groups had the highest scavenging activity, of 101.0±0.4%, while 10, 2, and 0.4mg/ml treated groups had 95.2±0.0%, 29.0±0.0%, and -9.6±0.2%, respectively (Fig. 3).

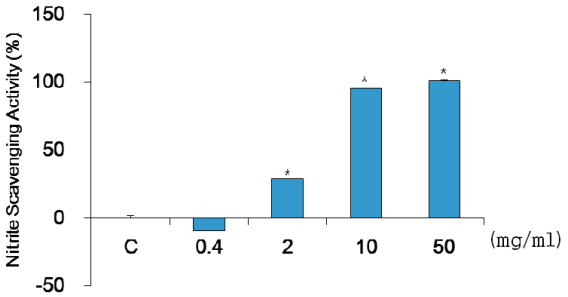

Fig. 3. Nitrite scavenging activity at pH 1.2.

C : control, distilled water treated group.
0.4, 2, 10, and 50 : AGHAE treated group (0.4, 2, 10, and 50mg/ml).
Data are expressed as the mean±SEM of three experiments.
* : significantly different from control, $p<0.05$.

D. Nitrite scavenging activity at pH 3.0

The nitrite scavenging activity of sample was measured at each concentration (0, 0.4, 2, 10, and 50mg/ml). A dose dependent nitrite scavenging activity was observed in sample treated groups. AGHAE 50mg/ml treated groups had the highest scavenging activity, of 56.9±0.0%, while 10, 2, and 0.4 mg/ml treated groups had 42.1±0.0%, 22.8±1.0%, and 7.4±1.0%, respectively (Fig. 4).

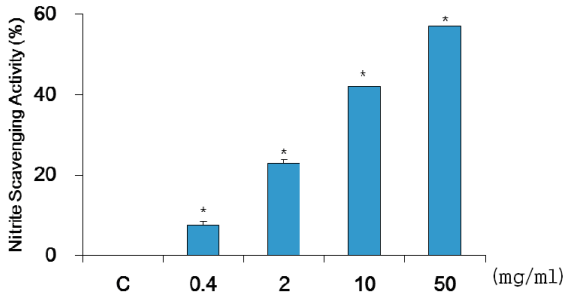

Fig. 4. Nitrite scavenging activity at pH 3.0.

C : control, distilled water treated group.
0.4, 2, 10, and 50 : AGHAE treated group (0.4, 2, 10, and 50mg/ml).
Data are expressed as the mean±SEM of three experiments.
* : significantly different from control, $p<0.05$.

E. Nitrite scavenging activity at pH 6.0

The nitrite scavenging activity of sample was measured at each concentration (0, 0.4, 2, 10, and

50mg/ml). A dose dependent nitrite scavenging activity was not observed at pH 6.0 environment. All concentrations of AGHAE treatment (0.4, 2, 10, and 50mg/ml) showed the significant effects. These scavenging effects are 72.0±0.3%, 69.5±0.2%, 72.2±0.2%, and 73.2±0.2%, respectively (Fig. 5).

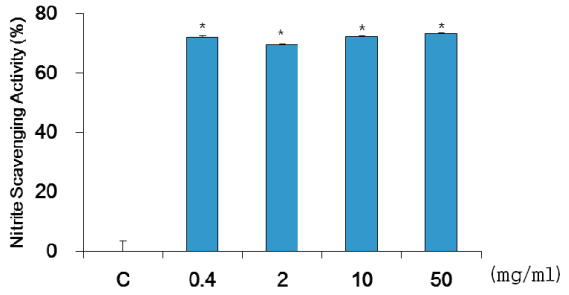

Fig. 5. Nitrite scavenging activity at pH 6.0
C : control, distilled water treated group.
0.4, 2, 10, and 50 : AGHAE treated group (0.4, 2, 10, and 50mg/ml).
Data are expressed as the mean±SEM of three experiments.
* : significantly different from control, $p<0.05$.

Ⅳ. Discussion

Angelicae Gigantis Radix is the root of *Angelica gigas* N_{AKAI}[16]. It's effects are to tonify blood for symptoms of heart and liver blood deficiencies including anemia, pale complexion, brittle nails, dry hair, dizziness, blurred vision, and palpitations. Other effects are to invigorate blood circulation and relieve pain for symptoms of menstrual disorders, blood deficiency, blood stagnation or qi stagnation all result in menstrual disorders such as irregular menstrual cycle, dysmenorrhea, amenorrhea and other gynecological disorders. The last effects are to moisten intestines and unblock the bowels for symptoms of constipation due to blood deficiency when the bowels are not properly nourished by blood, constipation or dry stools result[16,17]. Based on these effects, experimental studies were reported about *Angelicae Gigantis Radix* : anti-oxidant effect by Ahn[22], the effect on the immune-suppression by Hwang[23], the protective actions for toxipathic hepatitis by Kim[24], the improvement of ischemic stroke by Han[25].

Elastase breaks down elastin, an elastic fiber that, together with collagen, determines the mechanical properties of connective tissue. The neutrophil form breaks down the *Outer membrane protein A* (OmpA) of *E. coli* and other Gram-negative bacteria, and also breaks down Shigella virulence factors. This is accomplished through the cleavage of peptide bonds in the target proteins. The specific peptide bonds cleaved are those on the carboxy side of small, hydrophobic amino acids such as glycine, alanine, andvaline. Actually, elastase is the only enzyme that is capable of degrading elastin, an insoluble elastic fibrous protein in animal connective tissues. It is capable of hydrolyzing nearly all proteins, including supporting and structural proteins of the connective tissue such as collagen and elastin[26]. Elastin is the main component of the elastic fibers of the connective tissue and tendons. The elastic fibers in the skin, together with the collagenous fibers, form a network under the epidermis[27]. Elastase also plays a critical role in inflammatory processes. The enzyme has drawn much attention, primarily because of its reactivity and non-specificity. It is able to attack all major connective tissue matrix proteins, including elastin, collagen, proteoglycans, and keratins[28].

The serine proteinase elastase is located in the azurophil granules of mature circulating polymorphonuclear neutrophils. This neutrophil elastase is a potent non specific serine protease which plays a role as bactericidal agent and in the degradation of immune complexes by intraphagosomal processes. It promotes inflammation when the granule contents are secreted in the extracellular environment. In certain pathological circumstances, an imbalance between neutrophil elastase and its major plasmatic inhibitor alpha 1-PI (formerly, alpha 1-antitrypsin) leads to abnormal tissue destruction and disease development. Genetic or acquired alpha 1-PI deficiency is thought to be involved in the pathogenesis of pulmonary emphysema. A variety of degenerative

and degradative disorders are also associated to uncontrolled proteolysis by neutrophil elastase (rheumatoid arthritis, glomerulonephritis, adult respiratory distress symptom, psoriasis, cancer). Numerous inhibitors of neutrophil elastase have been reported. Various molecules are currently undergoing clinical trials for emphysema and other pulmonary diseases[29].

The defects of elastic matrix impair hypertension which is associated with alteration in the great arteries, arteries, and arterioles. Clinical studies for human pulmonary hypertension and systolic left ventricular failure are now in progress. An elastase inhibitor is currently being investigated in phase I clinical trials in patients with pulmonary hypertension owing to chronic obstructive pulmonary disease[8-11].

Experimental studies about elastase inhibition has been reported with various herbs : Lee[30] revealed *Rheum undulatum* has elastase inhibition effect, Jung[31] reported *Rehmanniae Radix*, *Aurantii Immaturus Fructus*, *Achyranthis Bidentatae Radix*, *Aurantii Fructus* have high elastase inhibition effect but *Acanthopanacis Senticosi Radix*, *Chelidonium majus L.*, *Lithospermi Radix*, *Acori Gramineri Rhizoma*, *Taraxaci Herba*, *Alpiniae Oxyphyllae Fructus*, *Uncariae Ramulus Et Uncus*, *Astragali Radix* do not. However, there has not been a single report about elastase inhibition effect of *Angelicae Gigantis Radix* until now.

In this study, inhibitory effect of AGHAE on elastase activity was determined according to the method. AGHAE showed the elastase inhibitory effect in dose dependent manner. AGHAE was found to inhibit elastase activity highly at a concentration of 10mg/ml.

The production of ROS was induced by neutrophil elastase[12,13]. They reported that neutrophil elastase enhancement of MUC5AC messenger RNA levels was dependent on the production of intracellular oxidants or an alteration in the redox state of the cell. It means that ROS may play a role in elastase mediated inflammation. Accordingly, antioxdative activities of AGHAE were also examined.

DPPH free radical scavenging activity of AGHAE was measured at each concentration (0, 4, 20, 100, and 500mg/ml). A dose dependent free radical scavenging activity was observed.

Nitrite plays an pivotal role in elastase mediated diseases[14,15]. So, nitrite scavenging activities were also examined. However, nitrite scavenging activity changes at various pH environments. Accordingly, nitrite scavenging activities at pH 1.2, 3.0, and 6.0 were measured in this study. Considering data at pH 1.2, 3.0, and 6.0 nitrite scavenging activity, suggesting it is pH dependent, was increased with increasing pH. It was the best environment for nitrite scavenging activity of AGHAE situation at normal pH.

These results suggest that AGHAE may have potential effects for pulmonary emphysema and pulmonary hypertension. I think further studies will be needed to unravel exactly under the clinical trial and molecular mechanisms.

Ⅴ. Conclusion

I investigated the effects of AGHAE on elastase activity. Anti-oxdative activities of AGHAE were also examined via measuring the DPPH free radical scavenging and nitrite scavenging activities.

The results are as following :

1. AGHAE was found to inhibit elastase activity highly at a concentration of 10mg/ml (52.5±2.1%).
2. AGHAE 100mg/ml treated groups had the highest scavenging capability, of 63.0±2.1%.
3. AGHAE 50mg/ml treated groups had the highest scavenging activity, of 101.0±0.4% at pH 1.2.
4. AGHAE 50mg/ml treated groups had the highest scavenging activity, of 56.9±0.0% at pH 3.0.
5. All concentrations of AGHAE treatment (0.4, 2, 10, and 50mg/ml) showed the significant

effects at pH 6.0.

6. Accordingly, it could be the best environment for nitrite scavenging activity of AGHAE at normal pH.

In conclusion, AGHAE showed the inhibiting effects on the elastase, and free radical scavenging activity of DPPH and nitrite.

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