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右歸丸對氣喘動物模式蛋白質體學研究及其藥物組合之分析探討

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中、英文摘要及關鍵詞

關鍵詞：右歸丸、塵蟎、氣喘、Th1/Th2 平衡、細胞激素、化學趨化因子、趨合分子 、蛋白質體學

氣喘是全球性的重大公共衛生問題，研究指出，引發氣喘最重要的因子是過敏反應，其罹患率與致死率在過去二十年中持續增加，氣喘於治療與預防有其重要性。

目前已知中藥對於免疫系統具調節功效，我們先前的研究也發現中藥方劑可以調節 Th1 與 Th2 細胞平衡，小青龍湯源自東漢‧張仲景《傷寒論》，是治療氣喘的名方。我們研究發現小青龍湯能夠降低塵蟎(Dermatophagoides pteronyssinus, Der p)所誘發之氣喘老鼠肺泡沖洗液的發炎細胞總數及嗜酸性白血球浸潤，調降過敏原誘發肺內淋巴球反應，及降低肺泡沖洗液和血清中前趨發炎細胞激素(proinflammatory cytokine)並可抑制肺組織中轉錄因子、細胞激素與化學驅化因子之表現。去年本研究室探討小青龍湯蛋白質體層次之研究，我們得到了更進一步的進展。

右歸丸是明 張景岳《景岳全書》氣喘緩解期調理氣喘的名方，我們發現右歸丸能夠降低塵蟎(*Dermatophagoides pteronyssinus*, Der p)誘發氣喘天竺鼠立即性與遲發性氣喘反應，亦能減少塵蟎所誘發之氣喘老鼠肺泡沖洗液的發炎細胞總數及嗜酸性白血球浸潤及降低肺泡沖洗液中 $CD4^{+}T$ 細胞族群。探討右歸丸在蛋白質體學層次的作用，並與右歸丸全方作用相比，了解各藥物在方中的作用與所扮演的角色，我門取老鼠肺泡沖洗液及肺組織進行蛋白質學研究，了解氣喘動物與正常鼠間右歸丸與各藥物治療氣喘蛋白質之變化，並從其中找尋更佳更有效的組合，進一步分析探討，以利未來新藥或新方配法之開發。

Asthma is a major public health problem worldwide, and asthma morbidity and mortality have increased over the last two decades.

There are detailed description of the clinical experiences and prescriptions of asthma in traditional Chinese medicine; Yo-Qui-Wang(YQW) is one of the Chinese herbal medicines used to treat bronchial asthma for the thousand-year clinical practice. In our previous research, we found YQW has bronchodilator and immunomodulatory effects on reducing bronchial inflammation in the allergen-induced murine model. Now, we want to evaluate the YQW in proteomic change in allergic-challenged mice and the subgroups effect of YQW on changes of the inflammatory cell infiltration、cytokines、chemokines、serum Ag-specific antibodies 、 Th1/Th2 balance 、 transcription factor and proteomic change in allergic-challenged mice to further understand the possible mechanisms on immune response. Those herbs maybe provide a good database to develop new formulae or drug in future.

報告內容

近年來許多研究傳統中醫藥經由調節免疫系統(例如 T 輔助型細胞[Th]中 Th1/Th2 細胞之調控等)，而達到“改善體質”之作用來達到預防與治療氣喘的目的，我們研究發現，中藥方劑可以降低氣喘天竺鼠呼吸道過度反應性、立即性與遲發性呼吸道阻力，調節發炎細胞浸潤與氣喘老鼠肺周邊淋巴結(draining lymph node ; DLN)中 Th2 細胞之百分比[1-6]。

氣喘致病機轉中，T-淋巴球在產生、調控及氣喘呼吸道慢性發炎上扮演重要角色，其暴露於過敏原後，肥大細胞(mast cell)開始了發炎之過程，淋巴球很可能提供氣喘活化繼而建立慢性且持續性發炎之訊息。我們的研究發現右歸丸可調控 T 淋巴球之浸潤[4]，而動物模式亦顯示由塵蟎(*Dermatophagoides pteronyssinus*, Der p)所激發之氣喘模型中，Th2 細胞($IL\text{-}4^+/CD3^+/CD4^+$)之百分比亦明顯增加[4]。由前人之研究中可看出輔助性 T 輔助型淋巴球依所分泌淋巴激素不同可分成 Th1 及 Th2，Th1 細胞產生 IL-2、IL-3、GM-CSF，INF-γ、IL-10、TNF-β 等細胞激素，Th2 細胞產生 IL-3、GM-CSF、IL-4、IL-5、IL-6、IL-10、IL-13 細胞激素，這二種次分類的 Th 細胞參與不同的免疫反應，Th1 細胞參與遲發性過敏反應 (delayed type hypersensitivity)而 Th2 細胞參與過敏性發炎反應(allergic inflammation)。

在 Th1 和 Th2 細胞之間的平衡關係中，研究顯示調節 Th1/Th2 細胞的平衡以使 Th2 比例減少而 Th1 比例增加，被認為是治療由 Th2 細胞為主的過敏疾病，因而 Th1/Th2 之免疫調節劑之發展乃為近年來研究對過敏氣喘預防與治療之重點之一，不少運用重組細胞激素(recombinant cytokine)或細胞激素拮抗劑(cytokine antagonist)，調節 Th1/Th2 平衡而改變疾病癒後的研究中發現，IL-12 在某些情況下可將已建立之 Th2 反應轉變為 Th1 反應優勢，被認為其可能用於治療過敏[7-8]，IFN-γ 或抗 IL-4 抗體或抗 IL-5 抗體會抑制過敏性老鼠肺內嗜酸性白血球症[9]，但是直接給予此類的細胞激素會造成令人憂心的副作用，此外由於它們缺少口服活性因而限制了療效。另一種調控細胞激素之方法是給予小分子如 AS-101 (tellurium-based compound) [10-11]和 OK-432 (streptococcal preparation) [12]來操作內部的 Th1/Th2 平衡，其因能調節 IL-10 或 IL-12 的產生而使得傾向 Th1 反應，但是它們口服無效。因此尋找能調節 Th1/Th2 細胞平衡的口服藥物是值得開發的方向。

過敏性氣喘是一種呼吸道疾病，是探討其成因、發展過程或尋找診斷指標和治療標的，毫無疑問的從肺組織直接取樣是最理想的方式。肺泡沖洗液是一種容易取得和能反映肺部病態的樣本，從肺泡沖洗液中不單可收集呼吸道肺泡中細胞，作細胞學檢查[13]，肺泡沖洗液中含有多種源自血液或由局部上皮或發炎細胞所釋放的蛋白，可以定性和定量分析[14]。許多肺部疾病的研究如氣喘和急性呼吸緊迫症候肺泡沖洗液中蛋白組成皆出現異常。Wathez 等利用 2-DE 分析人類肺泡沖洗液，研究 sarcoidosis、idiopathic pulmonary fibrosis 和 hypersensitivity pneumonitis[15]。另外有研究發表利用 2-DE 分析吸入性 α 1-protease inhibitor 對 cystic fibrosis 的療效評估[16]。

目前 2-DE 與氣喘相關研究並不多，我們試圖透過塵蟎誘發呼吸道發炎及過敏反應，來了解中藥對呼吸道微環境蛋白質的表現，進一步鑑定中藥在治療塵蟎過敏中涉及那些主要蛋白分子調控。

過敏性氣喘病理生理機轉非常複雜，包含很多細胞表現不同功能[17-18]，病理機轉之研究需深入了解不同蛋白質在正常肺狀態及特殊疾病過程中的功能角色，肺泡沖洗液包含不同細胞及源自於血液或上皮及發炎細胞所釋放出來之蛋白質[19] ，由於肺泡沖洗液中蛋白質有不同來源，分析肺泡沖洗液可得知重要的病理性介質及很多肺疾病在分子層次上更正確的特點，過敏原誘發氣喘動物模式，我們可了解正常老鼠及氣喘老鼠在過敏原激發與發炎及應之間關係。

目前已知中藥對於免疫系統具調節功效，研究中醫藥是否能調整 Th1/Th2 平衡乃為近年來研究重心之一。我們先前的研究也發現中藥方劑可以調節 Th1 與 Th2 細胞平衡，臨床上右歸常用於氣喘病人，其治療機轉本實驗室已獲得初步成果，本計劃將進一步探討右歸丸對氣喘老鼠呼吸道蛋白質體方面之變化，並依中藥配伍理論將右歸丸做不同的組合與右歸丸全方作用相比，了解各藥物在方中的作用與所扮演的角色，我們取老鼠肺泡沖洗液及肺組織進行蛋白質學研究，了解氣喘動物與正常鼠間與右歸丸全方及各藥物治療氣喘蛋白質之變化，並從其中找尋更佳更有效的組合，進一步分析探討，以利未來新藥或新方配法之開發。

四、研究方法：

一、藥品製備與購買

1. YQW preparation

YQW (KODA pharmaceutical Co., Ltd. in Taiwan) is composed of ten medicinal plants . Six hundred gram of fifteen components is soaked in 6 L of spring water with for 30 min at room temperature and then boiled for 50 min. Repeat two times of boiled extraction and harvest these two times of supernatant. This supernatant will be sequentially passed through No.1 filter paper for remove insoluble ingredients. The supernatant was concentrated to 600 ml. Hesperidin was purchased from Sigma.

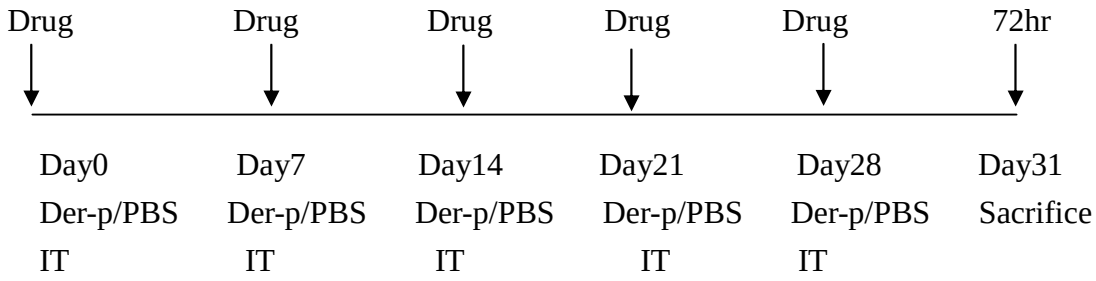
二、

Table 1.實驗分組：

組別	處理
Naïve 組	6-8 週 BALB/c 雄鼠不激發、不餵藥
Der p 組	BABL/c 小鼠，以 50 μl Der p（1mg/ml）氣管內接種激發 5 次，每次間隔一週，最後一次激發後 3 天犧牲。

Der p＋右歸丸組	BABL/c 小鼠，以 50 μl Der p（1mg/ml）氣管內接種激發 5 次，每次間隔一週，且於每次激發前 30 分鐘給 0.3ml 藥物，最後一次激發後 3 天犧牲。

三、氣喘動物模式建立簡圖：



四、實驗方法：

1. Mice and reagents

Specific pathogen-free, male, 6-to 8-wk-old BALB/c mice（Laboratory Animal Center ）were used in this study. The mice were housed in microisolator cages(Laboratory Products, Inc., Maywood, NJ, USA） fed sterile food and water ad libitum. All experimental animal care and treatment followed the guidelines set up by the National Science Council of the Republic of China. Lyophilized house-dust mite （Dermatophagoides pteronyssinus [Der p]） was purchased from Allergon （Engelholm, Sweden）. The crude mite preparation was extracted with ether. After dialysis with deionized water, the mite extract was lyophilized and stored at -80℃before use. LPS concentration of the Der p preparation was ＜0.96 EU/mg of Der p （Limulus amebocyte lysate test; E-Toxate; Sigma-Aldrich）.

2. Allergen challenge, assessment of blood eosinophilia and airway inflammation

Groups of five BALB/c mice were i.t. inoculated with five doses of Derp（1 mg/ml, 50 μl）in phosphate-buffered saline（PBS） at 1-wk intervals. At three days after the last challenge, the number of blood eosinophils was determined using diagnotic reagent system（Unopette test 5877; BD Biosciences, Rutherford, NJ）with blood samples collected via the orbital sinus. Mice were then killed by i.p. injection of xylazine （200 μg/mice）and ketamine（2 mg/mice）, serum samples were collected and stored at -80℃ until assay. BAL was performed （two washes of 1 ml of ice –cold endotxin-free PBS） according to the previously described procedure[7]. The BAL fluids were separated （1500 rmp, 10 min, 4 ℃）and stored at -80 ℃. After total leukocyte counting, differential counts were performed on cytospin preparations （1 × 10^5 cells/100 μl of BALF）stained with Liu stain（Biotech, Taiwan）in a blind manner. For TCM study, mice were given 1g/kg YGW at 1-wk intervals before each Der p inoculation and naïve mice were also included in the experiment for purposes of comparison.

3. Two-dimensional electrophoresis

Protein concentrations of the BAL fluid were determined using a commercial protein assay kit (Micro BCA, Pierce, Rockford, IL, USA) with bovine serum albumin as standard. The proteins were precipitated with TCA (10% final concentration) in an ice bath for 20 min, and subsequently centrifuged at 3500 rpm for 15 min and 4℃. The pellet was suspended in ice-cold acetone using a sonicator and centrifuged as described above. The pellet was air-dried for a few minutes and, finally, resuspended in the sample solution (9 M urea, 0.5% v/v Triton X-100, 2% v/v Pharmalyte 3-10 and 65 mM dithioerythritol (DTT)) by sonication.

In the first dimension, 250 μl of each sample were subjected to isoelectric focusing in a Pharmacia IPG strip (pH 3-10 L, 7 cm). The gels were rehydrated overnight in rehydration solution (8 M urea, 0.5% v/v Triton X-100, 2 mM acetic acid and 9.7 mM DTE). The first phase was set at 500 V for 5 h, the second phase was a linear gradient spanning from 500 V to 3500 V in 5 h, and the final phase was set at 3500 V for 14 h. After electrophoresis the strips were kept at -80oC or prepared directly for the second-dimensional electrophoresis.

4. Silver staining

The gels were fixed in 40% ethanol and 10% acetic acid in water overnight, and then incubated in a buffer solution containing 30% ethanol, 4.1% sodium acetate and 0.2% sodium thiosulfate for 30 min. After washing three times in water for 5 min each, the gels were stained in 0.1% silver nitrate solution containing 0.02% formaldehyde for 40 min. Development was performed for 15 min in a solution consisting of 2.5% sodium carbonate and 0.01% formaldehyde. EDTA solution (1.46%) was used to sto p the development and the stained gels were then washed three times in water for 5 min each.

5. In-gel enzymatic digestion

Protein spots were excised from gel with an Ettan Spot Picker(Version 1.0, Amersham Pharmacia Biotech), destained twice with 30mM potassium ferricyanide and 100mM sodium thiosulfate (1:1 v/v) and then equilibrated in 50mM NH_4HCO_3 to pH 8.0. After dehydrating with ACN and drying in N_2 at 37℃ for 20 min, the gel pieces were rehydrated in 15 μL trypsin solution (10 μg/mL in 25mM NH_4HCO_3) at 47℃ for 30 min and then incubated at 37℃ overnight. Peptides were then extracted twice using 0.1% TFA in 50% CAN and dried with N_2.

6. Image acquisition and analysis

The stained gels were scanned in an ImageScanner (Amersham Biosciences) operated by the software, LabScan 3.00, also from Amersham Biosciences. Intensity calibration was carried out using an intensity stepwedge prior to gel image capture. Image analysis including spot detection, quantification and normalization was carried out using the ImageMaster 2D Elite software 4.01c (Amersham Biosciences). The relative intensities of spots were used for comparison among the three groups and only those significantly different spots (two-fold

increase or decrease) were selected for analysis by MS.

7. MALDI-TOF-MS/MS identification

Digested peptides were dissolved with 0.8 μL saturated solution of CHCA in 50% v/v ACN/0.1%TFA, applied on a MALDI target plate and air-dried. MS analysis was performed using a 4700 Proteomics Analyzer (Applied Biosystems). Proteolytic peptides of standard Myoglobin were used for internal calibration and the 6 strongest peptides per spot were selected automatically for MS/MS analysis. PMF and sequence data were matched by searching the Swiss-Prot (http://us.expasy.org) database using MASCOTengi

8. Statistical analysis

Results are expressed as mean±SEM. One-way ANOVA was used for multiple group comparisons and the Student's *t*-test for others. Differences with $P<0.05$ were judged to be significant.

參考文獻

1. Kao ST. Yeh TJ. Hsieh CC. Yeh FT. Lin JG. Effect of San-Ao-Tang on immediate and late airway response and leukocyte infiltration in asthmatic guinea pigs. Immunopharmacology & Immunotoxicology. 22(1): 143-62, 2000
2. Kao ST. Hsieh CC. Chen TC. San-Qi-Wan modulate lung draining Th1/Th2 lymphocyte and regulated the inflammation in asthmatic animal model.
3. Kao ST. Hsieh CC. Lin CS. Hsieh WT. Shiau HB. Lin JG. Effects of Xiao-Qing-Long-Tang on bronchoconstriction and airway eosinophil infiltration in ovalbumin sensitized guinea pigs in vivo and vitro studies Allergy. 2001;69:1845-1496(SCI)
4. Kao ST. Wang SD. Wang JY. Yu CK. Lei HY. The effect of Chinese herbal medicine, Xiao-qing-long-tang (XQLT), on allergen-induced bronchial inflammation in mite-sensitized mice. Allergy 55: 1127-1133, 2000.
5. Kao ST. Yeh TJ. Hsieh CC. Shiau HB. Yeh FT. Lin JG. The effects of Ma-Xing-Gan-Shi-Tang on respiratory resistance and airway leukocyte infiltration in asthmatic guinea pigs. Immunopharmacology & Immunotoxicology. 23(2), 2001.
6. Kao ST. Chang JD. Hisue TR. Lai YS. Lin JG. The effect of Ma-Xing-Gan-Shi-Tang on *Dermatophagoides pteronyssinus*-induced asthmatic guinea pig. Mid Taiwan Journal of Medicine. 4(1): 62-8, 1999
7. Wynn TA. Eltoum I. Oswald IP. Cheever AW. Sher A. Endogenous interleukin 12 (IL-12) regulates granuloma formation induced by eggs of Schistosoma mansoni and exogenous IL-12 both inhibits and prophylactically immunizes against egg pathology. Journal of Experimental Medicine. 179(5): 1551-61, 1994.
8. Gavett SH. O'Hearn DJ. Li X. Huang SK. Finkelman FD. Wills-Karp M. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. Journal of Experimental Medicine. 182(5): 1527-36, 1995

9. Kung TT. Stelts DM. Zurcher JA. Jones H. Umland SP. Egan RW. Kreutner W. Chapman RW. Interferon-gamma and antibodies to interleukin-5 and interleukin-4 inhibit the pulmonary eosinophilia in allergic mice. Inflammation Research. 44 Suppl 2:S185-6, 1995.

10. Fujimoto T. Duda RB. Szilvasi A. Chen X. Mai M. O'Donnell MA. Streptococcal preparation OK-432 is a potent inducer of IL-12 and a T helper cell 1 dominant state. Journal of Immunology. 158(12): 5619-26, 1997.

11. Wattiez R, Hermans C, Cruyt C, Bernard A, Falmagne P. Human bronchoalveolar lavage fluid protein two-dimensional database: study of interstitial lung diseases. Electrophoresis 21:2703, 2000

12. Tanizaki Y, Sudo M, Kitani H, Kawauchi K, Mifune T, Takeyama H, Kohi F, Tada S, Takahashi K and Kimura I. Characteristic of cell components in bronchoalveolar lavage fluid (BALF) in patients with bronchial asthma classified by clinical symptoms. Arerugi 39:75, 1990

13. Magi B, Bini L, Perari MG, Fossi A, Sanchez JC, Hochstrasser D, Paesano S, Raggiaschi R, Santucci A, Pallini V and Rottoli P. Bronchoalveolar lavage fluid protein composition in patients with sarcoidosis and idiopathic pulmonary fibrosis: a two-dimensional electrophoretic study. Electrophoresis 23:3434, 2002

14. Griese M, von Bredow C, Birrer P. Reduced proteolysis of surfactant protein A and changes of the bronchoalveolar lavage fluid proteome by inhaled alaph 1-protease inhibitor in cystic fibrosis. Electrophoresis 22:165, 2001

15. Cohn,L Elisa, J A, and Chupp, G, L, (2004) Asthma: mechanisms of disease persistence and progression. Annu.Rev. Immunol.22,789-815.

16. Hamid, Q Tulic, M,K, Liu, M.C,and MoQbel, R(2003) Inflammatory cells in asthma: mechanisms and implications for therapy. J. Allergy Clin. Immunol.111,S5-S12.

17. Bowler, R.P; B.,Chan, E.D., Enghild,J.J, Wars,L.B., Matthay M. A. and Duncan, M.W.(2004)Proteomic analysis of pulmonary edema fluid and plasma in patients with acute lung injury. Am.J.Physiol.286,L1095-L1104.

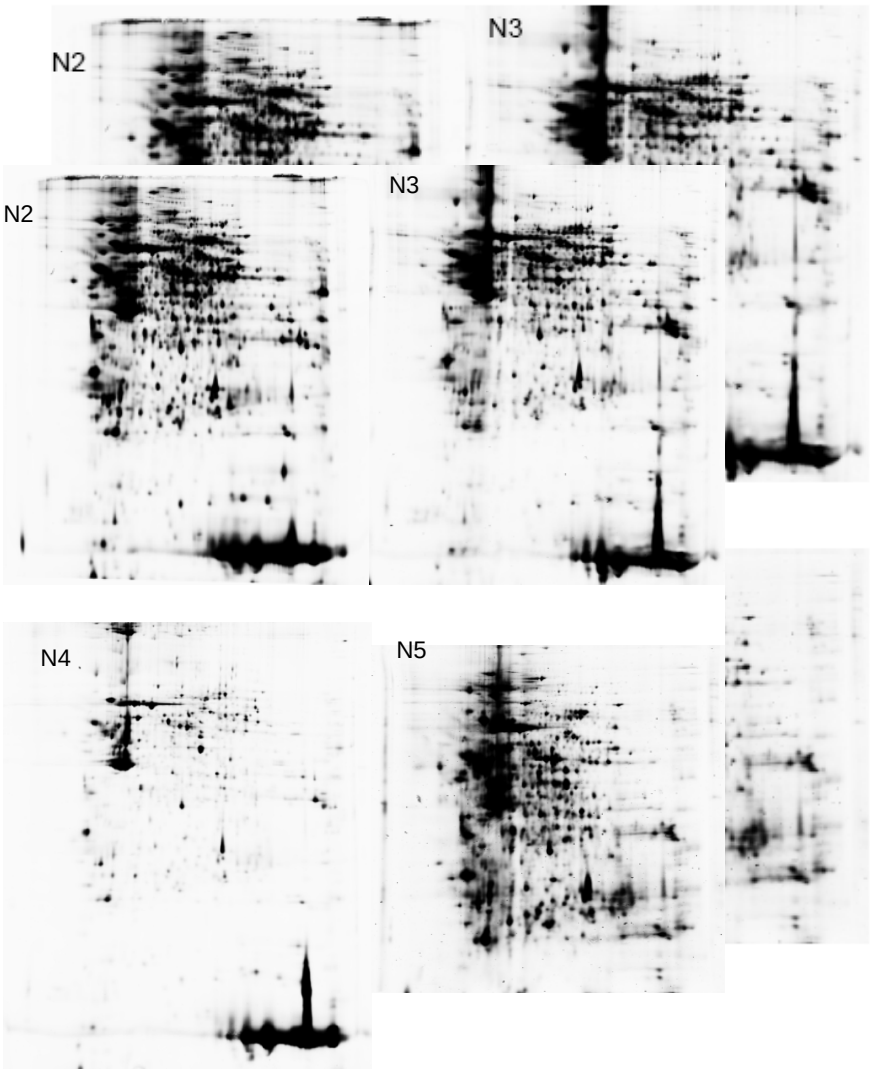
18. Nocker RE, van der Zee JS, Weller FR, van Overveld FJ, Jansen HM, Out TA.(1999) Segmental allergen challenge induces plasma protein leakage into the airways of asthmatic subjects at 4 hours but not at 5 minutes after challenge. J Lab Clin Med. 134:74-82.

19. Plymoth A, Lofdahl CG, Ekberg-Jansson A, Dahlback M, Lindberg H, Fehniger TE, Marko-Varga G.(2003) Human bronchoalveolar lavage: biofluid analysis with special emphasis on sample preparation. Proteomics 3,962-72.

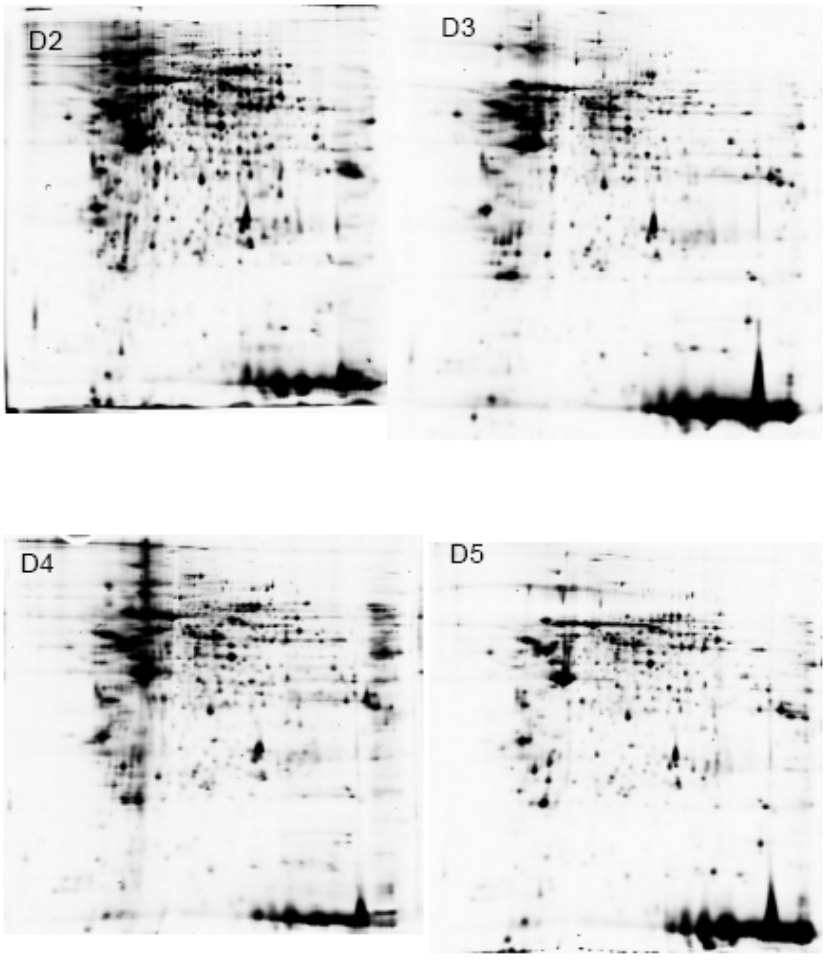
結果與討論

初步取得右歸丸的支氣管肺泡沖洗液，並進行 2-D 的實驗。經由初步比對 native 組、Der p 組、右歸丸拆方組-X、右歸丸拆方組-R、右歸丸拆方組-K，發現給予右歸丸不同拆方組的老鼠其支氣管肺泡沖洗液確實有不相同之處。

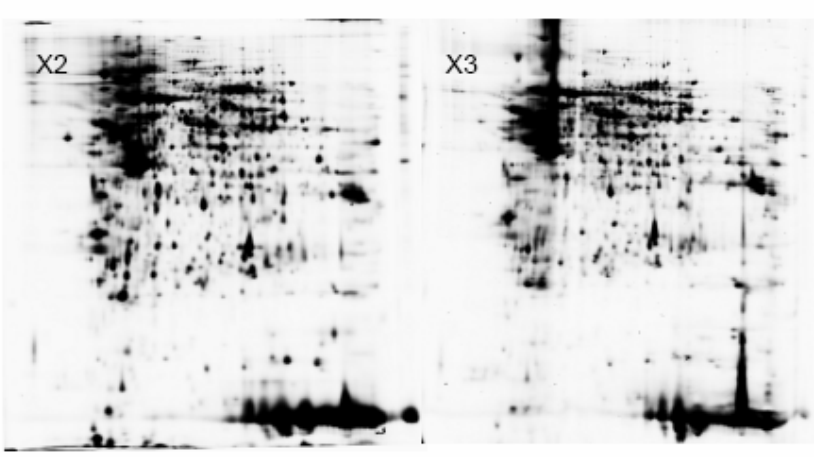
Result：native 組(4 隻老鼠)

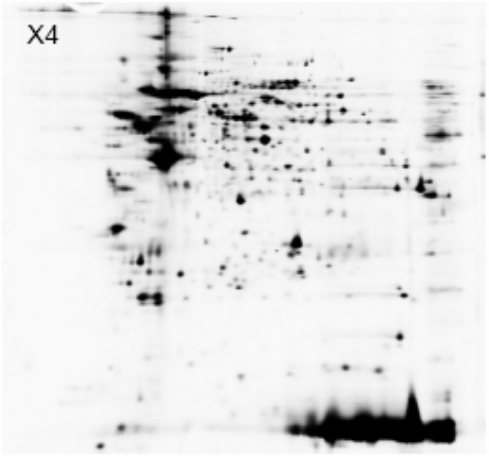


Der p 組 (4 隻老鼠)

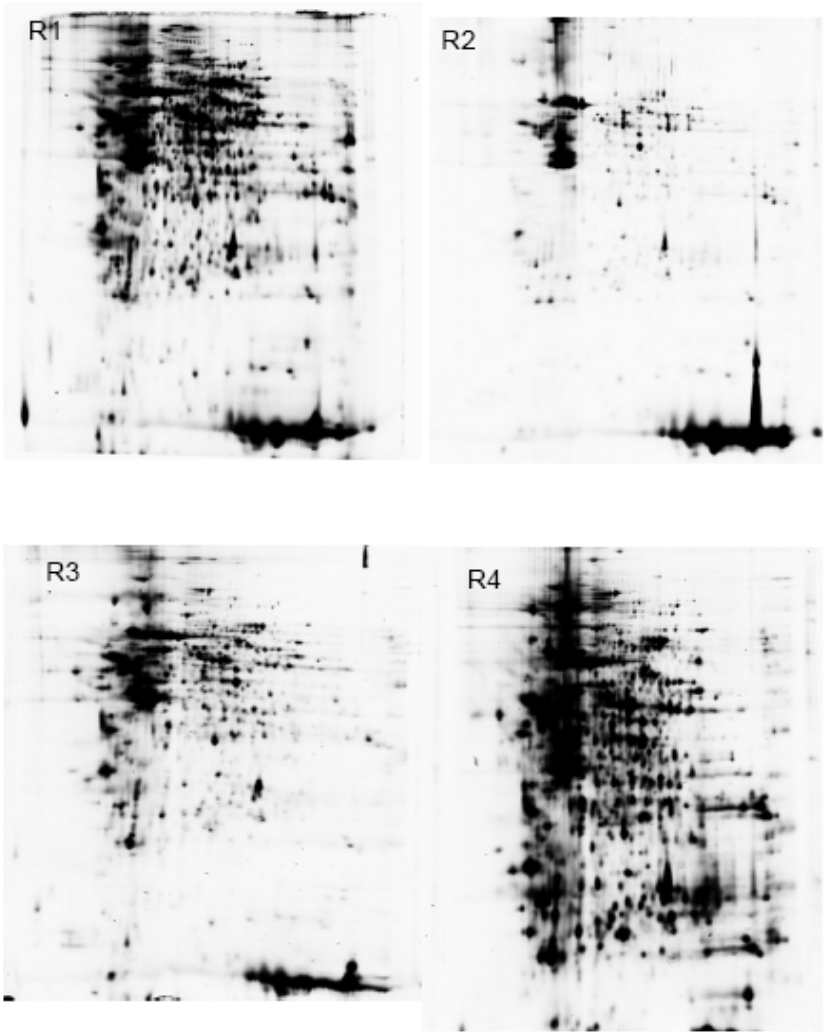


右歸丸拆方組-X (3 隻老鼠)

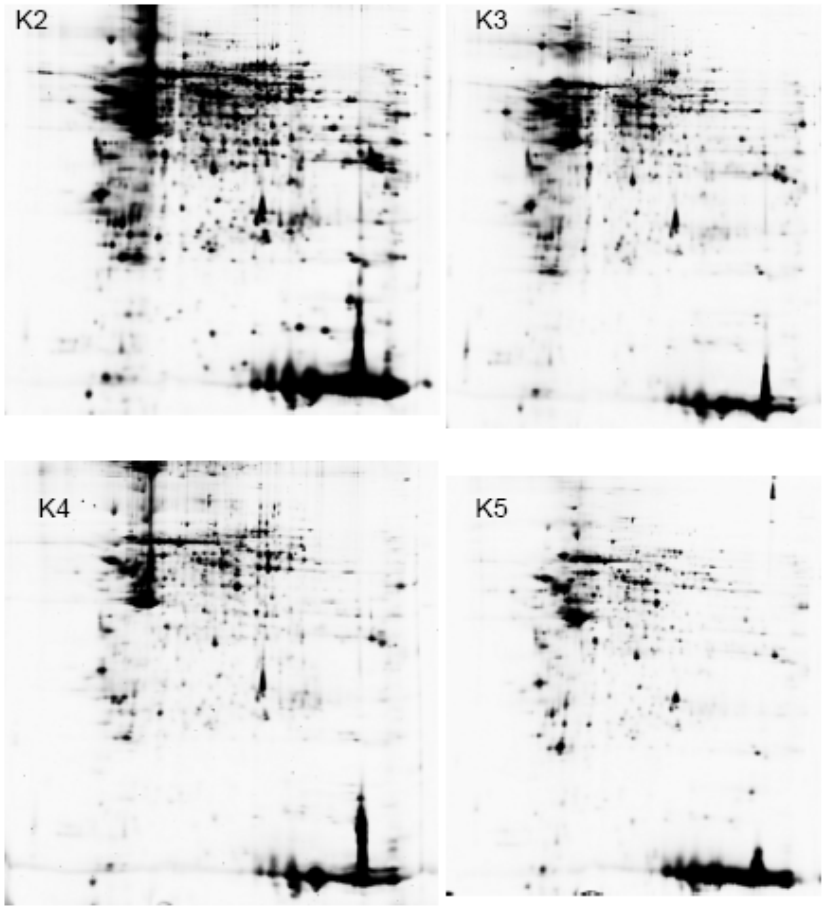




右歸丸拆方組-R (4 隻老鼠)



右歸丸拆方組-K (4 隻老鼠)



因此本實驗具有深入探討的價值，而此實驗結果將作為日後細部研究右歸丸各成分，在分子層面機制上，如何達到治療以及預防氣喘效果的重要參考指標。

計畫成果自評

研究結果已達到原計劃要探討的部份，亦達成果預期目標，預計短期內將研究成果發表於學術期刊。