嘉南藥理科技大學專題研究計畫成果報告

長期服用龍膽瀉肝湯對大鼠肝細胞色素 P450 的影響

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計畫主持人：楊竹茂 副教授

共同主持人：

計畫參與人員：

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中文摘要

「龍膽瀉肝湯」為中藥固有成方中最常被使用於治療肝臟疾病的方劑。本研究計畫旨在以大白鼠動物實驗配合微陣列晶片，初步探討長期服用「龍膽瀉肝湯」後對肝臟代謝酵素之影響。

經以每天三次連續灌食 1gm/kg 劑量之「龍膽瀉肝湯」達七天後，取出動物肝臟經一序列操作過程分離肝臟細胞微粒體，並萃取其中 RNA，在經純化製成 RNA 樣品供生物晶片分析測定之用。晶片試驗採用 ABC Rat UniversoChip 8K-1 微陣列晶片，RNA 試樣經螢光標誌(labeling)後與晶片上之 cDNA 雜交(hybridization)， 再以 GenePix 4000B 掃瞄[影像波長：635 nm (Treatment)及 532 nm (Reference)]，最後以 GenePix Pro 3.0.5.56 專家軟體分析掃瞄結果。

由比對治療組與對照組在晶片上的基因表現，平均值比率大於 2 而具有基因表現調高(up-regulation)者共有約 75 種，其中較具生理意義的有：beta-globin、peptidylarginine deiminase type IV、hemoglobin alpha chain、hemoglobin alpha subunit、lysozyme、Phosphatidylinositol 4-kinase、phosphatidylinositol-4-phosphate 5-kinas、Thymosin beta-10、Topoisomerase II、TPA、Cathepsins、Chloride channel protein (p64)、farnesyl pyrophosphate synthase、ubiquitin-homology domain protein (PIC1)、cytoplasmic beta actin、UDP-glucose dehydrogenase 及 UDP-glucuronosyltransferase 等。平均值比率小於 0.5 而具有基因表現調降(down-regulation)者共有約 30 種，其中較具生理意義的有：creatine kinase、Heparin-binding growth associated protein、NADH-ubiquinone oxidoreductase 及 Syntaxin 2 等。

有關長期服用「龍膽瀉肝湯」導致基因表現 up-regulation 或 down-regulation 與生理活性之關連性有待繼續進一步探討，然而在本研究中並未發現對細胞色素 P450 各種代謝酵素有顯著的影響。

關鍵字：龍膽瀉肝湯、微陣列晶片、基因表現

英文摘要

"Long-Dan-Shei-Gan-Tan", a traditional Chinese herbal formulation, has been used for the treatment of hepatic-related diseases in the orient countries for more than thousand years. This study was aimed to primarily demonstrate the influence of long-term use of this formulation on the hepatic enzymes in rats by microarray biochip analysis.

The preparation was given orally trice a day with the dosage of 1 gm/kg for consecutive seven days. Livers were dissected and the microsomes were prepared by a series of separating processes. RNA samples were extracted and purified from microsomes, and then the fluorescence-labeled probes were hybridized with the immobilized cDNAs on the rat chips (ABC Rat UniversoChip 8K-1) and scanned with GenePix 4000B scanner (and $_{532}$ were used for treatment and reference, respectively). The final images were eventually analyzed with the GenePix Pro 3.0.5.56 software.

The gene expressions of the "treatment" groups were compared with those of the "control" groups and an approximate of 80 genes were found up-regulated (Ratio of Means >2), among them, the genes with significant physiologic functions were beta-globin, peptidylarginine deiminase type IV, hemoglobin alpha chain, hemoglobin alpha subunit, lysozyme, Phosphatidylinositol 4-kinase, phosphatidylinositol-4-phosphate 5-kinase, Thymosin beta-10, Topoisomerase II, TPA, Cathepsins, Chloride channel protein (p64), farnesyl pyrophosphate synthase, ubiquitin-homology domain protein (PIC1), cytoplasmic beta actin, UDP-glucose dehydrogenase and UDP-glucuronosyltransferase; an approximate of 30 genes were found down-regulated (Ratio of Means <0.5), among them, the genes with significant physiologic functions were creatine kinase, Heparin-binding growth associated protein, NADH-ubiquinone oxidoreductase and Syntaxin 2.

The impacts on the pharmacological actions of long-term use of "Long-Dan-Shei-Gan-Tan" that leading to up-regulated or down-regulated gene expression should be further studied. However, the genes involved to cytochrome P450 enzymes were not found modified by the present formulation.

Key words: Long-Dan-Shei-Gan-Tan; Microarray biochip; Gene expression

計畫緣由與目的

「傳統中藥」的使用，近年來廣受國人及國際人士的重視。國人常使用中藥治療疾病或作為平時強身保健之用。傳統上國人常誤認中藥為副作用少且可長期服用之藥物，因此自行購藥長期服用者非常普遍。然而在使用中藥期間，病患也常併服「西藥」。有關「中、西藥」合併使用時可能發生的交互作用卻少見報告。日本學者曾做過研究舉出某些中藥可減少西藥的劑量與副作用 (Tani, 1989; Mizuno *et al*., 1988)；國內學者也曾經研究過定喘湯、還少丹、小青龍湯及補中益氣湯對茶鹼 (theophylline) 動力學的影響 (Lin *et al*., 1992; 1991) 。

本實驗室之前研究服用茵陳蒿對同時服用乙醯胺基酚的影響時，發現茵陳蒿對乙醯胺基酚所誘發的急性肝臟壞死之毒性作用不但無保護作用反而有加速惡化的現象：如死亡率增高；血清中 alanine aminotransferase (ALT) 及 aspartate aminotransferase (AST) 更上昇；加重肝組織壞死的程度等；且實驗結果指出預先服用茵陳蒿後，更會嚴重增強乙醯胺基酚對麩胱甘肽(glutathione)至近乎完全排空的程度 (Yang *et al*., 1998)。為了瞭解茵蔯蒿是否促進乙醯胺基酚在肝臟的代謝作用而加速 NAPQI 的形成，另外利用西方點墨法 (Western blotting) 分析服用茵蔯蒿前後細胞色素 P450 (CYP)酵素之變化，另外以螢光偏極化免疫分析法 (TDx) 及高壓液相層析法(HPLC)分析服用茵蔯蒿後對乙醯胺基酚藥動學的影響，以闡明其相互作用之機轉。研究結果發現，連續餵食年青大白鼠七天茵蔯蒿後，肝微粒體中 CYP1A2 及 CYP2E1 之蛋白表現皆上昇，同時由血中動力學參數顯示，茵蔯蒿能使乙醯胺基酚之代謝加快， 達到最高血中濃度時間(T_{max})由 1 小時降至 0.25 小時，平均最高血中濃度(C_{max})由 200.6 μg/ml 降至 115.6 μg/ml；然而對年老大鼠反而能顯著提升其最高血中濃度，結果顯示，茵蔯蒿能經由提高肝微粒體中 CYP1A2 及 CYP2E1 之蛋白表現而加速乙醯胺基酚之代謝，增加活性之代謝中間體，進而使肝臟之 glutathione 被耗盡，最後導致肝細胞壞死。

由於作用於肝臟的傳統中藥非常多，而且肝臟中之酵素種類也非常多，因此長期使用中藥後對肝臟內之酵素必會產生一定程度的影響，進而可能影響併服之西藥。本研究以「龍膽瀉肝湯」為實驗藥物，經七天連續口服給藥後，配合微陣列生物晶片之分析方法，擬從晶片上大約 8000 種基因表現中，初步找出受到影響的基因種類，以作為探討長期服用「龍膽瀉肝湯」可能的藥理作用基轉及與併服西藥可能產生的相互作用。

結果與討論

A. Microarry Experiment Information

Sample type: RNA　Organism: Rat　Tissue: Liver

A260/280: 1.842 (Control)　1.949 (Treatment)

Concentration: 2.82 μg/μl (Control)　4.26 μg/μl (Treatment)

Chip type: ABC Rat UniversoChip 8K-1

cDNA labeling: 20.02 μg (Control)　40 μg (Treatment)

Hybridization: 42℃, 15.5 hrs

B. Summary of Analysis Information

Scanned by: GenePix 4000B [82719]

Image wavelengths: 635 nm (the emission wavelength of treatment)

532 nm (the emission wavelength of Control)

PMT (V): 720, 760　Laser Power (V): 5.3, 3.3

Analyzed by GenePix Pro 3.0.5.56

C. Results

晶片試驗與掃描參數

			635 PMT Volts	532 PMT Volts
	NF ratio of median (ALL)	NF ratio of mean (ALL)		
R84,85,86	0.975499638	0.942346206	690	610

NF ratio of median = Normalization Factor of Ratio_of_Medians

NF ratio of mean = Normalization Factor of Ratio_of_Means, Base on All Genes (exclude Not found & Bad)

NF ratio of mean (HK) = Normalization Factor of Ratio_of_Means, Base on HouseKeeping Genes (exclude Bad)

試驗規劃設計

晶片使用	晶片操作	Sample Labeling
R84,85,86	百恩諾代客操作	Cy3: Reference
		Cy5: Treatment
		Ratio=(TREATMENT)/(CONTROL) Cy5/Cy3

1. The up-regulated genes　Ratio_of_Means > 2　and　(F635_Mean - B635) >= 1000

Name	Ratio of Mean	Name	Ratio of Mean
50 kDa dynein-associated polypeptide	2.143	insulin-induced growth response protein	2.431
actin, beta, cytoplasmic	2.127	lysozyme	7.850
acyl-CoA synthetase 5	2.067	M.musculus hypothetical protein H19	2.660
alpha-2-globin chain	7.154	mannose-6-phosphate receptor	2.267
beta-globin	9.103	matrix Gla protein	2.015
C.elegans hypothetical protein F36D4.5	2.438	microsomal epoxide hydrolase	2.369
C.elegans hypothetical protein R08D7.3	2.177	Moesin	2.300
Calreticulin	2.248	Na-K-Cl cotransporter	20.026
Cathepsin A	2.332	osteonectin	2.705
cathepsin B	3.400	p41-Arc	3.325
cathepsin C	2.464	palmitoyl-protein thioesterase	2.946
Cathepsin D	2.931	peptidylarginine deiminase type IV	9.271
cathepsin K	4.260	PHD-finger protein	6.162
cathepsin L	2.300	Phosphatidylinositol 4-kinase	3.629
cell adhesion inhibitor beta ig-h3	2.810	phosphatidylinositol-4-phosphate 5-kinase	8.703
cell-binding bone sialoprotein	2.875	pro-alpha-2(I) collagen	2.045
Chloride channel protein (p64)	2.614	profilin	2.233
chloride intracellular channel protein 1	2.058	Protein disulfide isomerase	2.010
Clusterin	2.800	protein tyrosine phosphatase-like protei	2.542
collagen alpha 3(VI) chain	2.283	putative steroid dehydrogenase KIK-I	3.621
cyclin A	7.555	Ran / TC4 GTP-binding nuclear protein	2.046
cystatin beta	2.127	RGICP19	2.502
cytokeratin 5	2.021	Ribosomal phosphoprotein P0	2.091
dihydropteridine reductase	2.000	squalene epoxidase	4.684
DIM1 protein homolog	5.301	syndecan	3.032
erb B-2	2.031	thymosin beta-10	2.038
far upstream element-binding protein 2	3.061	TIMP-2 (AI058866)	2.133
Farnesyl pyrophosphate synthetase	3.173	Topoisomerase II	2.837
fibronectin	3.872	TPA	3.198
galectin-7	6.906	transcription factor B-ATF	2.086
glucose-6-phosphate dehydrogenase	2.058	Tubulin, beta-15	2.277
glutathione peroxidase	2.067	tubulin, gamma	2.215
glutathione S-transferase	3.785	Ubiquitin	2.079

GTP-binding nuclear protein Ran / TC4	2.239	ubiquitin-homology domain protein	2.487
guanine nucleotide-binding protein, G(i)	2.118	UDP-glucose dehydrogenase	3.987
H.sapiens HT021	6.803	UDP-glucuronosyltransferase UGT1A7	2.950
hemoglobin alpha chain	7.762		
hemoglobin beta subunit, major form	7.776		
hypoxia-inducible factor-1 alpha	3.004		

2. The down-regulated genes: Ratio_of_Means < 0.5 and (F532_Mean - B532) >= 1000

Name	Ratio of Mean	Name	Ratio of Mean
3-hydroxy 3-methylglutaryl coenzyme A sy	0.466	MAP kinase kinase	0.317
Adenine phosphoribosyltransferase	0.347	mitochondrial	0.486
cardiac triadin	0.336	myosin light chain 2	0.270
cathepsin H	0.174	myosin light chain kinase	0.421
CCAAT-binding transcription factor I, su	0.355	NADH-ubiquinone oxidoreductase 18 kDa su	0.381
Chromogranin A	0.327	NADH-ubiquinone oxidoreductase NDUFS2 su	0.361
Collagenase	0.349	protein disulfide isomerase-related prot	0.369
creatine kinase	0.464	Ras protein, c-Ha	0.289
cystatin C	0.428	rat leukemia virus polymerase	0.118
cytochrome C oxidase polypeptide VIIb	0.478	ribosomal phosphoprotein P0	0.117
golgi stacking protein homolog GRASP55	0.331	Ribosomal protein S3	0.348
Heparin-binding growth associated protei	0.428	secreted apoptosis related protein 1	0.253
heparin-binding neurotrophic factor	0.370	Stat3	0.489
homeobox containing nuclear transcriptio	0.336	Syntaxin 2	0.211
immunoglobulin superfamily-like protein	0.294	transcobalmin II	0.486

結論：由比對治療組與對照組在晶片上的基因表現，平均值比率大於 2 而具有基因表現調高(up-regulation)者共有約 75 種，其中較具生理意義的有：beta-globin、peptidylarginine deiminase type IV、hemoglobin alpha chain、hemoglobin alpha subunit、lysozyme、Phosphatidylinositol 4-kinase、phosphatidylinositol-4-phosphate 5-kinas、Thymosin beta-10、Topoisomerase II、TPA、Cathepsins、Chloride channel protein (p64)、farnesyl pyrophosphate synthase、ubiquitin-homology domain protein (PIC1)、cytoplasmic beta actin、UDP-glucose dehydrogenase 及 UDP-glucuronosyltransferase 等。平均值比率小於 0.5 而具有基因表現調降(down-regulation)者共有約 30 種，其中較具生理意義的有：creatine kinase、Heparin-binding growth associated protein、NADH-ubiquinone

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