一贯煎影响 CCl_4 大鼠肝硬化形成期肝组织基因表达谱的效应机制研究*

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摘 要:目的:探讨一贯煎对 CCl_4 诱导大鼠肝硬化形成阶段肝组织基因表达谱的影响。方法:首次大鼠皮下注射 CCl_4 3mL/kg 此后以 50% CCl_4 橄榄油溶液 2mL/kg 皮下注射,每周 2次,共计 12周,自第 9周开始给与药物干预 4周,12周末杀鼠取材;检测肝功能,肝组织病理,肝组织羟脯氨酸含量,并采用基因芯片技术探讨一贯煎对模型大鼠肝组织基因表达谱的影响。结果:(1)模型大鼠 8周时呈慢性肝损伤肝纤维化的病理改变,12周时已形成典型的肝硬化;与正常大鼠比较,12周时模型大鼠精氨酸加压素 1A受体 (AVPR1A)、CYP3A13、β球蛋白 (Beta-glb)等基因表达显著下调;淋巴毒素 A(LTA)、MMP-23、RNA结合基序蛋白 3(RBM3)、血小板反应蛋白 2(TSP2)、AP1γ亚单位结合蛋白 1(AP1GBP1)、生长素释放素受体(GHRHR)、阿米洛利结合蛋白 1(ABP1)等基因表达显著上调。(2)与 12周模型对照组比较,一贯煎组 AVPR1A、CYP3A13、Beta-glo等基因表达显著上调;LTA、MMP-23、RBM3、TSP2、AP1GBP1、GHRHR、ABP1等基因表达显著下调。结论:一贯煎抑制 CCl_4 诱导大鼠肝硬化形成的作用机制与提高肝脏生物转化功能、抑制肝脏炎症反应、抑制肝细胞凋亡和肝星状细胞活化、抑制肝窦内皮损伤、改善肝脏糖代谢及水钠潴留等多种途径有关。

关键词:肝硬化 基因芯片 一贯煎

一贯煎载于《柳州医话》,由北沙参、麦门冬、当归、生地黄、枸杞子、川楝子等 6味药物组成,具有养阴疏肝之功,为临床治疗肝硬化的代表方剂。前期研究基于中医对肝硬化“瘀血阻络,气阴虚损,湿热内蕴”基本病机的认识,采用养阴疏肝的一贯煎、活血化瘀的下瘀血汤、益气的黄芪汤、清热利湿的茵陈蒿汤及小柴

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胡汤(对照方剂,购自日本ツムラ株式会社,日本学者研究认为该方具有较好的抗肝纤维化作用[1-3])于CCl4诱导大鼠肝硬化形成阶段进行干预,药效学结果显示一贯煎具有良好的综合干预作用,其显著的作用特点在于提高血清白蛋白含量[4]。进一步从促进增生纤维结缔组织的降解及保护肝细胞、抑制肝细胞凋亡等不同角度探讨一贯煎抑制大鼠肝硬化形成的作用机制的结果显示,一贯煎可显著抑制肝细胞凋亡及肝星状细胞(HSCs)活化;显著降低MMP-2/TMP-2比值;显著提高MMP-9/TMP-1比值及HGFa的蛋白表达[5]。

本文采用基因芯片技术,基于肝组织基因表达谱的变化分析探讨了一贯煎抑制CCl4诱导大鼠肝硬化形成的作用机制,现将结果报告如下。

一、材料与方法

1. 动物

Wistar雄性大鼠 56只,清洁级,体重 130~150g,购自中国科学院上海实验动物中心,许可证号:SCXK(沪)2003-0003。上海中医药大学实验动物中心饲养、造模和观察,自由饮食。

2 药物与试剂

一贯煎所含的生药均购自上海华宇药业有限公司,有明确的原产地,经生药学专家鉴定,按照原方比例和制法由上海中医药大学附属曙光医院国家中医药管理局中药制剂中心制备,干燥后冷藏保存。CCl4分析纯和橄榄油购自中国医药集团上海化学试剂公司;肝功能测定试剂盒分别购自卫生部上海生物制品研究所及南京建成生物工程研究所;羟脯氨酸标准品购自日本ナカティテスク株式会社,批号:MIR8282。

3 模型制备

首次以CCl4 3mL·kg^{-1}剂量皮下注射,以后以2mL·kg^{-1}剂量皮下注射50%的CCl4橄榄油溶液,每周2次,共12周。

4 分组与给药

造模4周、8周后,随机抽取正常及模型大鼠各6只,处死作动态观察。其余模型大鼠随机分为模型对照组(10)、一贯煎组(9)干预治疗。第9周开始,在继续造模的同时,药物干预组按成人(65kg)kg体重日用量的8倍,即一贯煎 2 682g·kg^{-1}(相当于生药 7.938g·kg^{-1}),用蒸馏水 10mL稀释灌胃,每日 1次,共计 4周。正常组(8)与模型对照组以同体积生理盐水灌胃。

5 样品的采集和处理

造模 12周结束,大鼠用 2%戊巴比妥钠以 2mL·kg^{-1}体重剂量腹腔注射麻醉后,仰卧位固定,打开腹腔,观察肝脾的色、质、形态、体积等情况。经下腔静脉采血,摘取肝脾,称重,立即从肝右叶固定位置切取约100mg肝组织液氮冷冻保存,抽提RNA进行基因芯片杂交实验,从肝右叶切取 1.0cm×0.8cm×0.3cm大小肝组织 1块,10%中性福尔马林固定,脱水、包埋,切片,HE及胶原染色,观察组织学变化;留取肝组织作羟脯氨酸含量测定。所采血液 4℃静置 3h后,3000rpm离心 30min,分离血清,检测各项血清学指标。

6 观测项目与方法

(1)一般情况:包括大鼠的死亡情况、体重、肝脏大体形态、肝脏及脾脏重量等。

(2)肝组织切片:HE及胶原染色,观察肝脏组织学变化。

(3)肝组织羟脯氨酸(hydroxyproline,Hyp)含量测定:按 Jamall等人的方法[6]。

(4)肝功能:血清丙氨酸氨基转移酶(ALT)、门冬氨酸氨基转移酶(AST)活性,赖氏法;白蛋白(Alb)含量,溴甲酚绿法;血清总胆红素(TBil),重氮法;谷氨酰转酞酶(GGT),按试剂盒说明方法测定。

7 RNA的抽提、芯片杂交和数据分析

基因芯片:Affymetrix Rat 230 2.0基因表达谱芯片,共载基因探针 31099个。RNA的抽提采用 Trizol试剂盒。用纯化的RNA合成双连 cDNA,生物素标记cRNA合成参考 BioArray High Yield RNA Transcript Labeling kit方法。将 cRNA片段杂交于 Affymetrix Rat 230 2.0芯片。

基因芯片的数据处理:首先将信号进行标准化和基于模型的表达指数(model-based expression index,MBEI)分析,得出校正确的数值,用于差异基因分析。

特征性基因表达谱分析采用聚类分析法。特征性基因的筛选采用 Fold change法, 两个芯片的每个基因之间倍数通过表达指数比值得出, 以识别特征性基因, 并用 MBEI的标准误差来计算倍数的可信区间, 本研究在组间进行比较时, 以倍数的可信下限 (lower bound)作为基因表达上调或下调的评判标准。

8 统计方法

计量资料采用方差分析, 两两比较采用 q检验。

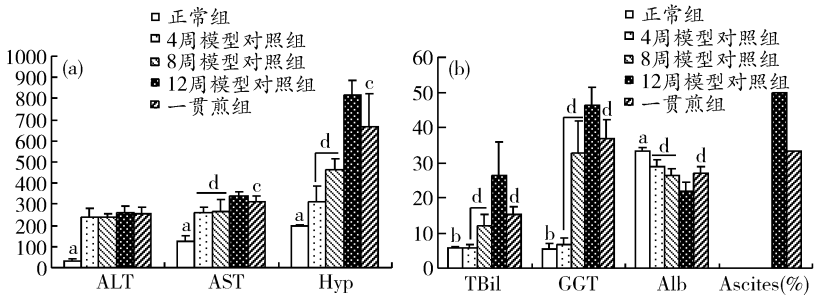
二、结 果

1. 肝功能、肝组织 Hyp含量及腹水变化

与正常组比较, 随着模型的加重, 模型组大鼠 ALT、AST、GGT、TBil、Hyp逐渐升高, 12周时达到高峰, 且 AST、GGT、TBil、Hyp显著高于 4周、8周模型对照组 ($P < 0.01$)。血清 Alb含量逐渐降低, 12周时显著低于 4周、8周模型对照组 ($P < 0.01$)。与 12周模型对照组比较, 一贯煎组 AST、GGT、TBil、Alb、Hyp显著改善 ($P < 0.05$或 0.01)。12周模型组的腹水发生率为 50%, 一贯煎组为 33.3% (图 1)。

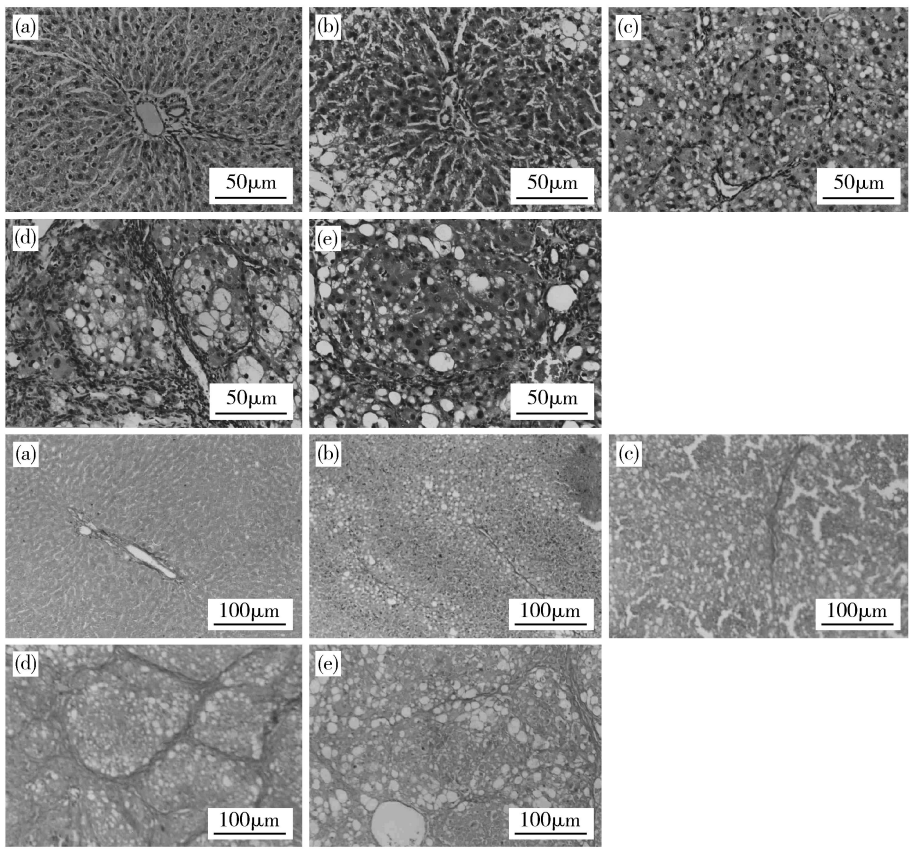
2 肝组织病理变化

HE及天狼星红染色显示, 4周末模型大鼠肝脏内可见以中央静脉为中心的肝细胞脂肪变性, 极菲薄的胶原纤维在脂肪变区域伸展。8周末模型大鼠



(a) ALT、AST、Hyp变化; (b) TBil、GGT、Alb、腹水发生率的变化 a与各时间点模型对照组比较, $P < 0.01$; b与 8周、12周模型对照组比较, $P < 0.01$; c与 12周模型对照组比较, $P < 0.05$; d与 12周模型对照组比较, $P < 0.01$

图 1 大鼠肝功能、Hyp含量及腹水发生率的变化



A HE染色; B天狼星红染色 (a)正常组; (b)4周模型对照组; (c)8周模型对照组; (d) 12周模型对照组; (e) 一贯煎组

图 2 肝组织病理变化

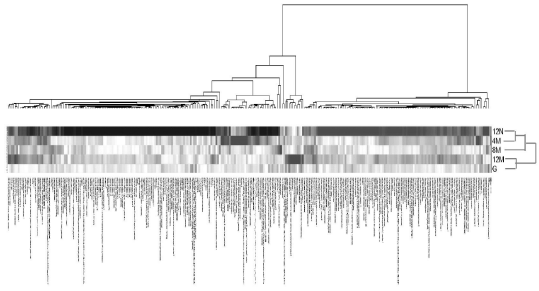
肝细胞脂肪变性累及整个肝小叶,可见较细的胶原纤维形成不完全包绕,间隔内可见较多的成纤维细胞和少量的炎性细胞浸润。12周末模型大鼠大量纤维结缔组织增生,形成大小不一的典型假小叶结构,间隔内可见大量的成纤维细胞和炎性细胞浸润,80% 大鼠(8/10)形成肝硬化;与 12周模型比较,一贯煎组大鼠肝细胞脂肪变性程度轻,纤维间隔明显减少,极少见紧密包绕的完整假小叶结构,仅有 1只大鼠(1/9,11.1%)形成肝硬化(图 2)。

3 大鼠肝组织特征性基因聚类分析

模型大鼠肝组织特征性基因来源于总的差异基因,采用单侧 Fisher确切概率法对模型组特征性基因表达数值的校正值进行聚类,比较方法为模型组不同时间点与正常组比较,一贯煎组与 12周模型对照组比较。聚类图的右半部分表示随着模型的进展,这部分基因表达逐渐下调;左半部分表示随着模型的进展,这部分基因表达逐渐上调(图 3)。

4 模型大鼠部分基因表达的动态变化及一贯煎组特征性差异表达基因

本文列出了与肝纤维化形成密切相关的部分基因,包括不同方剂组与 12周模型对照组比较后的基因仅在一贯煎组差异表达有统计学意义的所有基因就有(18个),并对一贯煎组有明确功能描述的特征性基因进行重点论述(表 1)。



12N:正常组; 4M: 4周模型组; 8M: 8周模型组; 12M: 12周模型组; G: 一贯煎组。

红色线条代表上调基因,蓝色线条代表下调基因,白色线条代表非差异基因;通过本图,可将差异基因区分为随时间变化有上调趋势(图的左半部分聚类)的基因和下调趋势(图的右半部分聚类)的基因

图 3 特征性基因聚类图

表 1 模型大鼠部分基因表达的动态变化及一贯煎组特征性差异表达基因

基因名称	Accession NO.	Summary	正常组(N)	4周模型对照组(4M)	8周模型对照组(8M)	12周模型对照组(12M)	一贯煎组(YGJ)	lower bound of FC(YGJ/12M
细胞离子稳态								
AVPR1A arginine vasopressin receptor 1A	NM_053019	encodes a receptor for arginine vasopressin	1676.61	822.3	517.16*	165.76* △	440.48▲	2.66↑
ABP1 amiloride binding protein 1	NM_022935	binds amiloride and some of its derivatives; may form an epithelial-specific Na+ channel	70.94	71.27	143.78	603.3* △	239.63▲	-2.28↓
炎症和免疫反应								
LTA lymphotoxin A	NM_080769	cytokine produced by lymphocytes	544.48A	253.89A	525.49A	468.84	195.34▲	-2.1↓
Cd44 CD44 antigen	BI302830	adhesion molecule involved in migration, cell fusion and resorption in osteoclasts that also plays a role in cellular metastasis	30.29	200.21*	313.11*	906.04* *	548.23	-1.55(↓)
Cd48 CD48 antigen	X13016	plays a role in mast cell activation and response to Mycobacterium tuberculosis	512.56	742.75	650.84	1207.78*	1001.86	-1.13(↓)
Cd53 CD53 antigen	NM_012523	a tetraspanin protein; may be involved in cell survival and growth regulation	229.17	570.75*	426.96	599.6*	558.59	-1.01(↓)

基因名称	Accession NO.	Summary	正常组 (N)	4周模型对照组 (4M)	8周模型对照组 (8M)	12周模型对照组 (12M)	一贯煎组 (YGJ)	lower bound of FC (YGJ/12M
Cd63 CD63 antigen	NM_017125	human homolog facilitates endocytosis of H, K- ATPase beta-subunit; may play a role in protein trafficking	591.89	1723.82*	1614.85*	2448.27*	1846.96	-1.25(↓)
细胞外基质及其代谢								
Col1a1 collagen, type I, alpha 1	BI285575		88.83	667.77*	882.31*	1850.28*△	1166.49	-1.51(↓)
Col3a1 collagen, type III, alpha 1	BI275716		1149.52	2831.2*	2961.62*	4670.16*	4322.93	-1.03(↓)
Col4a2 collagen, type V, alpha 2	AI179399		138.98	390.2*	408.69*	675.02*	584.19	-1(↓)
procollagen, type I alpha 2	BI282748		123.98	659.39*	537.07*	1182.96*	863.38	-1.25(↓)
procollagen, type IV, alpha 1	AI176393		188.16	505.87*	664.03*	888.71*	730.27	-1.14(↓)
procollagen, type VI, alpha 3	AI176126		132.96	233.4	259.19	652.03*	464.18	-1.25(↓)
procollagen, type XII, alpha 1	BE108345		32.98	199.46	145.37	378.9*	268.97	-1.32(↓)
Mgp matrix Gla protein	NM_012862	a vitamin K-dependent protein; part of the bone matrix	460.34	1031.6	1525.86*	4652.83*	2698.95	-1.62(↓)
MMP-23 Matrix metalloproteinase 23	NM_053606	play a role in degrading and remodeling components of extracellular matrix	188.86A	167.36	158.96A	424.41	155.28▲	-2.06↓
Mmp12 matrix metallopeptidase 12	NM_053963	may have a role in glomerular injury in a crescentic glomerulonephritis	54.43	523.28*	528.28*	783.42*	516.26	-1.44(↓)
TSP2 Thrombospondin 2	AI406660	No	139.49	326.94	360.09*	1367.82*△	620.44▲	-2.09↓
TIMP-1 tissue inhibitor of metalloproteinase 1	NM_053819	acts as an inhibitor of metalloprotease activity; may play a role in vascular tissue remodeling	194.57A	467.14	356.37	726.65	487.37	-1.49(↓)
促纤维化细胞因子								
AP1GBP1 AP1 gamma subunit binding protein 1	AI716461	may link the AP1 adaptor complex to other proteins	146.61A	56.04	51.3A	209.07	34.7▲	-4.43↓
GHRHR growth hormone releasing hormone receptor	NM_012850	binds growth hormone-releasing hormone; required for normal growth	540.19A	413.67	619.5A	940.69	375.34▲	-2.28↓
Ednrb endothelin receptor type B	X57764	No	296.89	364.1	490.51	837.81*	621.56	-1.22(↓)
Ctgf connective tissue growth factor	NM_022266	a mediator of TGFbeta1-driven matrix production and normal skeletal remodeling	270.64	648.92*	682.75*	1743.7*	1063.1	-1.57(↓)
Ltbp1 latent transforming growth factor beta binding protein 1	NM_021587	large subunit component of masking protein; neutralizes the activity of TGF-beta 1	93.19	172.41	236.25	741.23*	511.28	-1.36(↓)
Ltbp4 latent transforming growth factor beta binding protein 4	BG375362	No	154.85	208.09	279.57	490.69*	316.55	-1.35(↓)
Tbrg1 transforming growth factor beta regulated gene 1	BG666773	binds the TGFbeta receptor; plays a role in regulation of cell growth and proliferation; induces synthesis of extracellular matrix proteins and may play a role in fibrosis	633.32	1376.95	1304.87	1741.04*	1516.08	-1.11(↓)
Pdgfra platelet derived growth factor receptor, alpha polypeptide	AI232379	acts as a receptor tyrosine kinase for PDGF; may play a role in glial cell generation	310.97	441.74	555.72	1188.09*	756.11	-1.46(↓)
Pdgfrb platelet derived growth factor receptor, beta polypeptide	BM389426	binds platelet derived growth factor BB homodimer	78.47	115.13	140.55	267.13*	205.75	-1.13(↓)

基因名称	Accession NO.	Summary	正常组 (N)	4周模型对照组 (4M)	8周模型对照组 (8M)	12周模型对照组 (12M)	一贯煎组 (YGJ)	lower bound of FC (YGJ/12M
细胞凋亡与增值								
Casp12 Caspase 12	NM_130422	involved with the terminal stage of apoptosis	48.96	308.18	204.55	473.38*	413.32	-1.05(↓)
Hgf Hepatocyte growth factor	BF284043	plays a role in positive regulation of cell proliferation; may promote entry into the cell cycle	212.04	84.97	95.62	53.59*	56.89	0.41(↑)
异生化合物代谢								
CYP3A13 cytochrome P450, family 3, subfamily a, polypeptide 13	U46118	catalyzes the hydroxylation of progesterone at 6beta, 16alpha, and 21 positions	2154.45	986.45*	282.77*#	103.15*△	319.89▲	2.47↑
Cyp4f4 cytochrome P450 4F4	U39206	member of the cytochrome P450 CYP4F subfamily	1689.71	1349.86	1353.15	767.88*	968.68	1.14(↑)
Cyp2t1 cytochrome P450 monooxygenase CYP2T1	NM_134369	putative member of the cytochrome p450 monooxygenase enzyme family, which catalyze many reactions involved in drug metabolism and cholesterol biosynthesis	516.62	335.05	274.35	205.27*	241.76	0.92(↑)
Cyp4a12 cytochrome P450, 4a12	NM_031605	monoxygenase found in rat testes	183.35	135.59	100.77	51.15*	74.04	1.03(↑)
Cyp1a2 cytochrome P450, family 1, subfamily a, polypeptide 2	K02422	a monooxygenase that may play a role in xenobiotic metabolism	3958.65	5742.56	2375.25*#	1499.72*	2710.6	1.47(↑)
Cyp2d13 cytochrome P450, family 2, subfamily d, polypeptide 13	AB008424	member of the p450 xenobiotic-inducible superfamily that induces immunoreactivity in ventral mesencephalon	4469.19	2789.91	2800.96	1337.49*	1903.2	1.27(↑)
Cyp2f2 cytochrome P450, family 2, subfamily f, polypeptide 2	NM_019303	member of the p450 2F subfamily; forms styrene glycol from styrene in liver and lung microsomes	1347.24	938.71	716.12	405.84*	550.18	1.22(↑)
Cyp2j9 cytochrome P450, family 2, subfamily j, polypeptide 9	U39943	monooxygenase; responsible for the oxidation of endogenous arachidonic acid pools	1531.4	1355.22	1054.87	581.7*	860.11	1.33(↑)
Cyp8b1 cytochrome P450, family 8, subfamily b, polypeptide 1	NM_031241	endoplasmic reticulum membrane protein; plays a role in sterol metabolism	1934.17	1333.67	1753.03	841.15*	899.97	0.95(↑)
Cyp2c Cytochrome P450, subfamily IIC	NM_019184	most abundant member of the p450 xenobiotic-inducible superfamily in non-stimulated liver	6618.8	4027.31	358.93*#	266.32*	493.45	1.13(↑)
分类不明								
RBM3 RNA binding motif protein 3	AI598399	mouse homolog displays increased protein synthesis in response to mild hypothermia	330.1	923.47*	831.56*	2304.33*△	1070.33▲	-2.04↓
MGC72973 Beta-glo	BI287300		1622.31	895.63	1806	537.59*△	1279.36▲	2.14↑
Il2rg interleukin 2 receptor, gamma	AI178808	cytokine receptor which contributes to ligand binding and signal transduction	333.52	272.05	311.58	535.95	216.76▲	-2.11↓
Pkn1 Protein kinase N1	BE099509	displays phosphorylation dependent protein kinase activity	153.22	112.98	131.92	254.9	86.86▲	-2.41↓
hr hairless homolog	NM_024364	may play a role in development of the central nervous system	97.51	139.01M	171.98	270.09*	102.9▲	-2.62↓
Abr active BCR-related gene	BI282699		150.72	142.04	138.11A	301.13	129.37▲	-2.04↓

基因名称	Accession NO.	Summary	正常组 (N)	4周模型对照组 (4M)	8周模型对照组 (8M)	12周模型对照组 (12M)	一贯煎组 (YGJ)	lower bound of FC (YGJ/12M
Amy2 amylase 2, pancreatic	NM_031502		14.55	7.66	10.38	111.9*△	7.31▲	-9.06↓
hypothetical LOC303812	BF412161		252.84	245.36	284.17	552.62	187.12▲	-2.41↓
Ilvbl IlvB (bacterial acetolactate synthase)-like	BE109624		279.27	235.14A	207.77A	435.84	165.42▲	-2.09↓
Transcribed locus	AI010048		137.37	163.3	158.96	408.42*△	81.72▲	-3.14↓
Transcribed locus	BF523849		330.2(A)	191.55	238.17	464.78	191.33▲	-2.02↓

注: (A): 不表达; 与正常组相比, *, FC可信下限≥2或≤-2; 与4周模型组比较, #, FC可信下限≥2或≤-2; 与8周模型组比较, △, FC可信下限≥2或≤-2; 与12周模型组比较, ▲, FC可信下限≥2或≤-2; ↑和↓分别表示一贯煎干预后该基因表达显著上调或下调, 达到FC≥2或≤-2的筛选标准. (↑)和(↓)分别表示一贯煎干预后该基因表达上调或下调, 但未达到FC可信下限≥2或≤-2的筛选标准。

三、讨　论

中医方剂是在中医辨证论治原则指导下形成的复杂活性成分体系, 组成的复杂性决定了其作用靶点的多向性和难测性, 采用什么样的方法来探明复方的作用机理是长期以来困扰中医药的一大难题。本研究从肝功能、肝组织病理及肝组织基因表达谱的变化等方面动态地观察了CCl4诱导大鼠肝硬化形成过程中模型大鼠病理变化特点及养阴疏肝的一贯煎对其影响。结果显示模型4周时主要表现为肝组织的炎症反应, 8周时形成纤维化, 12周时已经形成典型的肝硬化。随着肝硬化的形成, 肝脏生物转化功能逐渐降低, 炎症反应、星状细胞活化、细胞外基质的合成逐渐增强, 肝实质细胞凋亡逐渐增加。复杂的病理改变可能成为一贯煎有效抑制肝硬化形成的病态学基础。

1. 抑制肝脏炎症反应、肝星状细胞活化及肝实质细胞凋亡

炎症反应、肝星状细胞活化及肝实质细胞凋亡是各种慢性肝病的共同特征[7]。本研究显示, 随着模型的进展, 肝组织炎性细胞浸润逐渐增加, ALT、AST活性逐渐增高。与HSCs活化和增殖密切相关的细胞因子如PDGFRA、PDGFRB、LTBP1、LTBP4、TBRG1、CTGF等基因表达均显著上调, 并发现生长素释放素受体(growth hormone releasing hormone receptor, GHRHR)及AP1γ亚单位结合蛋白(AP1 gamma subunit binding protein 1, AP1GBP1)基因表达亦显著上调。

GHRHR通过调节生长素释放素(growth hormone releasing hormone, GHRH)对生长素(growth hormone, GH)合成和分泌的刺激, 维持生长素细胞的正常功能[8-10]。GHRH由下丘脑释放后与垂体生长素细胞上特异的GHRHR相结合, 刺激垂体释放GH[11-13], GH进一步刺激胰岛素样生长因子Ⅰ(insulin-like growth factor 1, IGF-1)的生成[12,14]。IGF-1主要在肝脏合成, 是多种细胞的有丝分裂原之一, 与细胞的异常分化、肿瘤的发展及转移等有关。体内外研究表明GHRH是GHRHR mRNA表达的关键调节者[15], 这一点可GHRH特异性拮抗剂能够干扰垂体产生GH和肝脏合成IGF-1而抑制肿瘤的生长得到印证[14,16-21]。近期研究显示, 在人的正常组织(肝、肺、肾等)和肿瘤组织(胰腺癌、小细胞肺癌等)中也有垂体生长素释放素受体(pGHRH-R) mRNA和蛋白表达[22], 提示我们, 就CCl4诱导大鼠肝硬化而言, GHRHR基因在肝脏的高表达可能主要与肝星状细胞的活化和增殖有关。

AP1GBP1是与氧化应激相关的一类二聚体转录调控因子, 其活化在细胞增生、分化、转化、凋亡以及细胞外基质积聚中发挥重要作用, 具有增强AP-1功能的作用[23], 主要通过激活AP-1而促进HSCs的活化与增值, 且激活的AP-1可在转录水平上直接上调TIMP-1基因的表达[24,25]。前期研究中肝组织α-SMA、TIMP-1的蛋白表达水平、肝细胞凋亡指数以及本研究中肝功和以Ⅰ型前胶原、Ⅰ型胶原为主的多种胶原基因表达水平的变化, 提示炎症反应、星状细胞活化及肝细胞凋亡共同参与了CCl4诱导大鼠肝硬化的形成过程。

另外, RNA结合基序蛋白3(RNA binding motif protein 3, RBM3)在冷应激状态下可与60S的核糖体

亚单位结合,改变 microRNA 水平,提高球蛋白的生物合成[26]。但也有研究表明 RBM3 是机体对缺氧的适应性反应,体内外研究均显示在组织缺氧状态下 RBM3 mRNA 表达显著增加[27]。该基因表达水平在模型动态变化过程中逐渐上调可能说明由于肝窦毛细血管化及肝组织结构的改建,影响了肝窦血流和窦内外物质交换,进而引起肝组织缺氧的病理状态。

一贯煎可显著改善肝组织病理,抑制肝脏炎症反应,降低肝组织 Hyp 含量,提高血清白蛋白含量及 β 球蛋白(Beta-glo)的基因表达,降低肝组织 α-SMA 的蛋白表达及 GHRHR、AP1GBP1 的基因表达,抑制肝实质细胞的凋亡。这从不同层面上揭示了一贯煎具有抑制肝脏的炎症反应、肝星状细胞的活化和肝实质细胞的凋亡的综合作用,一贯煎的这些作用可能与其提高以 CYP3A13 为主的 CYP4F4、CYP2T1、CYP4A12、CYP1A2、CYP2D13、CYP2F2、CYP2J9、CYP8B1、CYP2C 等基因表达而提高肝细胞的生物转化功能有关,而干预后 RNA 结合基序蛋白 3(RBM3)基因表达水平的显著下调可能是该方对肝组织病理改善的重要反应。

虽然一贯煎对多种与胶原、促纤维化因子、部分生物转化等相关的基因的表达调控未达到统计学的设定值,但总体呈现改善趋势,提示尽管其对某一种基因的调控不够理想,但多靶点的作用途径可能使其发挥综合干预效应,从而有效抑制肝硬化的形成。

2 抑制肝窦内皮损伤

肝窦内皮损伤是肝纤维化的早期事件,许多蛋白酶被认为参与介导基质降解,而基质金属蛋白酶类(MMPs)在肝纤维化中的作用重大[28,29]。其中 MMP-2是目前较为公认的肝窦内皮损伤因子,MMP-2 酶原可能作为 HSCs 的自分泌生长因子,通过与 HSCs 表面的 MT1-MMP 结合而活化,在肝纤维化早期参与了基底膜胶原的降解,使基底膜内的生长因子(如 TGF-β,bFGF)释放,或破坏基底膜保持 HSCs 静止状态的能力,引起 HSCs 的活化,这与肝纤维化的发生密切相关[28-31]。前期研究显示,随着模型的进展,MMP-2活性逐渐增强,以肝硬化形成阶段(9-12周)尤为显著.

另外,血小板反应蛋白 2(Thrombospondin 2,TSP2)和基质金属蛋白酶-23(MMP-23)基因表达在模型动态变化过程中也呈现出上调趋势。TSP2 是一种细胞基质蛋白,是细胞外 MMP-2 的重要调节者。它主要参与组织损伤修复过程中血管发生和基质重构[32,33]。但 TSP2 需与 MMP-2 相互作用,形成 TSP2/MMP2 复合体,才能发挥正常作用。TSP2 缺失小鼠可发生 MMP-2 的活性增强和纤维原细胞的胶黏缺陷[34,35]。MMP-23 属Ⅱ型跨膜基质金属蛋白酶,其表达序列与 MMPs 相似,能够被单一的蛋白水解酶所裂解,使其具有活性和分泌作用,用埃希氏大肠杆菌进行重组的 MMP-23 虽然表达水平较低,但却具有显著的蛋白水解活性[36,37]。我们的研究发现 TSP2 基因表达水平与 MMP-2 活性变化趋势一致,提示在 CCl_4 诱导的大鼠肝硬化形成过程中,TSP2/MMP-2 复合物及 MMP-23 可能共同参与了基底膜胶原的降解和促进肝组织结构重建的过程,加速了肝纤维化向肝硬化的发展。一贯煎可显著降低 MMP-2 活性以及 TSP2 和 MMP-23 的基因表达,提示一贯煎抑制肝硬化形成机制可能与减轻肝窦内皮的损伤有关。

3 改善肝脏糖代谢功能

CCl_4 诱导大鼠肝硬化也伴随类似于肝硬化患者的糖代谢异常和胰岛素抵抗[38],肝细胞损伤是其关键细胞学基础。我们的研究结果显示,随着肝功能损害的加重,精氨酸加压素 1A 受体(arginine vasopressin receptor 1A,AVPR1A 或 V1aR)基因表达水平逐渐下降,V1aR 不仅可通过对循环血量和压力感受器反射敏感性的调节而稳定血压[39],而且与肝脏的糖代谢关系密切。研究表明大鼠肝实质细胞富含 V1aR[40],在出生后的第 21 天 V1aR mRNA 主要在中央静脉周围的肝细胞高表达,出生 60天后大部分肝实质细胞均表达 V1aR mRNA,与肝细胞 V1aR 在糖代谢调节中的作用一致[41],且 V1aR 的活化在颈动脉体感受器受钠氢化物刺激后中枢和周围高血糖反应中扮演关键角色[42]。近期研究还表明 V1aR 与肝脏脂质代谢有关,V1aR 缺乏则小鼠($V1aR^{-/-}$)脂质代谢增强,胰岛素信号转导被抑制[43],提示 V1aR 与肝硬化糖代谢异常和胰岛素抵抗有关。一贯煎可显著提高该基因的表达水平,表明其抑制肝硬化形成的作用机制可能通过保护

肝细胞、提高 V1aR 的基因表达而促进糖代谢和胰岛素功能恢复。

4. 改善水钠潴留

腹水以及自发性腹腔感染是晚期肝硬化患者常见并发症和死亡的主要原因之一,本研究结果显示 CCl_4 造模 12周时模型大鼠腹水发生率为 50%,肝组织病理呈现大小不一的假小叶改变,血清白蛋白水平较正常大鼠降低了 33.83%。基因芯片分析显示阿米洛利结合蛋白 1(amiloride binding protein 1, ABP1)基因表达在模型动态变化过程中逐渐上调,阿米洛利(amiloride)通过对上皮钠通道的阻止发挥利尿作用[44],阿米洛利结合蛋白从人的肾脏和大鼠的结肠克隆并发现在多种上皮组织表达[45,46],这种蛋白能够与阿米洛利及其衍生物结合,降低后者对 Na^+ 通道的阻滞作用,导致水钠潴留。一贯煎可显著提高血清白蛋白水平和降低 ABP1的基因表达水平及腹水发生率,提示抑制水钠潴留可能是一贯煎改善大鼠肝功能和抑制 CCl_4 大鼠肝硬化形成的又一重要机制。

需要说明的是,我们的研究还发现,白介素 2受体γ(interleukin 2 receptor, gamma, IL-2Rγ)在模型 4周、8周时表达有所下降,而 12周时较正常组升高,一贯煎可显著降低该基因的表达。IL-2Rγ是一个由 347个氨基酸构成的分子量为 64kD 的膜蛋白,起源于 X染色体[47],它不仅对 IL-2信号转导至关重要,而且也是 IL-4、7、9、15受体的信号调节所不可缺少的[48-53],主要表达于淋巴细胞[54]、单核细胞和嗜中性粒细胞[55,56],具有促进 T细胞和 B细胞的生成和功能的作用[57]。小鼠 IL-2Rγ的功能降低可引起免疫系统功能的改变[58]。而人类缺乏 L-2Rγ则表现出特有的成熟 T淋巴细胞缺乏[59],IL-2Rγ链的突变则引起 x染色体连锁严重联合免疫缺陷病(x-linked severe combined immunodeficiency, XSCID)[60,61],这些资料提示 L-2Rγ与机体的细胞免疫密切相关,至于为什么该基因在模型表达先下调而后上调,一贯煎显著抑制该基因表达的机制等问题目前尚不明确,有待于进一步探索。另外,蛋白激酶 N1(Protein kinase N1, Pkn1)、活化 BCR相关基因(active BCR-related gene, Abr)、胰淀粉酶 2(amylase 2, pancreatic; Amy2)、假设基因 LOC303812(hypothetical LOC303812)、IlvB(bacterial acetolactate synthase)-like, Transcribed locus (AI010048)、Transcribed locus(BF523849)等基因均在模型 12周时高表达,一贯煎可显著降低上述基因的表达。这些基因在肝硬化形成过程中的生物学功能目前尚不清楚,尚有待于进一步研究。

综上所述,一贯煎抑制 CCl_4 诱导大鼠肝硬化形成的作用机制是非常复杂的,从目前的研究结果分析看,这种机制的形成主要通过提高肝脏生物转化功能,抑制肝脏炎症反应、肝星状细胞活化、肝细胞凋亡、肝窦内皮损伤,改善肝组织缺氧状态、肝脏糖代谢及水钠潴留等多种途径,体现了中医复方作用的多途径、多靶点的综合优势,为中医经典方剂一贯煎治疗肝硬化提供了部分科学依据。当然,中医复方的成分及作用机制非常复杂,本文只是报道了部分初步研究结果,加之部分基因无肝纤维化相关文献报道或功能尚不明确,进一步验证工作尚在进行之中。

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Hepatic Gene Expression Analysis of Carbon Tetrachloride- Induced Rat Cirrhosis Identifies Distinct Effects of Yiguanjian Decoction

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This paper reports an investigation into the effects of Yiguanjian decoction on hepatic gene expression in carbon tetrachloride- induced rat cirrhosis. In the investigation, pure carbon tetrachloride was injected into rats subcutaneously at a dose of 3ml• kg^{-1} for the first time, and then 50% CCl_4- olive solution at a dose of 2ml• kg^{-1} twice a week for the following 12 weeks to induce cirrhosis. Yiguanjian decoction was administered orally starting from the 9th week for 4 weeks. Sera and liver tissues were collected at the end of 12th week to determine liver's functionalities, hepatic pathohistology, and hepatic hydroxyproline content. Meanwhile, oligonucleotides microarrays analysis was conducted to measure the effects of Yiguanjian decoction on hepatic gene expression. Histopathological examination showed that a chronic liver injury and liver fibrosis appeared in the 8th week, and typical cirrhosis formed in the 12th week for model group. Microarray analysis revealed that arginine vasopressin receptor 1A (AVPR1A), cytochrome P450 family 3, subfamily a, polypeptide 13 (CYP3A 13) and Beta- glo were down- regulated for model group in the 12th week, with another 7 genes up- regulated simultaneously, including lymphotoxin A (LTA), matrix metalloproteinase 23 (MMP- 23), RNA binding motif protein 3 (RBM3), thrombospondin 2 (TSP2), AP1 gamma subunit binding protein 1(AP1GBP1), growth hormone releasing hormone receptor (GHRHR), and amiloride binding protein 1(ABP1). It is worth mentioning that genes down- regulated in model group up- regulated significantly in Yiguanjian decoction- treated group, along with those noticeably up- regulated or down- regulated. It is concluded that Yiguanjian decoction inhibits CCl_4- induced cirrhosis in rat significantly, and the underlying pathogenesis involves an enhanced hepatic biotransformation, inhibiting inflammation, lowering hepatocyte apoptosis and hepatic stellate cell activation, reducing hepatic sinusoidal endothelial cell injury, and improving hepatic glucoregulation and sodium and water retention.

Keywords: cirrhosis; DNA microarray; Yiguanjian decoction

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we filled the collected RGB color data in CIELAB color space, using the latter as a platform. Then we measured the gamut of tongue display. We have created a numerical process for classifying tongue colors, based on the traditional process (for example: light white tongue, light red tongue, red tongue), and screened out typical colors for each tongue pattern. We believe that the novel approach has produced a good result for both description and classification of tongue colors, that can be compared with the results of human visions. It can be applied in clinical diagnosis.

Keywords: tongue diagnosis; tongue colors; CIELAB color space; classification; typical colors

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