

补骨脂“大燥伤肝”的客观性及生物学机制研究

张明亮^{1,2,3}, 赵旭³, 李伟霞², 王晓艳², 陈毓龙², 孔德鑫², 吴承钊¹, 陈小菲²,
柏兆方³, 牛明³, 王伽伯⁴, 赵艳玲^{3*}, 肖小河^{3*}, 唐进法^{2*}

(1. 成都中医药大学药学院, 四川 成都 611137; 2. 河南中医药大学第一附属医院, 河南 郑州 450000;
3. 中国人民解放军总医院第五医学中心, 北京 100039; 4. 首都医科大学中医药学院, 北京 100069)

摘要: 依据补骨脂“性本大燥”的药性理论, 寻找补骨脂致肝损伤的易感证候及生物标志物。分别构建肾阴虚证、肾阳虚大鼠模型, 所有动物实验操作和福利均遵循河南中医药大学第一附属实验动物伦理与动物福利委员会的规定(批号 YFYDW2020017)。结果发现补骨脂可显著降低肾阴虚证大鼠体质量、饮水量、尿量, 并增加其肝、睾丸、肾上腺、脾脏脏器指数及谷丙转氨酶(alanine aminotransferase, ALT)表达; 同时, 补骨脂还显著增加了肾阳虚证大鼠尿量, 并降低ALT、天冬氨酸氨基转移酶(aspartate aminotransferase, AST)表达; 苏木素-伊红(hematoxylin and eosin, HE)染色显示补骨脂明显加剧肾阴虚证大鼠肝细胞炎症浸润, 并减轻肾阳虚证大鼠肝损伤。超高效液相色谱-质谱(ultra-performance liquid chromatography-tandem mass spectrometry, UPLC-MS)检测血浆后共鉴定出17个补骨脂共同调控肾阴/阳虚证大鼠的具有显著差异的内源性代谢标志物。其中, 谷氨酰胺、喹啉酸、胆绿素、乳糖基酰基鞘氨醇等8个代谢物呈相反变化趋势, 主要涉及半胱氨酸和蛋氨酸代谢、酪氨酸代谢、色氨酸代谢、嘌呤代谢、鞘脂代谢、甘油磷脂代谢、谷氨酰胺代谢等通路。因此, 本研究推测肾阴虚证与肾阳虚证可能分别是补骨脂发挥肝损害与肝保护的易感证候, 这可能与其调控体内相关氨基酸代谢和鞘脂代谢等有关, 可为中医临床补骨脂的安全精准使用提供一定的实验数据支撑。

关键词: 补骨脂; 肝损伤; 肝保护; 肾阴虚证; 肾阳虚证; 代谢组学

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Study on the objectivity and biological mechanism of *Psoralea corylifolia* Linn.'s 'Great dryness damages the liver'

ZHANG Ming-liang^{1,2,3}, ZHAO Xu³, LI Wei-xia², WANG Xiao-yan², CHEN Yu-long²,
KONG De-xin², WU Cheng-zhao¹, CHEN Xiao-fei², BAI Zhao-fang³, NIU Ming³,
WANG Jia-bo⁴, ZHAO Yan-ling^{3*}, XIAO Xiao-he^{3*}, TANG Jin-fa^{2*}

(1. School of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, China; 2. The First Affiliated Hospital of Henan University of Traditional Chinese Medicine, Zhengzhou 450000, China; 3. The Fifth Medical Center of Chinese PLA General Hospital, Beijing 100039, China; 4. School of Traditional Chinese Medicine, Capital Medical University, Beijing 100069, China)

Abstract: According to the theory of 'Xingben Dazao' of *Psoralea corylifolia* Linn. (BL), the susceptible syndromes and biomarkers of liver injury caused by BL were searched. Rat models of kidney-yin deficiency syndrome (M_yin) and kidney-yang deficiency syndrome (M_yang) were established, and all animal experimental operations and welfare following the provisions of the First Affiliated Experimental Animal Ethics and Animal

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*通讯作者 Tel: 86-371-66233612, E-mail: zhaoyl2855@126.com; pharmacy302xxh@126.com; a0519@163.com

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Welfare Committee of Henan University of Traditional Chinese Medicine (No. YFYDW2020017). The results showed that BL significantly decreased the body weight, water intake, and urine weight of M_{yin} rats and increase the organ indexes of the liver, testis, adrenal gland, and spleen and the expression of alanine aminotransferase (ALT). Meantime, BL significantly increased the urine weight of M_{yang} rats and decreased the expression of ALT and aspartate aminotransferase (AST). Hematoxylin and eosin (HE) staining showed that BL could aggravate inflammatory infiltration of hepatocytes in rats with M_{yin} and alleviate liver injury in rats with M_{yang}. Metabolomics identified 17 BL co-regulated significant differential metabolic markers in M_{yin} and M_{yang} rats. Among them, 8 metabolites such as glutamine, quinolinate, biliverdin, and lactosylceramide showed opposite trends, mainly involving cysteine and methionine metabolism, tyrosine metabolism, tryptophan metabolism, purine metabolism, sphingolipid metabolism, glycerol phospholipid metabolism, glutamine metabolism, and other pathways. M_{yin}/M_{yang} may be the susceptible constitution of BL for liver damage or protection, which may be related to the regulation of amino acid metabolism and sphingolipid metabolism. The study can provide some experimental data support for the safe and accurate use of BL in the clinical practice of traditional Chinese medicine.

Key words: *Psoralea corylifolia* Linn.; liver injury; liver protection; kidney-yin deficiency syndrome; kidney-yang deficiency syndrome; metabolomics

补骨脂为豆科植物补骨脂 (*Psoralea corylifolia* Linn.) 的干燥成熟果实, 其性温, 味苦、辛, 归肾、脾经, 具有温肾助阳、纳气平喘、温脾止泻的功效, 临床主要用于治疗骨质疏松、骨关节炎、骨折等骨科疾病^[1], 且历代医书均未见记载补骨脂这一临床使用千年的补益药是否有毒。然而, 近年来先后出现多例补骨脂致肝损伤临床个案病例报道^[2,3], 国家药品监督管理局也先后通报了含补骨脂制剂(壮骨关节丸、仙灵骨葆胶囊、骨康胶囊等) 肝损伤的风险^[4], 而多个研究团队基于体内外实验也均证实了补骨脂可致肝损伤的客观属性^[5-7]。国际权威药物肝损伤信息网站 LiverTox (www.livertox.nih.gov) 也对补骨脂及其成分肝损害问题进行了收录。

尽管当前国内外多个研究团队已经部分阐明补骨脂毒性成分与潜在的致毒机制^[8-10], 但中医临床治疗讲究辨证用药, 药物偏性与机体病证相符则可产生较好的治疗效果, 而当中药的偏性与机体病证不符合时, 则可能会影响机体的阴阳气血津液失衡, 导致不良反应发生, 即所谓的“有故无殒也”。考虑到涉及中医证候样本, 尤其是具有难以预测特点的传统“无毒”中药所致药物性肝损伤的相关临床样本的难获取性, 导致目前尚无有关补骨脂肝毒性的中医证候证据, 且其肝毒性的研究基本停留在正常动物实验水平, 使得补骨脂所致肝损害是否与药不对证使用之间的因果关系一直悬而未解, 致使临床医生在使用补骨脂时依然存在抉择困难。因此, 确定适宜/禁忌的证候特征或许对于实现补骨脂在临床的安全、合理、精准使用具有重要意义。

《雷公炮炙论》指出“补骨脂, 性本大燥, 毒, ……”, 但该“毒”是“毒”还是“效”无从考证。历代医家均指出

阴虚火旺者忌服补骨脂, 对用药人群的证候特征作出了一定限制^[11]。但阴虚火旺者服用补骨脂到底会不会发生不良反应? 若发生, 该不良反应与肝损伤是否有关也尚不清楚。在此, 本实验基于性效相关理论, 选用肾阳虚证、肾阴虚证模型动物作为正反对照, 研究补骨脂对上述证候的改善或加剧情况及可能出现的肝保护/损害情况, 并借助代谢组学技术探讨补骨脂对不同证候发挥毒/效作用的代谢标志物网络, 为区分补骨脂适用/忌用人群提供科学依据。

材料与方

动物 48只雄性无特定病原体 (specific pathogen free, SPF) 级 Sprague Dawley (SD) 大鼠, 体质量 180~200 g, 由北京斯贝福提供, 许可证编号 SCXK (京) 2019-0010, 实验动物伦理由河南中医药大学第一附属医院伦理委员会批准 (批号 YFYDW2020017), 按昼夜节律自然照明, 于独立送回风净化笼饲养, 室温 22~26 °C, 相对湿度 40%~70%, 予 60 Co 辐照饲料和纯净水饲喂。

药品与试剂 补骨脂购自安徽普仁中药饮片有限公司 (批号 2102062), 经解放军第五医学中心肖小河研究员鉴定为正品, 凭证标本保存在安徽普仁中药饮片有限公司标本室, 粉碎机粉碎至 1.2 mm 左右, 过 80 目筛网, 备用。注射用氯化可的松冻干粉 (每支 0.1 g, 天津生物化学制药有限公司, 批号 022003012); 甲状腺素钠 (纯度 98%, 批号 S824029)、利血平 (纯度 98%, 批号 R817202) (上海麦克林生化科技有限公司); 谷丙转氨酶 (alanine aminotransferase, ALT)、天冬氨酸氨基转移酶 (aspartate aminotransferase, AST) (南京建成生物工

程研究所, 批号 20211209、20210913)。乙腈 (99.9%, 上海麦克林生化科技有限公司, 批号 A960504) 和甲醇 (99.9%, 阿拉丁公司, 批号 M116126) 为色谱纯试剂。

仪器 美国 Waters ACQUITY™ I-Class UPLC 系统, Xevo G2-XS Q/TOF 质谱仪, 配有 Lock-spray 接口, 电子喷雾离子源 (electron spray ion source, ESI), Masslynx 4.1 质谱工作站, ACQUITY UPLC® HSS T3 C18 色谱柱 (2.1 mm × 100 mm, 1.8 μm; 型号 176001398); 真空离心浓缩仪 (吉艾姆公司, 型号 CV200); 十万分之一电子天平 (梅特勒-托利多集团, 型号 XS205); 台式低温高速离心机 (上海力申科学仪器有限公司, 型号 Neofuge 1600R); 全自动酶标仪 (BioTel 公司, 型号 Synergy H1)。

分组与给药 48 只大鼠适应性喂养 7 天后, 按随机数字表法分为正常 (CON) 组、补骨脂 (BL) 组、肾阴虚证模型对照 (M_yin) 组、肾阴虚证模型+补骨脂 (MBL_yin) 组、肾阳虚证模型对照 (M_yang) 组、肾阳虚证模型+补骨脂 (MBL_yang) 组, 每组 8 只。补骨脂给药组每天上午给予 0.9 g·kg⁻¹ 用 0.5% 羧甲基纤维素钠 (sodium carboxymethyl cellulose, CMC-Na) 混悬的过 80 目筛的补骨脂生粉, 其余各组每天上午灌胃给予 CON 组、M_yin 组和 M_yang 组大鼠等体积 CMC-Na 溶液, 根据体质量变化调整灌胃量, 连续给药 21 天。

模型复制 肾阳虚模型: 从第 8 天开始, 除 CON 组外给予肾阳虚证各组大鼠每天皮下注射氢化可的松注射液 (25 mg·kg⁻¹), 每天 1 次, 连续注射 14 天^[12]。肾阴虚模型: 从第 15 天开始, 除 CON 组外给予肾阴虚证各组大鼠灌服甲状腺片溶液 (160 mg·kg⁻¹) + 利血平溶液 (1 mg·kg⁻¹), 每天 1 次, 连续灌胃 7 天^[13]。以大鼠肛温降低、抱团蜷缩、大便软烂为肾阳虚证模型复制成功标准^[12], 以大鼠肛温升高、多饮多尿、大便干结、暴躁易怒、拱背难抓取为肾阴虚证模型复制成功标准^[12]。

观察记录体质量、肛温、尿量、饮水量及脏器指数情况 造模过程中观察并记录各组大鼠的毛发色泽、行为状态、饮食、大便等外观方面的变化, 实验结束前测量记录各组大鼠肛温、24 h 饮水量与尿量情况, 取材时记录各组大鼠肝、肾、脾、肾上腺脏器指数变化情况。

肝功相关指标 末次给药后, 动物禁食不禁水过夜, 2% 戊巴比妥钠麻醉后, 分别用不含抗凝剂的空白管与含肝素钠的抗凝管进行腹主动脉采血, 3 500 r·min⁻¹ 4 °C 离心 10 min, 分别吸取血清、血浆冻存备用, 血清用来检测 ALT、AST 变化情况, 观测补骨脂对肾阴虚证、肾阳虚证模型大鼠肝脏功能的影响。

肝脏苏木素-伊红 (hematoxylin and eosin, HE) 染色 取肝脏组织 4% 多聚甲醛固定 48 h 后, 进行蜡

块包埋, 切片, 按照试剂说明操作要求进行染色。

血浆样本代谢组学检测 血浆样本处理如下, 取大鼠血浆与甲醇按 1:3 涡旋混匀, 4 °C、14 000 r·min⁻¹ 离心 10 min 使蛋白沉淀, 取 200 μL 上清进样分析。

色谱条件 流动相 A: 0.1% 甲酸水, 流动相 B: 0.1% 甲酸乙腈。梯度洗脱: 0~9 min, 5%~70% B; 9~16 min, 70%~100% B; 16~17 min, 100% B; 17~17.5 min, 100%~5% B; 17.5~20 min, 5% B。柱温 40 °C, 流速 0.3 mL·min⁻¹, 进样量 2 μL。

质谱条件 电喷雾离子源 (electrospray ionization source, ESI) 以 MSE Continuum 模式进行数据采集。准确质量测定采用亮氨酸脑啡肽 (L-enkephalin, LE) (ESI⁻ *m/z* 554.261 5, ESI⁺ *m/z* 556.277 1) 溶液为锁定质量溶液。毛细管电压 ESI⁻ 2.5 kV、ESI⁺ 3.0 kV, 离子源温度 100 °C, 脱溶剂气温度 250 °C, 锥孔电压 40 V, 锥孔气流量 50 L·h⁻¹, 脱溶剂流量 600 L·h⁻¹, 碰撞能量 10~45 V, 间隔扫描时间 0.2 s。质量扫描范围 50~1 200 *m/z*。

多元数据分析和统计学处理 质谱数据采用 Waters 公司的 MassLynx 软件 (v 4.1) 进行采集, 得到的原始数据导入 Progenesis QI 软件 (v 2.4) 中进行色谱峰对齐、峰提取及归一化等处理, 记录其保留时间。采用 SIMCA v14.1 对提取的数据进行主成分分析 (principal component analysis, PCA) 和正交-偏最小二乘判别法 (orthogonal-partial least squares discriminant method, OPLS-DA) 分析。运用变量对分类的重要程度 (variable importance in the projection, VIP) 来预测各个数据对模型的贡献值, 并将 VIP > 1 且 *P* < 0.05 的变量认作对模型有显著贡献。之后将相关差异代谢物置于 ChemSpider (<http://www.chemspider.com>)、Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.kegg.jp/>) 及 Human Metabolome Database (HMDB) (<https://hmdb.ca/>) 等数据库进行分子比对鉴定, 并将差异代谢物导入 MetaboAnalyst 5.0 在线数据库进行通路分析。

统计学处理 实验数据采用统计软件 GraphPad Prism 8.01 进行统计分析, 以 $\bar{x} \pm s$ 表示, 计量资料多组间比较采用单因素方差分析 (one-way ANOVA), 2 组间比较用 *t* 检验, *P* < 0.05 记为差异有统计学意义。

结果

1 补骨脂对肾阴虚证、肾阳虚证模型大鼠体质量、肛温、饮水量、尿量的影响

与 CON 组大鼠相比, BL 组大鼠体质量、肛温、饮水量均无显著变化; M_yang 组大鼠肛温、尿量均显著

降低 ($P < 0.05$), 符合肾阳虚的症状; M_yin 组大鼠体质量显著降低 ($P < 0.05$), 肛温、饮水量、尿量则均显著增加 ($P < 0.01$), 符合肾阴虚的症状。与 M_yang 组大鼠相比, MBL_yang 组大鼠尿量显著增加 ($P < 0.05$); 与 M_yin 组大鼠相比, MBL_yin 组大鼠体质量、饮水量、尿量均显著降低 ($P < 0.05$), 见表 1。

2 补骨脂对肾阴虚证、肾阳虚证模型大鼠脏器指数的影响

与 CON 组大鼠相比, M_yang 组大鼠仅肝脏指数显著升高 ($P < 0.01$), M_yin 组大鼠肝、肾、脾脏器指数均显著增加 ($P < 0.05$); 与 M_yang 组大鼠相比, MBL_yang 组大鼠脏器指数均无显著变化; 与 M_yin 组大鼠相比, MBL_yin 组大鼠肝、肾、睾丸、肾上腺、脾脏脏器指数均显著增加 ($P < 0.05$), 见表 2。

3 补骨脂对肾阴虚证、肾阳虚证模型大鼠血清 ALT、AST 的影响

与 CON 组大鼠相比, BL 组大鼠 ALT、AST 水平均无明显变化, M_yang 组大鼠 ALT、AST 水平则均显著升高 ($P < 0.01$), M_yin 组大鼠 AST 表达也显著增加 ($P < 0.001$)。与 M_yang 组大鼠相比, MBL_yang 组大鼠 ALT、AST 表达均显著降低 ($P < 0.01$); 与 M_yin 组大鼠相比, MBL_yin 组大鼠 ALT 表达显著增加 ($P < 0.01$)。HE 病理染色结果显示, CON 组与 BL 组大鼠肝组织无明显病理改变, M_yang 组大鼠肝组织有一定程度损伤, M_yin 组大鼠肝组织有一定程度炎症浸润, 与 M_yang 组大鼠相比, MBL_yang 组大鼠肝损伤明显减轻; 与 M_yin 组大鼠相比, MBL_yin 组大鼠肝细胞炎症

浸润明显加剧, 见图 1。

4 血浆代谢组学分析

等量混合所有被检样本作为质控样本, PCA 结果分析发现, 正、负离子模式下, 质控样本均可较好地聚集在一起, 表明本实验构建的大鼠血浆代谢物谱的 UPLC-MS 检测方法稳定, 重复性良好。PCA 降维分析发现在正、负离子模式下, M_yang 组、MBL_yang 组、BL 组及 CON 组均可较好地聚为一类, 而 M_yin 组及 MBL_yin 组则与这上述 4 组明显分离, 见图 2, 提示 M_yin 组及 MBL_yin 组大鼠体内的血浆代谢物产生了明显的变化。采用 OPLS-DA 方法, 对补骨脂干预组分别与 M_yin 组及 M_yang 组大鼠血浆代谢指纹谱数据进行两两比较分析, 发现在正、负离子模式下, M_yin 组与 M_yang 组均可与补骨脂干预组被较好地分开 (所有 $R^2Y > 0.90$, $Q^2 > 0.60$), 见图 2。通过检索匹配 HMDB、KEGG、ChemSpider 在线数据库, 以 $VIP > 1$ 与 $P < 0.05$ 为条件, 共鉴定出 24 个内源性代谢物, 含 17 个补骨脂可共同显著扰动 M_yin 组大鼠与 M_yang 组大鼠的差异内源性代谢标志物, 4 个补骨脂可特异性显著扰动 M_yin 组大鼠差异内源性代谢标志物, 2 个补骨脂可特异性显著扰动 M_yang 组大鼠差异内源性代谢标志物, 见表 3, 图 3。

5 差异代谢物感受性曲线 (ROC) 分析

为进一步评估上述差异代谢标志物在区分补骨脂分别干预 MBL_yin 组与 MBL_yang 组的敏感性与特异性, 采用 GraphPad Prism 8.01 软件进行了 ROC 曲线分析, 发现 3-(甲硫基)丙酸酯 [3-(methylthio)propanoate]、

Table 1 Effects of *Psoralea corylifolia* Linn. (BL) on body weight, anal temperature and water consumption, and urine volume of rats with kidney-yin deficiency syndrome (M_yin) and kidney-yang deficiency syndrome (M_yang). CON: Rats in the control group were supplied with sodium carboxymethyl cellulose; MBL_yang: Rats in the M_yang group were supplied with BL; MBL_yin: Rats in the M_yin group were supplied with BL. $\bar{x} \pm s$, $n = 8$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs CON group; $^{\Delta}P < 0.05$ vs M_yang group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs M_yin group

Group	Dose/g·kg ⁻¹	Weight/g	Anal temperature/°C	Water intake/g	24 h urine weight/g
CON	-	319.76 ± 20.85	36.37 ± 0.38	19.58 ± 9.63	9.76 ± 1.89
BL	0.9	321.39 ± 15.00	36.30 ± 0.38	23.17 ± 8.25	8.98 ± 1.10
M_yang	-	312.04 ± 21.56	35.94 ± 0.25*	22.77 ± 8.91	6.65 ± 0.88**
MBL_yang	0.9	313.59 ± 17.84	36.19 ± 0.31	20.56 ± 8.78	10.50 ± 3.58 $^{\Delta}$
M_yin	-	304.01 ± 23.11*	37.79 ± 0.51***	42.68 ± 13.24**	23.23 ± 6.54**
MBL_yin	0.9	269.00 ± 24.95 $^{\#\#}$	38.01 ± 0.40	22.23 ± 7.33 $^{\#}$	11.63 ± 5.21 $^{\#}$

Table 2 Effect of BL on organ index of rats with M_yin and M_yang. $\bar{x} \pm s$, $n = 8$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs CON group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs M_yin group

Group	Dose/g·kg ⁻¹	Liver/%	Kidney/%	Testis/%	Adrenal gland/%	Spleen/%
CON	-	2.64 ± 0.12	0.65 ± 0.08	0.98 ± 0.08	0.016 ± 0.002	0.22 ± 0.03
BL	0.9	2.89 ± 0.12	0.67 ± 0.04	0.97 ± 0.10	0.015 ± 0.002	0.22 ± 0.02
M_yang	-	3.07 ± 0.20**	0.70 ± 0.09	1.00 ± 0.10	0.016 ± 0.002	0.20 ± 0.02
MBL_yang	0.9	3.25 ± 0.20	0.72 ± 0.06	1.01 ± 0.08	0.015 ± 0.004	0.19 ± 0.02
M_yin	-	2.97 ± 0.13*	0.82 ± 0.05**	0.98 ± 0.05	0.020 ± 0.002	0.28 ± 0.03***
MBL_yin	0.9	3.78 ± 0.42 $^{\#\#\#}$	0.90 ± 0.10 $^{\#}$	1.14 ± 0.10 $^{\#\#}$	0.029 ± 0.006 $^{\#\#\#}$	0.35 ± 0.04 $^{\#\#}$

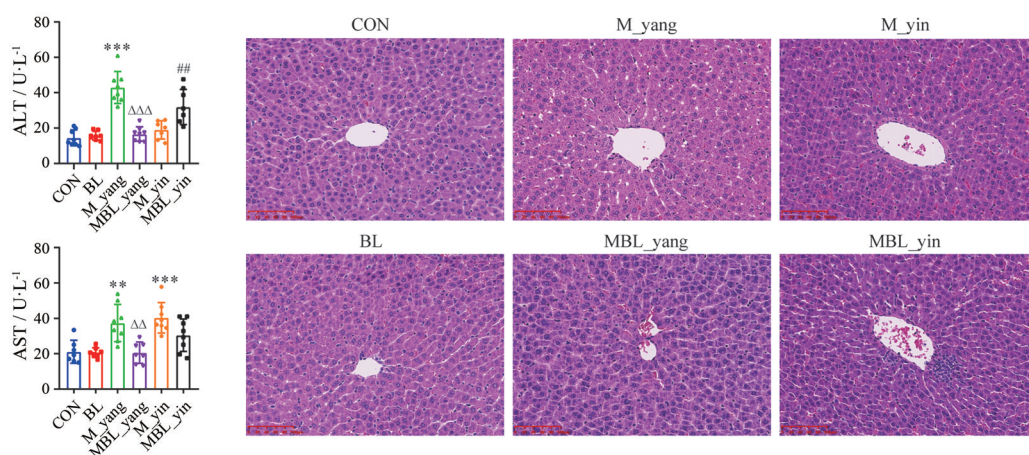


Figure 1 Detection of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) expression in serum, and hematoxylin and eosin (HE) staining for the liver pathological examination (scale bar = 100 μm), $n = 8$, $\bar{x} \pm s$. $**P < 0.01$, $***P < 0.001$ vs CON group; $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ vs M_yang group; $^{\#}P < 0.01$ vs M_yin group

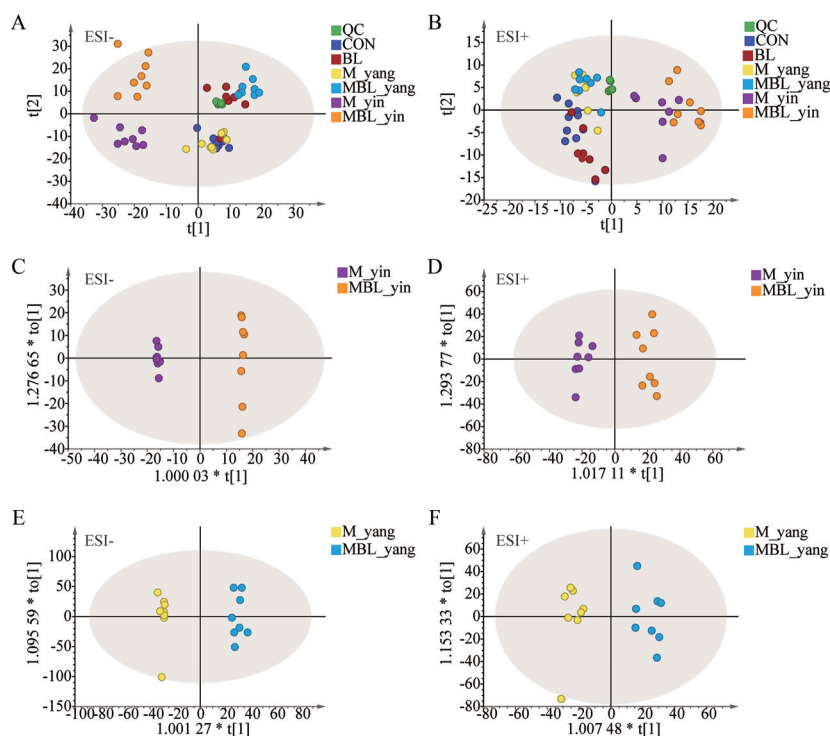


Figure 2 Analysis of plasma metabolites principal components analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) in different groups of rats ($n = 8$). (A, B: Scores scatter plot of liver metabolites in all groups determined by PCA in negative electrospray ion source (ESI-) mode (A) and negative electrospray ion source (ESI+) mode (B); C, D: Scores scatter plot of liver metabolites between the MBL_yin group and M_yin group determined by OPLS-DA in ESI- mode (C) and ESI+ mode (D); E, F: Scores scatter plot of liver metabolites between the MBL_yang group and M_yang group determined by OPLS-DA in ESI- mode (E) and ESI+ mode (F)

3-羟基-L-酪氨酸 (3-hydroxy-L-tyrosine)、三磷酸腺苷 (ATP)、多巴胺 (dopamine)、叶酸 (folate)、乳糖基酰基鞘氨醇 (lactosylceramide)、磷脂酰胆碱 (phosphatidylcholine)、磷脂酰乙醇胺 (phosphatidylethanolamine)、喹啉酸 (quinolinate) 在补骨脂干预 M_yin 与 M_yang 组大鼠具有较好的区分性, ROC 曲线下面积 (area under the

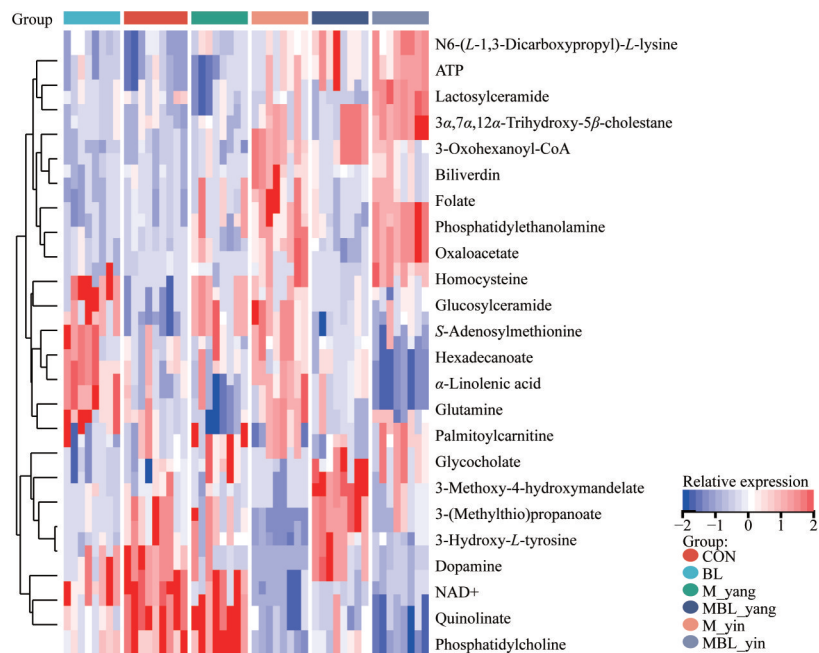
ROC curve, AUC) 均 ≥ 0.9 , 可作为潜在的临床用于诊断区分服用补骨脂患者是否会发生肝损伤的生物标志物, 见图 4。

6 代谢物与 ALT、AST 关联分析

对 ALT、AST 与补骨脂共同扰动代谢物变化进行关联分析, 发现叶酸、磷脂酰乙醇胺变化均与 ALT、

Table 3 Identification results and change trend of plasma metabolic spectrum of rats with M_yin and M_yang model interfered by BL. ↑: Increased; ↓: Decreased; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

ESI	Retention time/min	<i>m/z</i>	KEGG_ID	Metabolite	M_yin vs M_yang	MBL_yin vs M_yin	MBL_yang vs M_yang	MBL_yin vs MBL_yang
-	0.81	145.061 3	C00064	Glutamine	↑***	↓*	↑*	↑
-	0.90	166.017 2	C03722	Quinolate	↓***	↑*	↓***	↓**
-	1.31	487.972 7	C00002	ATP	↑***	↑*	↓*	↑***
-	3.95	178.050 2	C00355	3-Hydroxy- <i>L</i> -tyrosine	↓***	↑*	↑*	↓**
-	3.95	134.060 3	C03758	Dopamine	↓***	↑**	↑*	↓**
-	10.11	321.131 3	C00449	N6-(<i>L</i> -1,3-Dicarboxypropyl)- <i>L</i> -lysine	↑***	↑**	↑*	↑*
-	15.98	612.384 7	C00350	Phosphatidylethanolamine	↑***	↑**	↓*	↑***
-	20.20	457.316 3	C05454	3 α ,7 α ,12 α -Trihydroxy-5 β -cholestane	↑***	↓**	↑**	↑
-	23.13	860.170 6	C05269	3-Oxohexanoyl-CoA	↑***	↓*	↑*	↑**
-	23.20	440.134 7	C00504	Folate	↑***	↓**	↓*	↑***
-	23.21	179.036 5	C05584	3-Methoxy-4-hydroxymandelate	↑***	↓*	↑**	↑*
-	18.62	277.216 8	C00249	Hexadecanoate	↑***	↓**	↑	↑
-	20.30	398.131 8	C00019	<i>S</i> -Adenosylmethionine	↑**	↓**	↓	↑
-	0.77	112.985 1	C00036	Oxaloacetate	↑***	↓	↑**	↑***
-	23.36	180.032 8	C00155	Homocysteine	↑***	↑	↓	↓***
+	0.86	138.055 5	C08276	3-(Methylthio)propanoate	↓***	↑**	↑***	↓***
+	7.52	815.670 9	C00157	Phosphatidylcholine	↓***	↓**	↓**	↓***
+	13.73	400.342 4	C02990	Palmitoylcarnitine	↑***	↓**	↓*	↑*
+	20.28	900.566 1	C01290	Lactosylceramide	↑*	↑***	↓*	↑***
+	23.06	764.536 5	C01190	Glucosylceramide	↑**	↓**	↓*	↓
+	23.11	605.234 8	C00500	Biliverdin	↑**	↓*	↑***	↑
+	18.59	279.232 2	C06427	α -Linolenic acid	↑**	↓***	↑	↓***
+	16.66	466.328 9	C01921	Glycocholate	↓**	↑**	↑	↓
+	7.15	703.074 3	C00003	NAD ⁺	↑	↓	↑*	↓***

**Figure 3** Heatmap analysis of metabolites in plasma of BL interference model rats with M_yin and M_yang

AST表达成显著正相关。棕榈酰肉碱 (palmitoylcarnitine)、3-氧代十六烷基辅酶 A (3-oxohexanoyl-CoA)、葡萄糖神经酰胺 (glucosylceramide) 变化与 AST 表达呈显著正相关, 3-羟基-*L*-酪氨酸、3-(甲硫基)丙酸酯变化、多巴胺与 AST 表达呈显著负相关, 见图 5。

7 代谢物轮廓分析

为明确补骨脂调控 M_yang、M_yin 模型大鼠的差异代谢物变化之间的关联性, 分别对上述差异代谢物在进行了统计分析, 发现补骨脂分别干预 M_yang、M_yin 大鼠后, 谷氨酰胺 (glutamine)、喹啉酸、磷脂

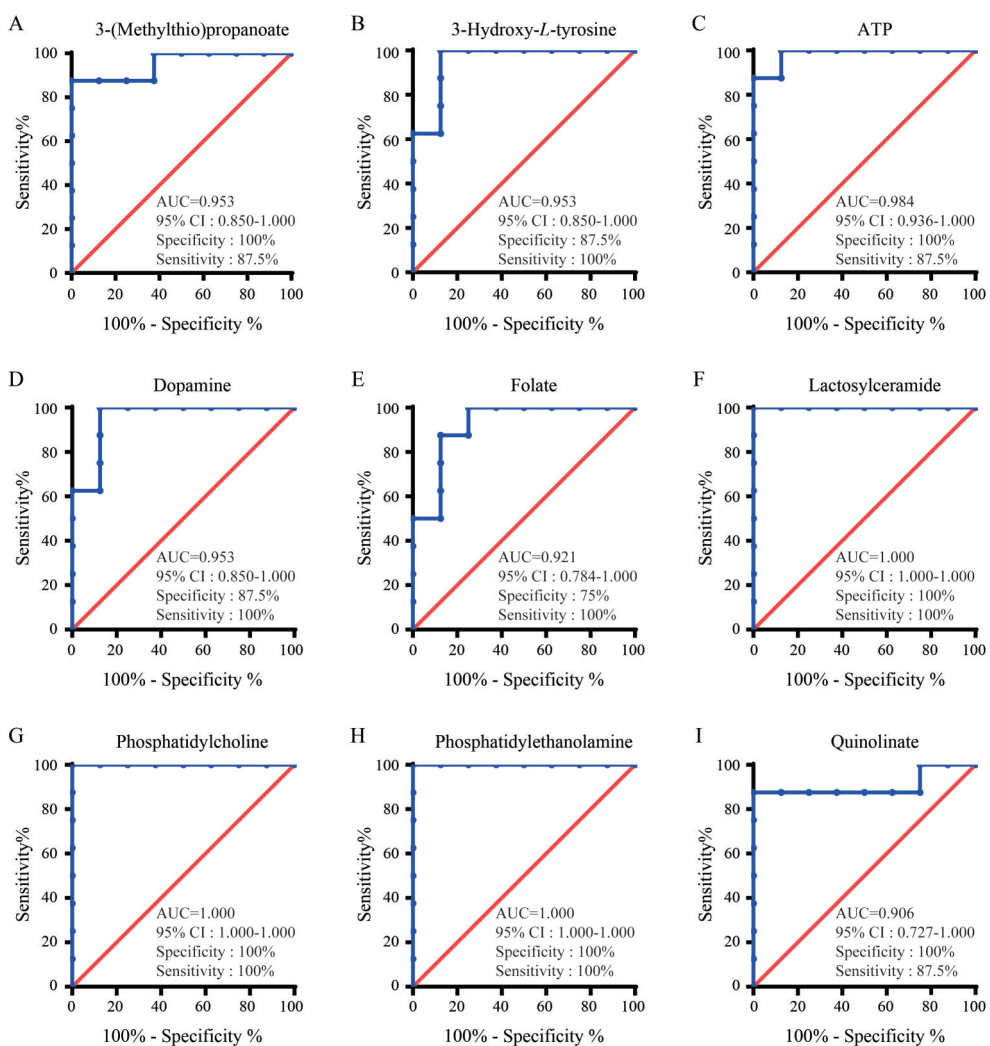


Figure 4 ROC curve of metabolites differentially expressed in M_yin and M_yang models regulated by BL. A: 3-(Methylthio)propanoate; B: 3-Hydroxy-L-tyrosine; C: ATP; D: Dopamine; E: Folate; F: Lactosylceramide; G: Phosphatidylcholine; H: Phosphatidylethanolamine; I: Quinolate. ROC: Receiver operating characteristic; AUC: Area under the ROC curve; CI: Confidence interval

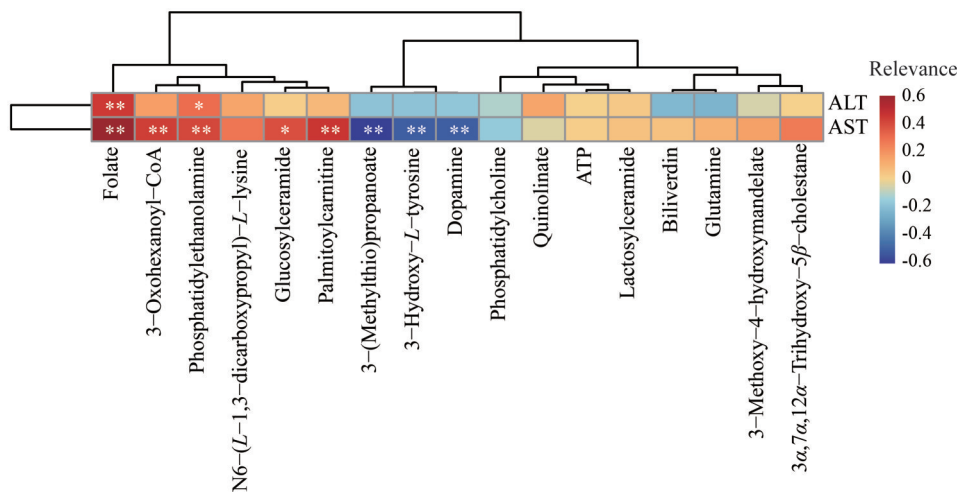


Figure 5 Heatmap of correlation between serum biochemistry (ALT, AST) and metabolites co-regulated by BL ($n = 8$), color depth represents correlation strength, the red color represents positive correlation, the blue color represents negative correlation, * $P < 0.05$, ** $P < 0.01$, the significance of correlation with ALT and AST

酰乙醇胺、 $3\alpha,7\alpha,12\alpha$ -三羟基- 5β -胆甾烷 ($3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β -cholestane)、 3 -氧代十六烷基辅酶 A (3 -oxohexanoyl-CoA)、 3 -甲氧基- 4 -羟基扁桃酸酯 (3 -methoxy- 4 -hydroxymandelate)、乳糖基酰基鞘氨醇、胆绿素 (biliverdin) 8 个代谢物呈相反的变化趋势。进一步对上述差异代谢物进行 KEGG 功能富集分析发现, 补骨脂差异扰动 M_yang、M_yin 代谢通路主要为半胱氨酸和蛋氨酸代谢、叶酸合成、酪氨酸代谢、色氨酸代谢、嘌呤代谢、卟啉与叶绿素代谢、亚油酸代谢、丝氨酸降解、鞘脂代谢、甘油磷脂代谢、 D -谷氨酰胺和 D -谷氨酸代谢、脂肪酸延伸等代谢通路, 并基于已识别的差异代谢产物和丰富的代谢途径构建了代谢网络, 见图 6。

讨论

本研究以中医证候为理论依据, 以补肾壮阳中药补骨脂为例, 构建了经典的肾阳虚证与肾阴虚证大鼠模型。结果发现, 补骨脂可明显减轻肾阳虚证大鼠肝组织损伤程度, 并加剧肾阴虚证大鼠肝组织炎症细胞浸润程度, 提示肾阳虚证、肾阴虚证或许是补骨脂分别发挥肝保护或肝损伤作用的 2 种易感证候, ALT、AST 表达变化也支持了这一现象。为寻找补骨脂发挥上述不同功效作用的潜在分子机制, 本课题组借助代谢组学手段检测、筛选并鉴定出 17 个补骨脂可共同调控肾阴虚证、肾阳虚证模型大鼠的血浆差异代谢标志物。

其中, 谷氨酰胺、喹啉酸、磷脂酰乙醇胺、乳糖神经酰胺、胆绿素、 3 -氧代十六烷基辅酶 A、 3 -甲氧基- 4 -羟基扁桃酸酯、 $3\alpha,7\alpha,12\alpha$ -三羟基- 5β -胆甾烷在补骨脂干预肾阴虚证、肾阳虚证模型大鼠后表现出反向上调或下调的变化趋势。

胆绿素可抑制 Toll 样受体 4 (toll-like receptor 4, TLR4) 与一氧化氮 (nitrous oxide, NO) 表达而降低脂多糖 (lipopolysaccharide, LPS) 对巨噬细胞的炎性诱导^[14], 而 LPS 诱导的免疫炎症可致肝损伤发生, 提示胆绿素含量降低或可表征肝损伤的发生, 本实验中补骨脂分别降低与增加肾阴虚证、肾阳虚证大鼠胆绿素表达, 间接说明胆绿素可能是补骨脂对肾阴虚证、肾阳虚证模型大鼠发挥肝损伤/保护的內源物之一。胆绿素可较好降低猪肝脏缺血再灌注损伤^[15]也侧面支持了胆绿素有一定肝保护作用。

喹啉酸作为色氨酸-犬尿氨酸的一种代谢产物, 可选择性激动兴奋性谷氨酸受体亚型之一的 N -甲基- D -门冬氨酸 (N -methyl- D -aspartate, NMDA) 受体, 引起细胞膜内外离子失衡, 激活神经毒性信号转导途径, 释放系列神经毒性物质, 导致精神类疾病的发生^[16]。同时, 在炎症或病毒感染条件下, 喹啉酸表达也明显升高, 且伴随烟酰胺腺嘌呤二核苷酸 (nicotinamide adenine dinucleotide, NAD⁺) 的紊乱^[17]。已有研究证实喹啉酸可剂量依赖性损害孕鼠子代肝细胞^[18]。本实验中补骨脂可分别促进肾阴虚证、肾阳虚证模型大鼠喹啉酸含

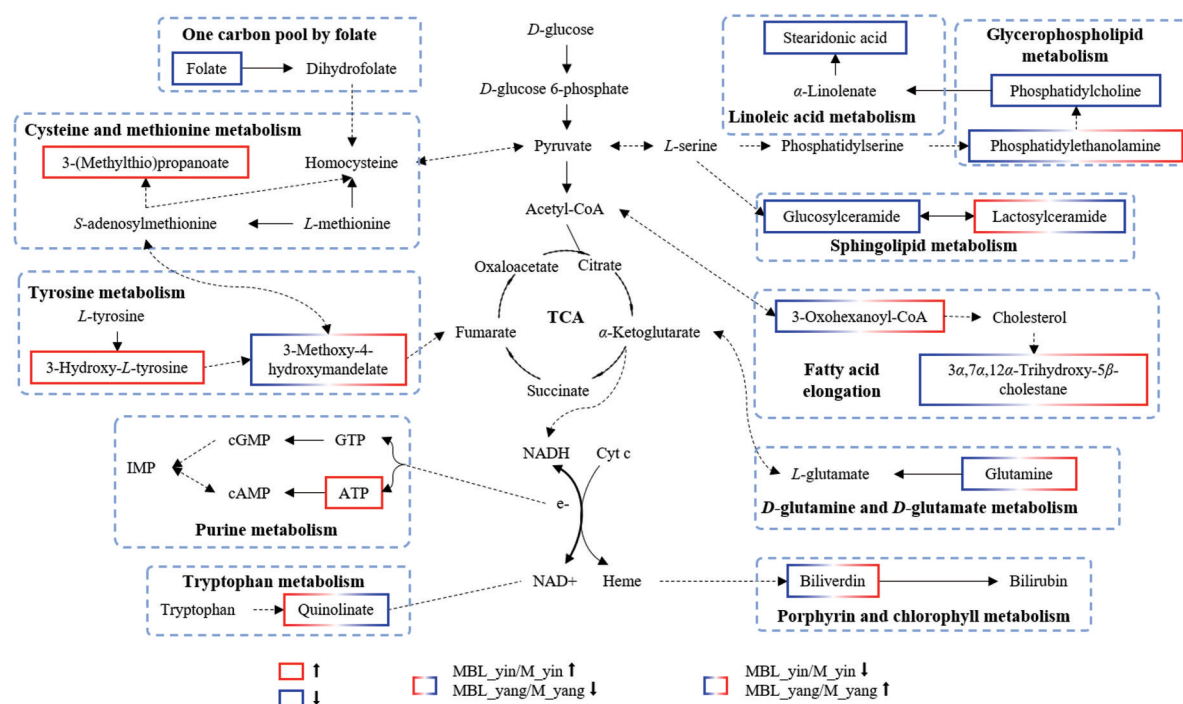


Figure 6 BL jointly disturbs the metabolic pathway and metabolite network of rats with M_yin and M_yang

量的升高与降低,说明喹啉酸或许是补骨脂对上述两种模型发挥毒/效不同作用的内源性物质基础之一。

谷氨酰胺具有一定的肝保护作用,主要表现在谷氨酰胺可抑制四氯化碳诱导的小鼠肝纤维化及转化生长因子- β 1 (transforming growth factor- β 1, TGF- β 1) 介导的小鼠肝细胞上皮-间充质转化与凋亡^[19],还可通过表皮生长因子受体 (epidermal growth factor receptor, EGFR) 预防酒精性肠道通透性、内毒素血症和肝损伤^[20]。谷氨酰胺还可通过抑制核转录因子 (nuclear transcription factor, NF- κ B) 并激活核因子-E2相关因子2 (nuclear factor E2-related factor 2, Nrf2) 发挥抗氧化、抗炎作用来减轻硫代乙酰胺诱导的重症急性肝衰竭大鼠肝损伤与肝脏缺血/再灌注损伤程度^[21,22]。因此,本实验中补骨脂降低肾阴虚证大鼠血浆中谷氨酰胺水平或与其引发的肝损伤有关,而补骨脂上调肾阳虚证大鼠谷氨酰胺水平则可能是其发挥肝脏保护作用的原因之一。

乳糖酰基鞘氨醇,在外界不良环境刺激下,可激活烟酰胺腺嘌呤磷酸二氢酶 (nicotinamide adenine phosphodihydrogenase, NADPH) 氧化酶,生成活性氧物 (reactive oxygen species, ROS) 并营造高度“氧化应激”环境,进而诱导线粒体功能障碍,触发信号分子和通路级联,引发炎症、动脉粥样硬化、心血管疾病和糖尿病等症状或疾病^[23]。补骨脂干预肾阴虚证大鼠乳糖酰基鞘氨醇显著增加,肾阳虚证组大鼠则与之相反,暗示乳糖神经酰胺或许也是补骨脂对上述两种证候发挥毒/效不同作用的内源性物质基础之一。也有文献^[24]称预给药乳糖酰基鞘氨醇可抑制刀豆蛋白诱导的肝损伤,提示乳糖酰基鞘氨醇具有双向作用。

3-甲氧基-4-羟基扁桃酸酯作为酪氨酸一种代谢产物,既可与半胱氨酸与S-腺苷蛋氨酸相互作用生成同型半胱氨酸,又可与3-氧代十六烷基辅酶A共同参与还原型烟酰胺腺嘌呤二核苷酸 (nicotinamide adenine dinucleotide reduced, NADH) 向NAD⁺转化,而3 α ,7 α ,12 α -三羟基-5 β -胆甾烷则作为NAD⁺的氧化还原酶,可将NAD⁺还原为NADH,上述内源物变化均可影响三磷酸腺苷 (adenosine triphosphate, ATP) 生成^[25]。尽管本实验结果发现服用补骨脂后,肾阴虚证、肾阳虚证大鼠血浆中3-甲氧基-4-羟基扁桃酸酯、3-氧代十六烷基辅酶A、3 α ,7 α ,12 α -三羟基-5 β -胆甾烷水平变化趋势不一致,但ATP水平均显著增加,说明补骨脂均可促进肾阴虚证、肾阳虚证大鼠能量代谢,这也可能是两组大鼠体温升高的原因之一。

此外,本研究还鉴定出4个补骨脂可特异性显著扰动肾阴虚证大鼠差异内源性代谢标志物,其中棕榈

酸 (hexadecanoate)^[26]与 α -亚麻酸 (α -linolenic acid)^[27]具有较好的抗炎作用,S-腺苷蛋氨酸 (S-adenosylmethionine) 可治疗四氯化碳诱导的肝损伤^[28],甘氨酸则被报道与非酒精性脂肪性肝病与肝纤维化的发生呈正相关^[29]。此外,本研究还鉴定出2个补骨脂可特异性显著扰动肾阳虚证大鼠差异内源性代谢标志物,草酰乙酸 (oxaloacetate) 可通过减少氧化应激和增强能量代谢改善双氧水 (H₂O₂) 与四氯化碳 (CCl₄) 诱导的肝损伤^[30],NAD⁺也可通过增强抗氧化能力和减少DNA损伤降低多柔比星诱导的小鼠肝损伤^[31]。而上述被补骨脂特异调控的代谢物在肾阴虚证大鼠中,向加剧肝损伤发展的方向呈对应的显著增加或降低趋势,在肾阳虚证大鼠中则朝保肝方向呈显著增加趋势。

综上所述,肾阴虚证或许补骨脂导致肝损伤的易感证候之一,而肾阳虚证则可能是补骨脂发挥肝保护作用的疗效证候之一,切合“补骨脂,阴虚火旺者忌服”这一历代医家古籍的提示,且补骨脂发挥不同功效可能与调控肝脏谷氨酰胺、色氨酸、嘌呤等能量代谢相关通路有关,也符合补骨脂的温燥之性。本研究将为临床安全合理使用补骨脂提供一定的基础实验证据,也为其他传统无毒中药毒性研究提供可供借鉴的研究方法,但补骨脂“性本大燥”致肝损伤/保护作用的易感证候特征仍需临床报道数据来进一步证实。

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