人脐带间充质干细胞及其外泌体改善肝纤维化的机制研究

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摘要: 基于间充质干细胞 (mesenchymal stem cells, MSCs) 和人脐带间充质干细胞衍生外泌体 (human umbilical cord MSC-derived exosomes, hUC-MSCs-Exos) 的疗法已被广泛视作肝纤维化治疗的潜在策略, 本研究旨在探讨人脐带间充质干细胞 (human umbilical cord MSCs, hUC-MSCs) 和hUC-MSCs-Exos对肝纤维化的作用, 并揭示其潜在的机制。通过构建四氯化碳 (carbon tetrachloride, CCl_4) 诱导的肝纤维化动物模型, 观察组织形态和病理变化, 结合肝功能指标、炎症因子水平及过氧化物含量的变化, 综合评价hUC-MSCs和hUC-MSCs-Exos的抗纤维化能力。同时, 采用miRNA测序技术 (miRNA sequencing, miRNA-seq) 筛选差异表达基因, 借助蛋白免疫印迹法 (Western blot, WB) 验证hUC-MSCs和hUC-MSCs-Exos对Wnt/*β*-catenin信号通路相关蛋白的调控作用。所有方案和程序均由新疆医科大学动物实验中心动物实验伦理委员会批准, 且所有实验程序均符合中国有关实验动物的法规 (批准号: IACUC-JT-20240627-26)。结果显示, hUC-MSCs和hUC-MSCs-Exos均能通过减少胶原堆积、改善肝功能、抑制炎症、降低氧化应激反应有效缓解肝纤维化, 且hUC-MSCs-Exos治疗肝纤维化的效果优于hUC-MSCs; 同时, 二者均可下调肝纤维化组织中Wnt/*β*-catenin通路组分 (Wnt3a、*β*-catenin、WISP1/CCN4、cyclin D1) 的表达。此外, miRNA-seq结果进一步表明miRNA-184对该通路有显著调控作用。本文研究结论证实, hUC-MSCs及hUC-MSCs-Exos均能改善大鼠肝纤维化, 且hUC-MSCs-Exos抗纤维化作用可能与Wnt/*β*-catenin信号通路有关, 这为治疗肝纤维化提供了有效的治疗策略。

关键词: 人脐带间充质干细胞; 外泌体; 肝纤维化; Wnt/*β*-catenin信号通路; miRNA-184

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Mechanisms of human umbilical cord mesenchymal stem cells and their exosomes in ameliorating liver fibrosis

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Abstract: Mesenchymal stem cells (MSCs) and human umbilical cord MSC-derived exosomes (hUC-MSCs-Exos) based therapy has been widely regarded as a potential strategy for the treatment of hepatic fibrosis, and the aim of the present study was to investigate the effects of human umbilical cord MSCs (hUC-MSCs) and hUC-MSCs-Exos on hepatic fibrosis and to reveal their potential mechanisms of action. By constructing an animal

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model of carbon tetrachloride (CCl_4)-induced hepatic fibrosis, observing the histomorphological and pathological changes, and combining the changes in liver function indices, inflammatory factor levels and peroxide content, we comprehensively evaluated the anti-fibrotic ability of hUC-MSCs and hUC-MSCs-Exos. Meanwhile, miRNA sequencing (miRNA-seq) technology was used to screen for the differentially expressed genes, and the role of hUC-MSCs and hUC-MSCs-Exos on the regulation of proteins related to the Wnt/β-catenin signaling pathway was verified with the help of protein immunoblotting (Western blot, WB). All protocols and procedures were approved by the Animal Ethics Committee of the Animal Experiment Center of Xinjiang Medical University, and all experimental procedures complied with Chinese regulations concerning laboratory animals (approval No.: IACUC-JT-20240627-26). The results showed that both hUC-MSCs and hUC-MSCs-Exos could effectively alleviate hepatic fibrosis by reducing collagen accumulation, improving liver function, inhibiting inflammation, and decreasing oxidative stress, and hUC-MSCs-Exos was more effective than hUC-MSCs in treating hepatic fibrosis; at the same time, both of them could down-regulate the expression of Wnt/β-catenin pathway components (Wnt3a, β-catenin, WISP1/CCN4, cyclin D1) in the hepatic fibrosis tissues. In addition, miRNA-seq results further indicated that the expression of miRNA-184 was significantly involved in the regulation of this pathway. The findings in this paper confirmed that both hUC-MSCs and hUC-MSCs-Exos improved liver fibrosis in rats, and the antifibrotic effect of hUC-MSCs-Exos may be related to the Wnt/β-catenin signaling pathway, which provides an effective therapeutic strategy for the treatment of liver fibrosis.

Key words: human umbilical cord mesenchymal stem cell; exosome; liver fibrosis; Wnt/β-catenin signaling pathway; miRNA-184

肝纤维化是慢性肝病的常见结果, 每年导致约200万人死亡, 占全球死亡率的3.5%[1]。其特征是细胞外基质过度沉积、免疫稳态失衡和血管新生异常[2]。肝移植是治疗肝纤维化的有效方法, 但面临免疫排斥、供体短缺、手术并发症和高医疗费用等挑战[3]。因此, 开发新的治疗策略至关重要。

在治疗肝脏疾病的新兴治疗方式中, 基于间充质干细胞 (mesenchymal stem cells, MSCs) 的治疗是一种很有前景的肝纤维化治疗策略[4]。MSCs已被证明具有促进肝细胞再生、延缓肝纤维化、减轻肝脏炎症等作用[5]。由于MSCs治疗存在成本较高、处理细胞时间延迟等问题[6], 越来越多的证据支持MSCs旁分泌机制介导的治疗作用[7]。外泌体 (30～150 nm) 作为干细胞旁分泌膜结合纳米囊泡, 参与复杂的细胞间通讯[8], 不仅含有细胞蛋白和脂质, 还含有宿主细胞的mRNA和miRNA[9]。相较于干细胞, 外泌体体积更小, 结构更简单[10], 便于保存, 能够特异性靶向组织[11], 不会导致肿瘤风险或免疫排斥等。因此, 源自间充质干细胞外泌体的治疗有望为肝脏疾病治疗提供一种创新、无创、低免疫原性且无毒的替代策略, 同时为肝细胞修复提供重要的新机制信息。

在纤维化疾病的靶点挖掘与相关信号通路解析的领域中, 其复杂的信号调控网络长期以来未得到足够重视, 亟需通过系统性的分子通路研究来深入理解疾病机制[12]。肝纤维化的进展与多种综合信号通路相关, 包括Wnt[13]、PI3K/Akt[14]和JNK[15]等。值得关注的是, 诸多研究证据显示, Wnt/β-catenin信号通路的异常激活, 会对肝纤维化的发生与发展起到调控作用[16]。这种对肝纤维化的影响, 在一定程度上进一步推动了肝癌的发生和 (或) 进展[17]。然而, 目前关于人脐带间充质干细胞 (human umbilical cord MSCs, hUC-MSCs) 及人脐带间充质干细胞衍生外泌体 (human umbilical cord MSC-derived exosomes, hUC-MSCs-Exos) 如何通过调控Wnt/β-catenin信号通路缓解肝纤维化的具体分子机制仍未得到全面探究。因此, 本研究将通过CCl_4诱导建立大鼠肝纤维化模型, 探讨hUC-MSCs及hUC-MSCs-Exos对大鼠肝纤维化的治疗效果, 并进一步分析hUC-MSCs及hUC-MSCs-Exos调控Wnt/β-catenin信号通路抗纤维化的作用机制。本研究提示hUC-MSCs-Exos或可为肝纤维化疾病的治疗提供新的途径。

材料与方法

药品与试剂 分化簇 (cluster of differentiation, CD) 90抗体 (328108)、CD73抗体 (344016)、CD19抗体 (302206)、CD105抗体 (800508)、CD11b抗体 (301310)、人类白细胞抗原-DR分型 (human leukocyte antigen-DR isotype, HLADR) 抗体 (307610) 购自深圳市达科为生物技术股份有限公司; 凋亡相关基因相互作用蛋白X (apoptosis-linked gene-interacting X protein, Alix) 抗体 (AB275377)、肿瘤易感基因101蛋白 (tumor susceptibility gene 101 protein, TSG101) 抗体

(AB125011)、CD63 抗体 (AB134045)、ECL 试剂盒 (ab65623) 购自 Abcam 公司; CCl_4 (CI4528579) 购自上海麦克林生化科技股份有限公司; 橄榄油 (olive oil, F27HS176630) 购自上海源叶生物科技有限公司; 秋水仙碱 (colchicine, Col) (H2130067) 购自宝信生物科技 (新疆) 有限公司; 泰镍二氨基联苯胺彩色试剂盒 (DAB, DA1016)、蛋白上样缓冲液 (4×, p1016) 购自 Solarbio 公司; 谷丙转氨酶 (alanine aminotransferase, ALT) 试剂盒 (140123010)、谷草转氨酶 (aspartate aminotransferase, AST) 试剂盒 (140223010)、白蛋白 (albumin, ALB) 试剂盒 (148323007) 购自深圳迈瑞生物医疗电子股份有限公司; 羟脯氨酸 (hydroxyproline, Hyp) 试剂盒 (LZ-R10375)、丙二醛 (malondialdehyde, MDA) 试剂盒 (LZ-R10312)、肿瘤坏死因子-α (tumor necrosis factor-α, TNF-α) 试剂盒 (LZ-R3436)、白细胞介素 2 (interleukin-2, IL-2) 试剂盒 (LZ-R3916)、白细胞介素 10 (interleukin-10, IL-10) 试剂盒 (LZ-R3930)、α-平滑肌肌动蛋白 (alpha-smooth muscle actin, α-SMA) 抗体 (LZ-E01201)、I 型胶原蛋白 (collagen type I, collagen I) 抗体 (LZ-E035679)、III 型胶原蛋白 (collagen type III, collagen III) 抗体 (LZ-E032268)、无翅型 MMTV 整合位点家族成员 3A (wingless-type MMTV integration site family, member 3A, Wnt3a) 抗体 (LZ-E02013)、β-连环蛋白 (beta-catenin, β-catenin) 抗体 (LZ-E02089)、WNT1 诱导信号通路蛋白 1 (WNT1-inducible signaling pathway protein 1, WISP1) 抗体 (LZ-E02354)、细胞周期蛋白 D1 (cyclin D1) 抗体 (LZ-E01153)、甘油醛-3-磷酸脱氢酶 (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) 抗体 (LZ-E01025) 购自上海联组生物科技有限公司; TransZol Up plus RNA Kit 提取试剂盒 (ER-501)、TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix 试剂盒 (AT311-02)、PerfectStart Green qPCR SuperMix 试剂盒 (AQ601-02) 购自北京全式金生物技术股份有限公司; VAHTSTM Small RNA Library prep Kit for Illumina (NR801-02)、VAHTSTM DNA Clean Beads (N411-03) 购自南京诺唯赞生物科技股份有限公司; PMSF 蛋白酶抑制剂 (AR1182)、RIPA 裂解液 (18D191302)、二抗 (BST18CBB18054) 购自武汉博士德生物工程有限公司; BCA 试剂盒 (2761051) 购自 AAT Bioquest 公司; PVDF 膜 (2019-21175) 购自 Millipore 公司; 苏木素−伊红 (hematoxylin and eosin staining, H&E)、马松 (Masson)、天狼星红 (Sirius red) 染色试剂盒购于四川赛因斯特生物科技有限公司; Ki-67 抗体 (27309-1-AP) 购自 Proteintech 公司; 肝细胞核因子 4α (hepatocyte nuclear factor 4α, HNF-4α) 抗体 (AF6297) 购自 Affinity 公司。

仪器 透射电镜 (JEM-F200) 购自日本 JEOL 公司; 纳米颗粒跟踪分析仪 (NTA, NanoSight300) 购自美国 Malvern Instruments Ltd. 公司; 显微镜 (Nikon Eclipse E100) 购自日本尼康公司; 病理切片扫描仪 (Pannoramic SCAN Ⅱ) 购自匈牙利 3DHISTECH Kft; Nanodrop2000 (Nanodrop2000)、酶标仪 (MULTISKAN FC) 购自美国赛默飞公司; 凝胶成像仪 (4800Muti) 购自中国上海 Tanon 公司; CX9 全自动分析仪 (CX9) 购自美国 BECKMAN 公司; 流式细胞仪 (NovoCyte®) 购自美国安捷伦公司; 常规 PCR 仪 [TC-96-G-H(b)B] 购自杭州博日科技股份有限公司; 荧光定量 PCR 仪 (SLAN-96S) 购自上海宏石医疗科技有限公司。

hUC-MSCs 的制备、培养和鉴定 检查脐带完整性后, 称重、量长度, 浸入 75% 酒精 10 s, 用生理盐水冲洗 2 或 3 次。撕开脐带外皮, 暴露华通氏胶, 去除血管, 将胶组织切成 3～5 mm 小块, 用含 10% 胎牛血清的培养基培养, 隔天换液, 12～14 天收获细胞。传代细胞以每平方厘米 3×10^6 个细胞接种, 37 ℃、5% 二氧化碳条件下培养。本研究使用第 3～5 代细胞[18,19]。

采用流式细胞术对 hUC-MSCs 进行鉴定, 并评估其分化成骨、成脂和成软骨细胞的能力。在相应诱导培养基中培养 21 天后, 用茜素红、油红 O 和阿尔新蓝染色确认分化。

hUC-MSCs-Exos 的提取和表征鉴定 将 hUC-MSCs 培养至 80% 的密度后更换无血清培养基, 48 h 后收集培养基。300 ×g (4 ℃) 离心 10 min, 去除细胞沉淀。过滤后, 2 000 ×g (4 ℃) 离心 20 min。采用超速离心法将上清液经 10 000 ×g (4 ℃) 30 min 及 100 000 ×g (4 ℃) 90 min 离心后, 去上清液。PBS 重悬管中颗粒, 100 000 ×g (4 ℃) 离心 90 min, 丢弃上清液, 每管用 50～100 μL PBS 重悬微球 (外泌体)。所有程序均在 4 ℃下进行, 获得的外泌体保存在−80 ℃或立即使用[20]。在进一步使用前用 BCA 蛋白测定试剂盒定量。本研究使用的脐带间充质干细胞来自 5～6 代。

将外泌体溶液置于铜网, 用磷钨酸染色后通过透射电镜观察其形态和大小。利用纳米颗粒跟踪分析仪检测外泌体的浓度、粒径和纯度。用 Western blot (WB) 鉴定外泌体的标志物 Alix、TSG101 和 CD63。

实验动物造模及样本采集 Sprague Dawley (SD) 大鼠 (8 周龄, 雄性, 200 g) 55 只, 购自新疆医科大学动物实验中心, 合格证号: SCXK (新) 2023-0001。所有方案和程序均由新疆医科大学动物实验中心动物实验伦理委员会批准。所有实验程序均符合中国有关实验动物的法规 (批准号: IACUC-JT-20240627-26)。

实验分预实验和正式实验两阶段: 15只用于预实验, 分为control组、model组、hUC-MSCs-Exos低剂量组、hUC-MSCs-Exos高剂量组、hUC-MSCs组, 每组3只, 旨在摸索造模条件及确定外泌体给药剂量 (附图1); 正式实验40只, 随机分为5组 (control组、model组、hUC-MSCs-Exos组、hUC-MSCs组、Col组), 每组8只。

大鼠适应性喂养1周后处理如下: control组给予普通食水喂养; model组、hUC-MSCs组、hUC-MSCs-Exos组及Col组大鼠腹腔注射30% CCl_4-橄榄油混合液造模 (3 mL·kg^{-1}, 2次/周, 连续7周)。造模结束后, control组每只大鼠给予PBS 100 μL, hUC-MSCs治疗组每只大鼠给予含1×10^{6}个hUC-MSCs混悬液100 μL, hUC-MSCs-Exos治疗组每只大鼠给予0.3 μg·μL^{-1} (附图1) hUC-MSCs-Exos混悬液100 μL, Col组每只大鼠给予Col 0.4 mg·kg^{-1}; model组继续予上述造模液腹腔注射。所有治疗均经尾静脉注射, 2次/周, 共4周。

正式实验中, 部分大鼠因造模应激、自然死亡脱落, 最终每组随机选取6只纳入统计分析。4周后治疗结束, 抽取大鼠左心室动脉血, 离心, 取上层血清。切除的肝组织快速冷冻在液氮中。全血离心分离小鼠血清和血浆进行肝功能检测, 取肝组织进行病理分析和进一步分析。

肝脏组织学评价 将肝组织固定于4%多聚甲醛, 石蜡包埋并切片。切片经H&E、Masson和Sirius red染色后, 按标准程序处理。每组切片随机选取6个视野 (20×), 使用Image-pro plus 6.0分析软件量化纤维化区域, Ishak评分系统评估肝纤维化程度。

免疫组化 (IHC) 免疫组化染色时, 加Ki-67、HNF-4α一抗4 ℃培养过夜, 二抗37 ℃孵育1 h。泰镍DAB试剂盒显色后, 用显微镜观察, 病理切片扫描仪扫描及图像采集后, 用Image pro plus 6.0软件量化分析。

血液生化测定 采用CX9全自动生化分析仪检测血清中ALT、AST、ALB肝功能指标。

肝脏Hyp和MDA含量的定量分析及炎症因子检测 采用酶联免疫吸附 (ELISA) 试剂盒检测各实验组大鼠血清中Hyp和MDA及TNF-α、IL-2和IL-10的含量。在450 nm处测量光密度 (A) 值, 绘制标准曲线并计算样品含量。

miRNA文库建立与miRNA-seq数据分析 取control组、model组、hUC-MSCs组和hUC-MSCs-Exos组大鼠肝脏标本 (n = 3) 进行核酸提取, 并用Nanodrop2000检测RNA浓度, 1.0%琼脂糖凝胶电泳评估RNA质量。合格的RNA样本用VAHTSTM Small RNA Library prep Kit构建文库, 经PCR纯化和切胶回收后, 使用Illumina Novaseq6000测序。利用R语言的corrplot软件包 (版本: 4.4.1) 分析基因表达相关性, DESeq2进行差异表达分析, 筛选标准为 $|\log_2FC| \geqslant 0.5$, P value < 0.05。最后, 采用ggplot2和heatmap软件包绘制差异基因的火山图和热图。

实时荧光定量聚合酶链式反应 (real-time PCR, RT-qPCR) 检测RNA表达水平 每组取3只大鼠的肝组织各50 mg (n = 3), 采用商用RNA纯化试剂盒提取总RNA。以总RNA为模板, 使用TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix制备第一链cDNA。后续qPCR反应根据PerfectStart Green qPCR SuperMix试剂盒进行。每个样本重复检测3次, 结果采用$2^{-\Delta\Delta CT}$方法计算, GAPDH被用作检测mRNA表达的内参基因, 而U6则被用作检测miRNA表达的内参基因。PCR引物由生工生物工程 (上海) 股份有限公司设计合成, 具体序列详见附表1。

WB检测 取肝组织约50～100 mg, 剪碎后加入含蛋白酶抑制剂的RIPA裂解液 (1 mL/100 mg组织), 用电动匀浆器冰上匀浆至无明显组织块。充分裂解后用BCA试剂盒测定蛋白质浓度。将蛋白与4× loading buffer按比例 (3∶1) 混合, 沸水浴10 min, 进行蛋白变性。用等量变性的蛋白上样, 进行10% SDS-PAGE电泳, 转移至PVDF膜上, 阻断非特异性信号后, 与一抗 (Alix、TSG101、CD63、α-SMA、collagen I、collagen III、Wnt3a、β-catenin、WISP1、cyclin D1和GAPDH) 4 ℃孵育过夜。二抗室温孵育90 min, 用ECL试剂盒通过凝胶成像仪显示条带, 用Image J软件测定各条带灰度值。

统计学分析 所有数据均采用GraphPad Prism 6.0软件、Image J软件和ImagePro Plus软件进行统计学分析。两组间比较用t检验, 多组间比较用ANOVA检验。P < 0.05视为有统计学差异。

结果

1 hUC-MSCs和hUC-MSCs-Exos的特性分析

本研究通过贴壁法制备和培养hUC-MSCs (图1A), 并对其进行鉴定, 以提供外泌体。hUC-MSCs呈纺锤形或梭形贴壁生长, 传代后细胞增殖明显 (图1B)。检测hUC-MSCs的成骨、成脂和软骨分化能力, 结果显示hUC-MSCs具有多谱系分化能力 (图1C)。流式细胞术分析显示, hUC-MSCs符合间充质干细胞特征, 高表达CD73、CD90、CD105 (> 95%), 低表达CD11b、CD19、HLADR (< 2%) (图1D)。

此外, 研究采用经典的超高速离心法制备hUC-MSCs来源的外泌体 (图2A), 并对hUC-MSCs-Exos开展了形态学和浓度评估。结果显示, hUC-MSCs-Exos超微结构为圆形或椭圆形囊泡结构, 颗粒状, 直径约

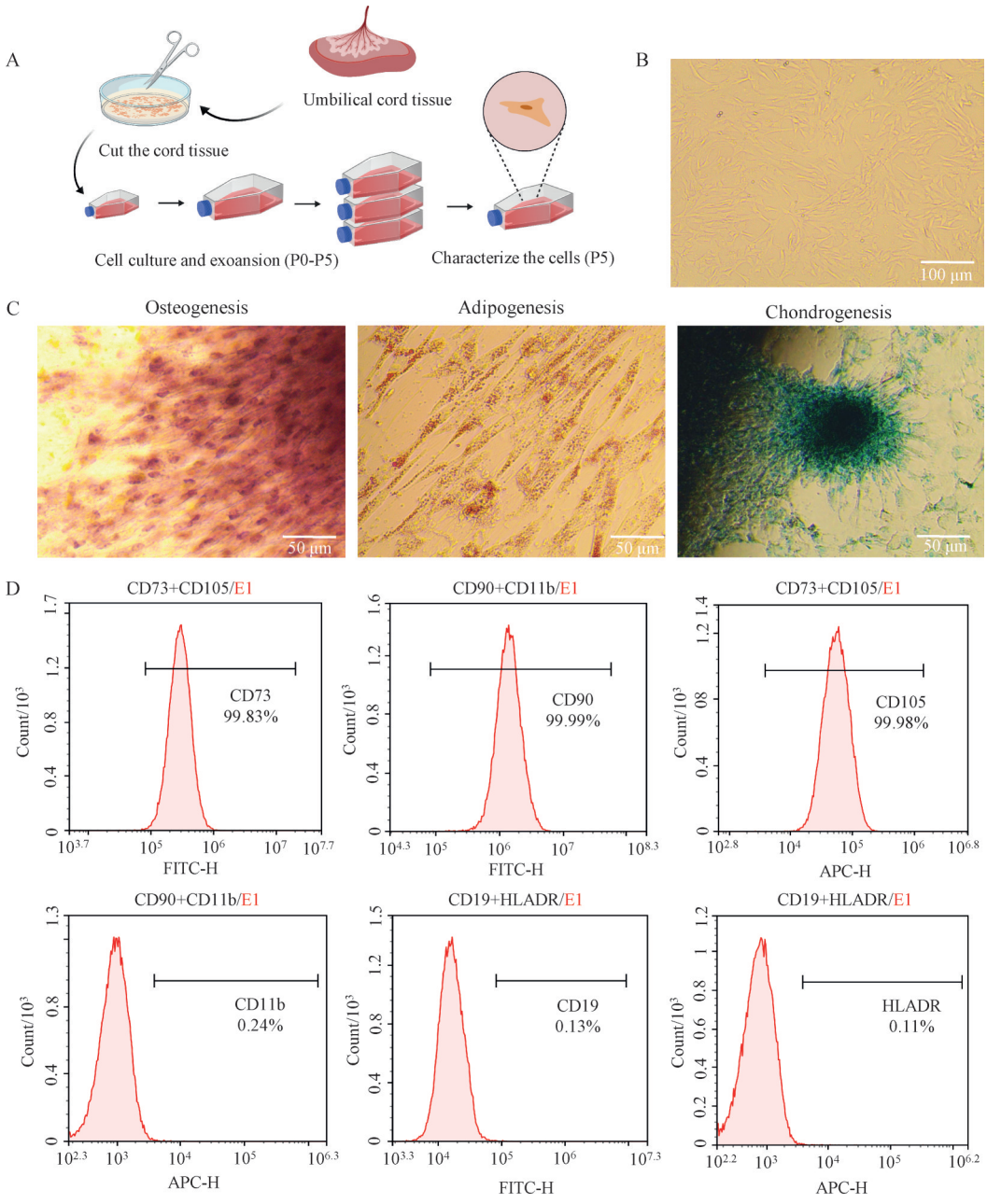


Figure 1 Culture and identification of hUC-MSCs. A: hUC-MSCs culture flow chart; B: Morphology of hUC-MSCs under inverted optical microscope (20×); C: Potential differentiation of hUC-MSCs; D: Flow cytometry for nanoparticle analysis of hUC-MSCs. hUC-MSCs: Human umbilical cord mesenchymal stem cells; CD: Cluster of differentiation; HLADR: Human leukocyte antigen-DR isotype

30～150 nm, 有相对完整的膜结构 (图2B)。NTA表征结果显示, 外泌体的平均粒径为122.5 nm, 每毫升含有9.2×10^{11}个颗粒, 符合外泌体粒径的物理特征 (图2C)。WB检测显示外泌体特异性表达Alix、TSG101和CD63 (图2D)。

2 hUC-MSCs和hUC-MSCs-Exos可缓解CCl_4诱导的大鼠体内肝纤维化

为评估hUC-MSCs-Exos和hUC-MSCs对肝纤维化大鼠的治疗效果, 本研究建立了CCl_4诱导的纤维化模型, 并通过组织学观察及指标分析进行验证 (图3A)。实验结果显示, 治疗组 (hUC-MSCs组、hUC-MSCs-Exos组、Col组) 大鼠体重增加, 而model组体重下降 (图3B)。WB检测证实了model组大鼠肝纤维化*α*-SMA、collagen I、collagen III蛋白表达显著增加 (图3C、D, $P<0.05$, $P<0.01$, $P<0.001$), 肝纤维化模型成功。

通过H&E、Masson和Sirius red染色评估肝纤维化程度。经解剖后发现, model组大鼠肝脏表面发黑, 质地硬, 出现纤维化, 而经hUC-MSCs和hUC-MSCs-Exos治疗的大鼠肝脏颗粒结节较少, 颜色较鲜红 (图4A)。H&E染色显示正常组大鼠肝细胞形态正常,

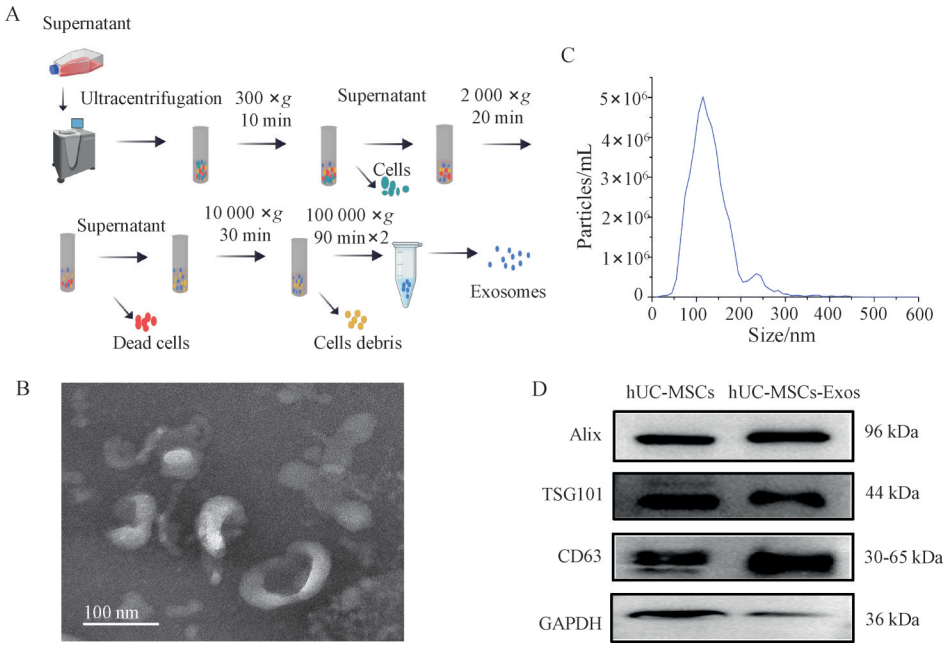


Figure 2 Extraction, separation and identification of hUC-MSCs-Exos. A: Schematic diagram of extraction and separation process of hUC-MSCs-Exos; B: Representative TEM image of hUC-MSCs-Exos (100 nm); C: Particle size distribution and concentration of hUC-MSCs-Exos analyzed by NTA; D: WB analysis of Alix, TSG101, CD63 and GAPDH in hUC-MSCs-Exos and whole-cell lysis of hUC-MSCs. hUC-MSCs-Exos: Human umbilical cord MSC-derived exosomes; TEM: Transmission electron microscope; NTA: Nanoparticle tracking analysis; WB: Western blot; Alix: Apoptosis-linked gene-interacting X protein; TSG101: Tumor susceptibility gene 101 protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

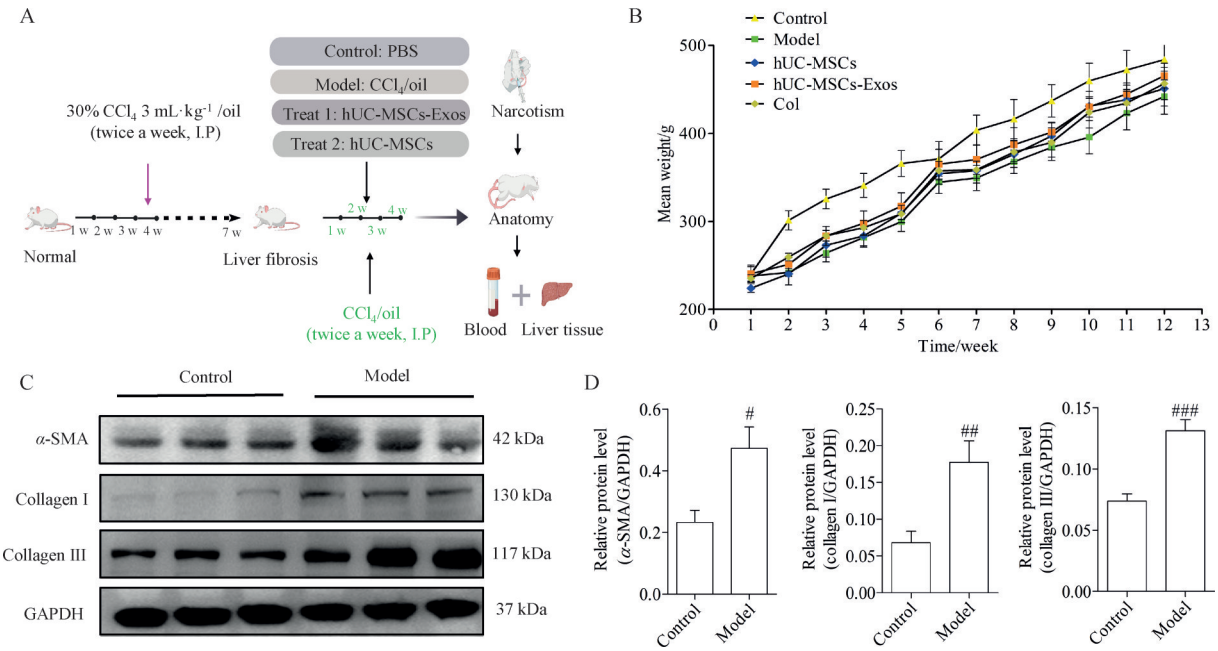


Figure 3 Successful modeling of liver fibrosis induced by CCl_4. A: Diagram of experimental scheme; B: Body weight in rats; C: Identification of rat liver fibrosis protein markers α-SMA, collagen I and collagen III; D: Quantitative analysis of rat liver fibrosis protein markers α-SMA, collagen I and collagen III. α-SMA: α-Smooth muscle actin; Collagen I: Collagen type I; Collagen III: Collagen type III. $n = 6$, mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* control group

model组肝细胞核排列紧密, 有色素沉着和炎症细胞浸润。治疗组 (hUC-MSCs 组、hUC-MSCs-Exos 组、Col组) 大鼠肝细胞形态恢复, 炎症减少, 肝纤维化改善 (图4A)。Masson染色及Sirius red染色显示治疗组

(hUC-MSCs 组、hUC-MSCs-Exos 组、Col 组) 干预后可显著性降低胶原面积百分比, 治疗组之间无明显差异 (图 4A、B, $P < 0.01$, $P < 0.001$)。Ishak 评分 (图 4B) 显示 model 组评分升高 ($P < 0.001$), 治疗组 (hUC-MSCs 组、hUC-MSCs-Exos 组、Col 组) 评分与 model 组相比明显降低 ($P < 0.001$)。以上结果表明, hUC-MSCs 组和 hUC-MSCs-Exos 组的治疗效果与阳性药物组相当, 能改善肝纤维化。

为了进一步评估 hUC-MSCs 及 hUC-MSCs-Exos 治疗是否能减少肝损伤, 促进肝再生, 本实验采用 IHC 方法对 Ki-67 和 HNF-4α 进行定量分析 (图 4C、D)。与 model 组相比, hUC-MSCs 组和 hUC-MSCs-Exos 组中 Ki-67 细胞核表达占比显著减少 ($P < 0.01$, $P < 0.001$); 且 hUC-MSCs 组和 hUC-MSCs-Exos 组能明显提高 HNF-4α 的细胞占比 ($P < 0.01$), 这一变化与肝纤维化程度的减轻存在关联。这提示 hUC-MSCs 及 hUC-

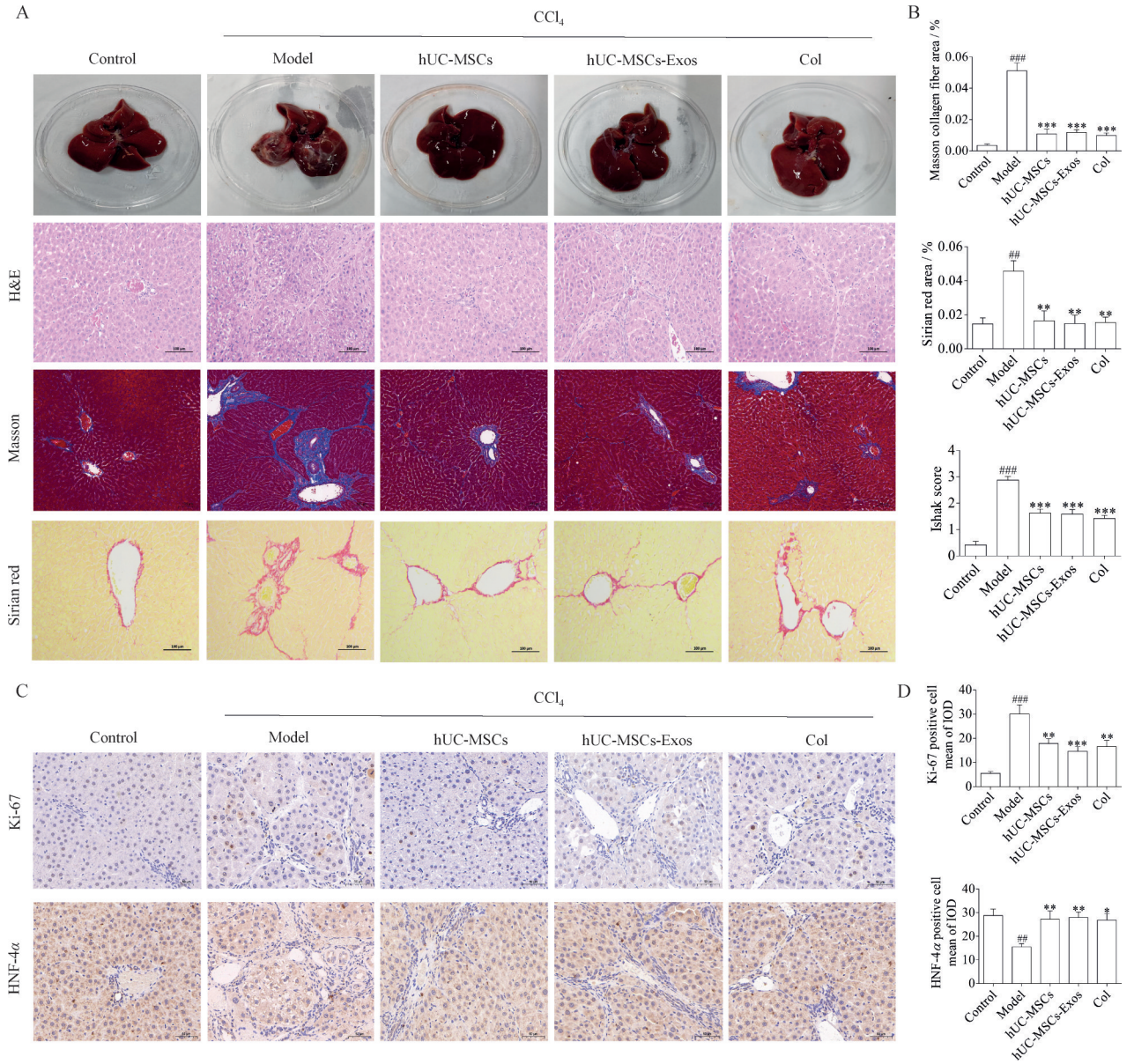


Figure 4 Effects of hUC-MSCs and hUC-MSCs-Exos on liver fibrosis in rats. A: Representative photographs of livers from rats in each group and histopathological images of liver sections were evaluated using H&E, Masson trichrome staining and Sirius red staining in animals with CCl_4-induced cirrhosis (20×, 100 μm); B: Evaluate the area ratio of collagen fibers stained with Masson trichrome and Sirius red in the livers of rats, and grade the liver histopathology according to the Ishak fibrosis score; C: Immunohistochemical staining was performed to detect the protein expressions of Ki-67 and HNF-4α in the injured liver of rats treated with hUC-MSCs and hUC-MSCs-Exos (20×, 50 μm); D: The nuclear proportion of Ki-67 and HNF-4α was evaluated. Ki-67: Ki-67 antigen; HNF-4α: Hepatocyte nuclear factor 4α. $n = 6$, mean ± SEM. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* model group

MSCs-Exos的抗纤维化作用可能涉及对细胞增殖活性 (Ki-67)[21]和肝细胞功能维持 (HNF-4α)[22]的调控, 但其究竟是通过直接调控Ki-67/HNF-4α, 还是经由上游通路间接影响, 仍需进一步实验验证。

3 hUC-MSCs和hUC-MSCs-Exos治疗可减轻肝脏炎症, 改善肝功能

通过生化分析评估hUC-MSCs和hUC-MSCs-Exos治疗后肝功能恢复情况。两组血清ALT和AST水平显著降低, ALB水平升高, 显示hUC-MSCs和hUC-MSCs-Exos均一定程度上恢复肝脏功能 (图5A, $P < 0.05$, $P < 0.01$, $P < 0.001$)。

通过ELISA法检测肝脏组织中炎症因子表达, 与model组相比, hUC-MSCs组和hUC-MSCs-Exos组炎症因子IL-2、IL-10、TNF-α表达均显著降低 (图5B, $P < 0.05$, $P < 0.01$, $P < 0.001$), 且hUC-MSCs-Exos组更能有效降低这些炎症因子含量, 表明hUC-MSCs-Exos抗炎效果更佳。

为了评估hUC-MSCs和hUC-MSCs-Exos对肝脏胶原沉积和脂质过氧化的影响, 本研究对大鼠血清中反映肝纤维化进展活性的Hyp[23]和标志氧化应激与肝细胞损伤程度的[24]MDA进行了检测。结果显示, hUC-MSCs和hUC-MSCs-Exos组Hyp和MDA水平显著降低, hUC-MSCs-Exos组则更低 (图5C, $P < 0.05$, $P < 0.01$), 表明hUC-MSCs-Exos在调节胶原沉积和氧化应激方面优于hUC-MSCs。

综上, hUC-MSCs和hUC-MSCs-Exos治疗均能显著降低大鼠肝纤维化引发的肝脏氧化应激、炎症反应, 并改善肝功能指标, 且hUC-MSCs-Exos治疗效果更佳。

4 hUC-MSCs-Exos通过调控Wnt/β-catenin信号通路抑制大鼠肝纤维化

为研究脐带间充质干细胞及其外泌体治疗肝纤维化的机制, 本研究采用miRNA-seq技术进行分析。首先, 通过样本相关性系数研究, 确认样本可靠性 (附图2), 适合进行差异分析。通过比较model和control组, 发现65个上调基因和109个下调基因。进一步比较hUC-MSCs、hUC-MSCs-Exos与model组, 分别有36和55个上调基因, 27和68个下调基因 (图6A)。为筛选治疗肝纤维化的潜在关联基因, 本研究分析了model与control组的基因变化, 并与hUC-MSCs、hUC-MSCs-Exos组的基因变化进行对比, 识别出9个相交基因 (图6B)。通过热图分析, miRNA-184在model组中表达最高, 但在治疗组中显著下降 (图6C、D)。已有研究表明, miRNA-184参与Wnt/β-catenin信号通路调控[25,26], 结合本研究中miRNA-184的表达变化与治疗效果的关联, 提示miRNA-184可能是hUC-MSCs及hUC-MSCs-Exos发挥抗肝纤维化作用的潜在参与分子, 但其具体调控机制 (包括是否由外泌体直接传递该miRNA介导) 尚需进一步验证。

WB及RT-qPCR结果显示, hUC-MSCs和hUC-

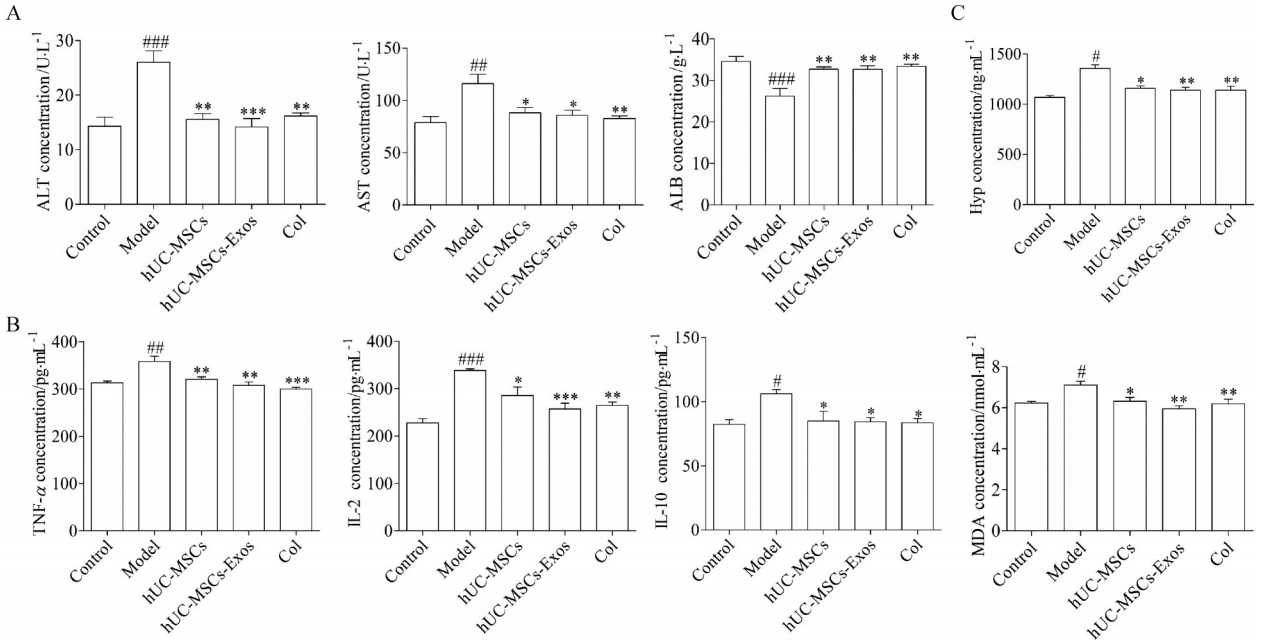


Figure 5 Effects of hUC-MSCs and hUC-MSCs-Exo on liver function, inflammatory factors and oxidative stress markers. A: Serum levels of AST, ALT, and ALB in different groups; B: The relative inflammatory gene expression for IL-2, IL-10 and TNF-α; C: Hyp and MDA contents in different groups. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; MDA: Malondialdehyde; Hyp: Hydroxyproline. $n = 6$, mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* model group

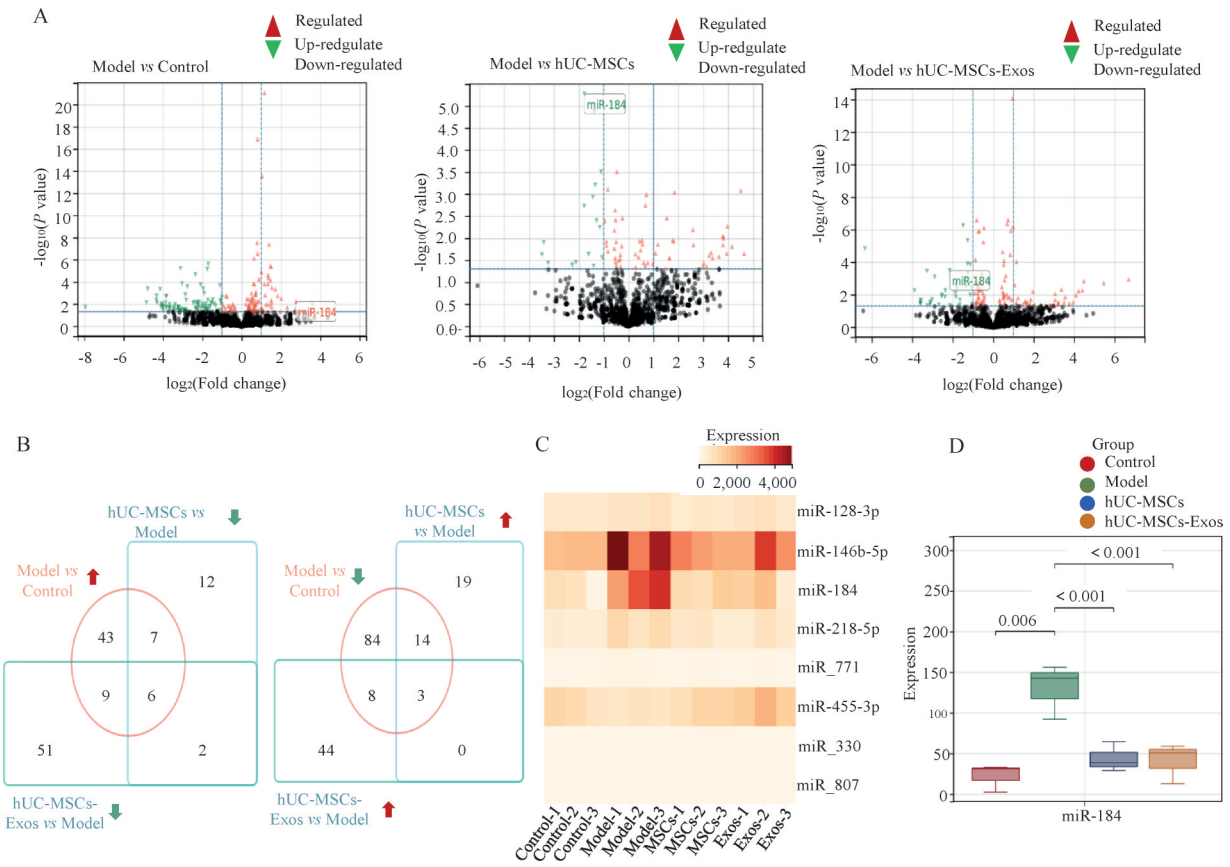


Figure 6 miRNA sequencing data analysis. A: Significant gene analysis of volcano map among control, model, hUC-MSCs and hUC-MSCs-Exos groups. Red represents up-regulated genes and green represents down-regulated genes; B: hUC-MSCs and hUC-MSCs-Exos improve the display of miRNAs related to liver fibrosis in Venn diagrams; C: Differential expression analysis heat map of RNA sequencing data of control, model, hUC-MSCs and hUC-MSCs-Exos; D: Box plot shows the expression of miRNA-184

MSCs-Exos 治疗均能显著降低 Wnt3a、β-catenin、WISP1 和 cyclin D1 的表达 (图 7), 且 hUC-MSCs-Exos 的治疗效果与 hUC-MSCs 相当, 提示 Wnt/β-catenin 信号通路在 hUC-MSCs-Exos 及 hUC-MSCs 的治疗作用中扮演了重要角色。已有研究表明, 异常激活的 Wnt/β-catenin 通路通过核内 β-catenin 上升激活 WISP1、cyclin D1 等下游靶基因的转录表达, 最终促进细胞异常增殖与纤维化进展[27,28]。WISP1 也被归为典型 β-catenin 靶基因之一, 在多种纤维化疾病中上调[29]。本研究结果进一步证实, hUC-MSCs 及 hUC-MSCs-Exos 很可能通过靶向调控 Wnt/β-catenin 通路, 发挥抗肝纤维化的生物学功能。

讨论

据报道, 间充质干细胞衍生的外泌体对肝脏疾病具有特异性疗效, 是肝病治疗极具前景的策略[30]。而肝纤维化作为各类慢性肝脏疾病进展至肝硬化、肝癌的关键前期病理阶段, 其有效干预对阻断肝病恶化具有重要意义[31]。该疾病核心发病机制为肝星状细胞 (hepatic stellate cell, HSC) 激活后增殖并转化为肌成纤维细胞, 上调 α-SMA 表达, 促使 collagen I、collagen III 等细胞外基质 (extracellular matrix, ECM) 合成增多且降解减少, 导致肝纤维化形成[32]。CCl_4 诱导啮齿类动物肝损伤是肝纤维化治疗研究的经典模型[33], 本研究采用该方法成功构建大鼠的肝纤维化模型, 其表现与文献中报道一致[34]。并且通过检测肝组织中 α-SMA、collagen I 及 collagen III 的蛋白表达水平, 从蛋白层面验证了肝纤维化模型的成功构建。

间充质干细胞外泌体在促进肝脏修复和抗纤维化方面与其亲本干细胞一样有效[35]。本研究病理结果显示, hUC-MSCs 与 hUC-MSCs-Exos 均可显著减少肝组织胶原纤维沉积, 抑制炎症细胞迁移和肝细胞凋亡, 缩小门静脉纤维化大小, 同时降低 Ki-67 阳性细胞率, 提高 HNF-4α 的阳性细胞占比, 且两组均能有效降低 AST 和 ALT 水平, 恢复 ALB 水平, 与阳性药物 (Col) 组治疗效果无显著差异。然而, Col 虽可通过抑制微管聚合发挥抗纤维化作用, 但存在潜在不良反应[36]; 人脐带来源 MSCs 虽具备自我更新、多向分化等特性[37], 但

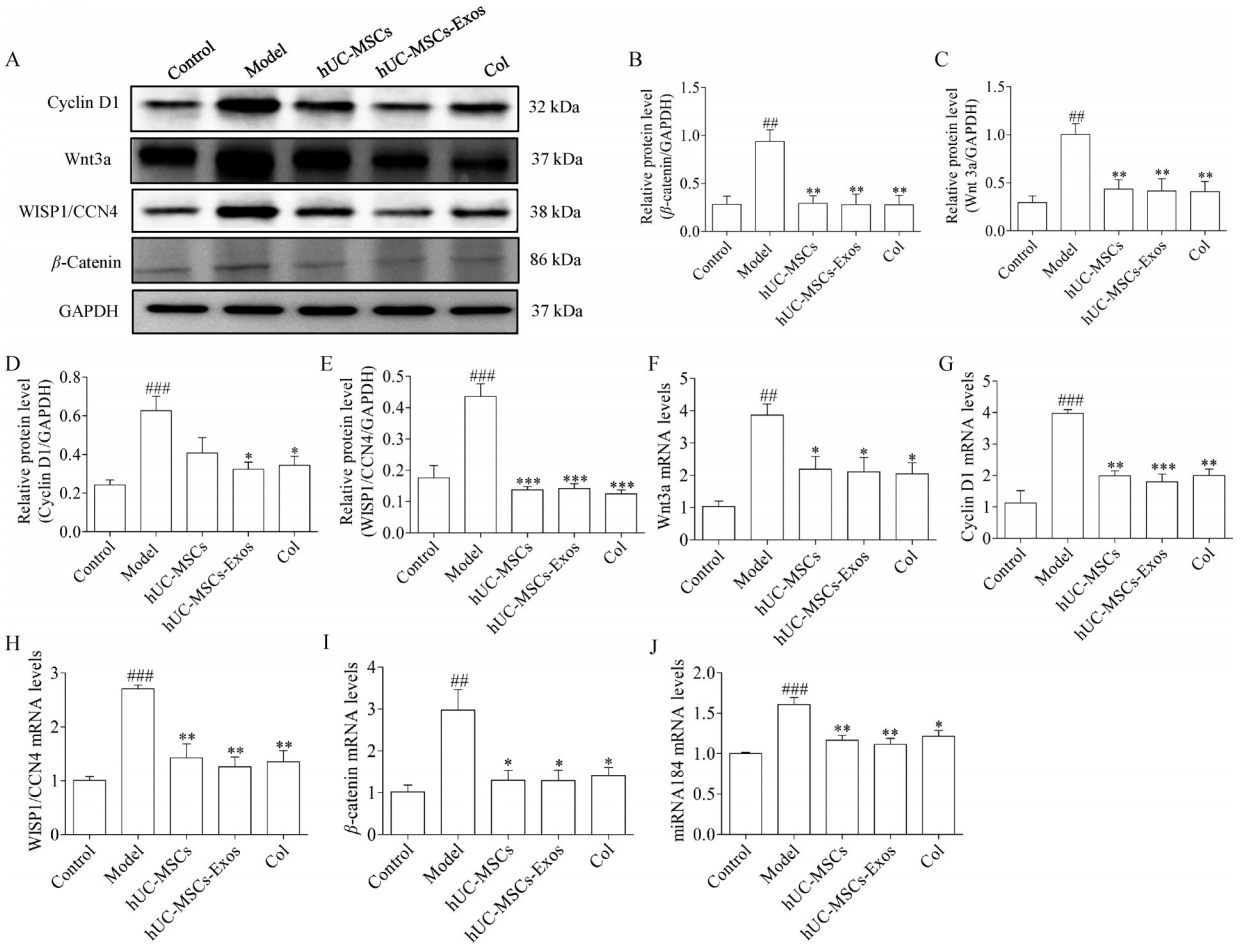


Figure 7 hUC-MSCs and hUC-MSCs-Exos inhibit hepatic fibrosis through Wnt/β-catenin signaling pathway. A: The protein expression of cyclin D1, Wnt3a, WISP1/CCN4 and β-catenin in rat liver tissues; B−E: Quantitative analysis of β-catenin (B), Wnt3a (C), cyclin D1 (D) and WISP1/CCN4 (E) protein in each group; F−J: RT-qPCR was performed to detect the expression levels of Wnt3a (F), cyclin D1 (G), WISP1/CCN4 (H), β-catenin (I), and miRNA-184 (J) genes in each group. n = 6, mean ± SEM. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* model group

静脉输注后可能影响其在肝脏的植入率和保留率[38], 降低治疗效果。相较于传统的抗纤维化药物, 外泌体兼具亲本干细胞的治疗益处, 且具有易与脂质体融合[39]、免疫原性低、体积小、易于储存和运输的优势[40], 利于异体应用[41], 相较于干细胞更适合肝纤维化治疗[42]。

氧化应激标志物MDA[43]的表达与炎症因子释放及肝纤维化进展密切相关[44], 大鼠血清检测结果显示, 在抑制炎症反应和氧化应激方面, hUC-MSCs和hUC-MSCs-Exos均可降低炎症因子及氧化产物含量, 且hUC-MSCs-Exos组效果优于hUC-MSCs组。这一结果与“间充质干细胞对纤维化的作用是通过减少炎症介导的, 而不是直接促进降解”[45]的观点一致。

为了进一步探索hUC-MSCs-Exos对肝纤维化治疗的机制, 本研究采用miRNA-seq技术对其进行了差异基因筛查, 并发现miRNA-184表达显著, 推测hUC-MSCs-Exos的抗肝纤维化作用可能与miRNA-184的表达有关。研究表明, miRNA-184在肝纤维化中起关键作用: 可通过调节HSC的增殖和凋亡减缓纤维化[46], 抑制ECM胶原蛋白合成并促进基质金属蛋白酶 (matrix metalloproteinases, MMPs) 表达[47], 抑制肝纤维化; 此外, miRNA-184还参与调控炎症信号通路, 减少炎症细胞活化和因子释放[48], 减轻肝脏炎症, 间接抑制肝纤维化。另外, 已有文献表明, Wnt/β-catenin信号通路是抗肝纤维化潜在治疗靶点[49], 且miRNA-184参与Wnt/β-catenin信号通路调控[50]。在本研究中, miRNA-184在肝纤维化中表达显著, hUC-MSCs-Exos干预可下调其表达, 并抑制Wnt/β-catenin信号通路中Wnt3a、β-catenin、WISP1、cyclin D1的表达。据此推测, hUC-MSCs-Exos抑制肝纤维化可能与miRNA-184参与Wnt/β-catenin信号通路调控有关。

综合文献及本研究结果推测: 在肝损伤纤维化过

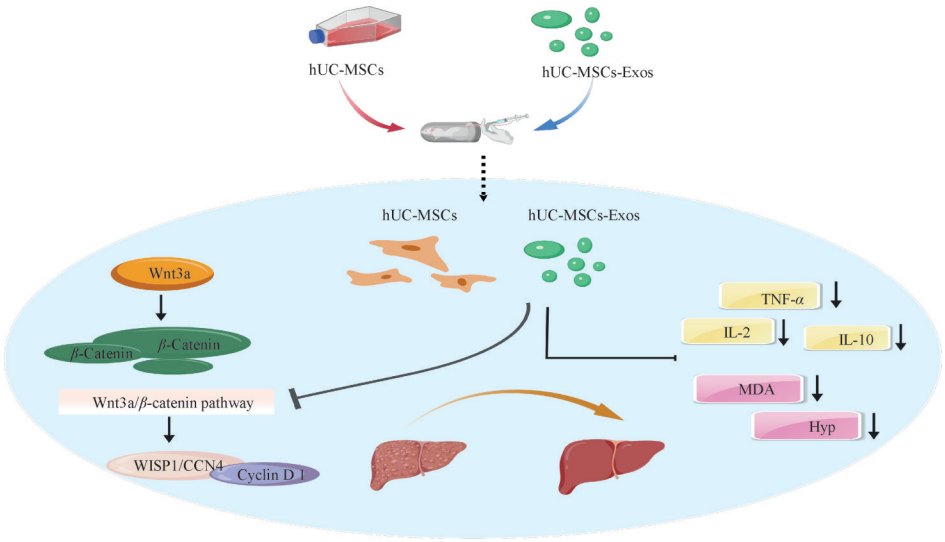


Figure 8 Mechanism diagram of hUC-MSCs and hUC-MSCs-Exos regulating liver fibrosis in rats through the Wnt/β-catenin signaling pathway

程中, miRNA-184表达发生改变, Wnt/β-catenin信号通路被激活, β-catenin降解受到抑制, 并大量积聚转移入核, 细胞核内的β-catenin水平增高, 促使WISP1、cyclin D1等下游相关基因表达[51]。同时, Wnt/β-catenin信号通路介导炎性因子(TNF-α、IL-2、IL-10)的产生和释放以及氧化应激反应(MDA); hUC-MSCs和hUC-MSCs-Exos进行干预, 降低了炎症因子的水平, 减轻了氧化应激反应, 从而改善了大鼠肝脏功能, 抑制了肝纤维化的发展(图8)。

综上所述, 本研究证实hUC-MSCs及hUC-MSCs-Exos可显著改善肝纤维化大鼠肝功能, 减少胶原沉积, 减轻炎症及氧化应激反应; 通过miRNA-seq技术结合后续验证实验进一步发现, hUC-MSCs及hUC-MSCs-Exos可通过阻断Wnt/β-catenin信号通路相关蛋白的表达发挥抗肝纤维化作用, 且hUC-MSCs-Exos治疗效果更优于hUC-MSCs, 这为治疗肝纤维化提供了一种新的、有效的途径。鉴于肝纤维化病理过程的复杂性, miRNA-184调控Wnt/β-catenin信号通路的关键靶基因及具体分子机制仍需深入阐明, 因此hUC-MSCs及hUC-MSCs-Exos的抗纤维化潜力还有待进一步探索。

作者贡献: 张亚杰负责文中实验方案设计、实验实施、数据收集、结果分析、文章撰写等工作; 李继洋、吕昕负责参与实验中动物实验造模给药及收集工作; 李子沐、许一丹、郭晶负责提供文章思路, 在文章写作方面进行指导和修改; 谷欣欣、黎虎负责图形、文章修改等工作; 潘琦、张黎敏对整个实验内容给予监督, 并对文章进行修改完善。

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