

大黄酚维持线粒体稳态并抑制巨噬细胞M1型极化减轻脓毒症相关急性肾损伤

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[摘要] 目的: 脓毒症相关急性肾损伤(sepsis-associated acute kidney injury, SA-AKI)是重症患者的重要致死原因。巨噬细胞极化失衡在SA-AKI进展中发挥关键作用, 其中经典活化的M1型巨噬细胞通过释放促炎细胞因子加剧肾损伤, 而线粒体稳态失衡是驱动巨噬细胞炎症表型转换的关键。中药单体大黄酚(chrysophanol, CHR)可通过调控巨噬细胞极化方向改善SA-AKI。本研究探讨CHR在SA-AKI微环境中是否通过维持巨噬细胞线粒体稳态来抑制M1型极化, 旨在阐明其发挥抗炎及肾脏保护作用的具体机制。方法: 采用盲肠结扎穿孔法(cecal ligation and puncture, CLP)构建C57BL/6小鼠SA-AKI模型, 并设立假手术(Sham)组、模型(CLP)组及CHR治疗(CLP+CHR)组。体外实验使用人单核巨噬细胞(human monocytic-leukemia cells, THP-1)和人肾小管上皮细胞(human renal tubular epithelial cells, HK-2)构建2种细胞模型: 1)建立M1型巨噬细胞与脂多糖(lipopolysaccharide, LPS)刺激的HK-2细胞的Transwell共培养体系并给予CHR干预, 评估CHR在SA-AKI中的整体保护效应; 2)对单层M1型巨噬细胞给予CHR处理, 聚焦CHR对线粒体稳态的保护作用。采用苏木精-伊红(hematoxylin and eosin, HE)染色观察肾脏病理变化; 检测血尿素氮(blood urea nitrogen, BUN)和肌酐(creatinine, Cr)水平以评价小鼠肾功能情况; 采用酶联免疫吸附试验(enzyme linked immunosorbent assay, ELISA)测定血清及共培养上清中白细胞介素(interleukin, IL)-6和肿瘤坏死因子 α (tumor necrosis factor alpha, TNF- α)水平; 采用细胞计数试剂盒8(cell counting kit-8, CCK-8)法评估HK-2细胞活力; 采用TUNEL检测评估凋亡情况; 采用蛋白质印迹法检测凋亡相关蛋白表达。采用透射电镜观察线粒体超微结构; 检测三磷酸腺苷(adenosine triphosphate, ATP)水平、线粒体膜电位(mitochondrial membrane potential, MMP)及烟酰胺腺嘌呤二核苷酸磷酸氧化型/还原型(nicotinamide adenine dinucleotide phosphate oxidized/reduced, NADP⁺/NADPH)。采用实时荧光定量聚合酶链反应(real time fluorescent quantitative polymerase chain reaction, qPCR)检测线粒体生物合成关键基因过氧化物酶体增殖物激活受体 γ 辅激活因子1 α (peroxisome proliferator-activated receptor gamma coactivator 1 α , PGC-1 α)、线粒体转录因子A(mitochondrial transcription factor A, TFAM)、核呼吸因子1(nuclear respiratory factor 1, NRF1)及M1型极化标志物白细胞分化抗原86(cluster differentiation 86, CD86)的mRNA表达, 并采用蛋白质印迹法验证其蛋白表达; 采用细胞免疫荧光染色观察CD86的表达强度。结果: 与Sham组相比, CLP组模型小鼠肾组织出现明显的肾小管扩张、上皮细胞坏死脱落及管型形成, 损伤评分显著升高($P<0.05$); 经CHR干预可显著减轻肾组织病理损害。在体外模型中, CHR干预可显著逆转LPS刺激导致的HK-2细胞活力下降($P<0.01$)。与Sham组相比, CLP组模型小鼠血清BUN、Cr、IL-6、TNF- α 水平均升高, 差异均有统计学意义(均 $P<0.01$); CHR干预能显著改善小鼠肾功能, 降低血清及共培养上清中促炎细胞因子水平(均 $P<0.05$), 抑制组织与细胞中的细胞凋亡。透射电镜结果表明, CLP组线粒体嵴断裂, 膜结构模糊; 经CHR干预后, 线粒体超微结构得到明显修复。CHR能显著提升SA-AKI

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模型中降低的ATP水平($P<0.001$), 改善M1型巨噬细胞线粒体膜电位, 降低 $\text{NADP}^+/\text{NADPH}$, 恢复线粒体抗氧化平衡($P<0.05$)。蛋白质印迹法和qPCR证实: CHR能显著上调受损模型中线粒体相关基因的mRNA和蛋白表达水平(均 $P<0.05$), 显著下调M1型巨噬细胞标志物的蛋白与mRNA表达(均 $P<0.05$)。免疫荧光结果显示M1组CD86荧光强度高, CHR组荧光强度减弱, 差异均有统计学意义(均 $P<0.05$)。结论: CHR可显著减轻SA-AKI模型小鼠肾脏病理损伤并改善肾功能, 其发挥抗炎及肾脏保护作用的机制可能与维持巨噬细胞线粒体能量与氧化还原稳态、抑制巨噬细胞向M1型极化相关。

[关键词] 大黄酚; 脓毒症相关急性肾损伤; 线粒体稳态; 巨噬细胞; 脂多糖; M1型极化

Chrysophanol alleviates sepsis-associated acute kidney injury by maintaining mitochondrial homeostasis and inhibiting M1 macrophage polarization

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ABSTRACT

Objective: Sepsis-associated acute kidney injury (SA-AKI) is a major cause of mortality in critically ill patients. Imbalanced macrophage polarization plays a crucial role in the progression of SA-AKI, in which classically activated M1 macrophages aggravate renal injury by releasing pro-inflammatory cytokines, whereas mitochondrial homeostasis disruption is a key driver of macrophage inflammatory phenotypic switching. Chrysophanol (CHR), a monomeric active component derived from traditional Chinese medicine, has been shown to ameliorate SA-AKI by regulating macrophage polarization. This study aimed to investigate whether CHR suppresses M1 macrophage polarization by maintaining mitochondrial homeostasis in the SA-AKI microenvironment, thereby elucidating its anti-inflammatory and renoprotective mechanisms.

Methods: A SA-AKI model was established in C57BL/6 mice using cecal ligation and puncture (CLP), and mice were assigned to sham, CLP, and CHR treatment (CLP+CHR) groups. In vitro experiments were performed using human monocytic leukemia cells (THP-1) and human renal tubular epithelial cells (HK-2). Two cell models were established: 1) A Transwell co-culture system of M1 macrophages and lipopolysaccharide (LPS)-stimulated HK-2 cells treated with CHR to evaluate the overall protective effects of CHR in SA-AKI; and 2) a monoculture model of CHR-treated M1 macrophages to specifically assess its effects on mitochondrial homeostasis. Renal pathological alterations were examined by hematoxylin and eosin (HE) staining. Blood urea nitrogen (BUN) and serum creatinine (Cr) levels were measured to evaluate renal function. Enzyme-linked immunosorbent assay (ELISA) was used to quantify interleukin (IL)-6 and tumor necrosis factor- α (TNF- α) levels in serum and co-culture supernatants. HK-2 cell viability was assessed using cell

counting kit-8 (CCK-8), and apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay and Western blotting. Mitochondrial ultrastructure was observed by transmission electron microscopy. Adenosine triphosphate (ATP) levels, mitochondrial membrane potential (MMP), and nicotinamide adenine dinucleotide phosphate oxidized/reduced (NADP⁺/NADPH) ratios were measured. Quantitative polymerase chain reaction (qPCR) was performed to determine the mRNA expression of mitochondrial biogenesis-related genes, including peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC-1α), mitochondrial transcription factor A (TFAM), nuclear respiratory factor 1 (NRF1), and the M1 polarization marker cluster differentiation 86 (CD86), with protein expression validated by Western blotting. CD86 expression was further evaluated by immunofluorescence staining.

Results: Compared with the sham group, mice in the CLP group exhibited marked renal tubular dilation, epithelial necrosis and detachment, tubular cast formation, and significantly increased renal injury scores ($P<0.05$), whereas CHR treatment markedly alleviated these pathological changes. In vitro, CHR significantly reversed the LPS-induced reduction in HK-2 cell viability ($P<0.01$). Serum levels of BUN, Cr, IL-6, and TNF-α were significantly elevated in the CLP group compared with the sham group (all $P<0.01$). CHR treatment significantly improved renal function, reduced pro-inflammatory cytokine levels in serum and co-culture supernatants (all $P<0.05$), and suppressed apoptosis in both tissues and cells. Transmission electron microscopy revealed disrupted mitochondrial cristae and blurred membrane structures in the CLP group, which were markedly restored following CHR treatment. CHR significantly increased the reduced ATP levels in the SA-AKI model ($P<0.001$), improved mitochondrial membrane potential in M1 macrophages, decreased NADP⁺/NADPH ratios, and restored mitochondrial redox balance ($P<0.05$). Western blotting and qPCR demonstrated that CHR significantly upregulated mitochondrial-related gene and protein expression in the injured model (all $P<0.05$), while significantly downregulating M1 macrophage marker expression at both the mRNA and protein levels (all $P<0.05$). Immunofluorescence analysis showed strong CD86 fluorescence intensity in the M1 group, which was significantly attenuated after CHR treatment (both $P<0.05$).

Conclusion: CHR significantly alleviates renal pathological injury and improves renal function in SA-AKI model mice. Its anti-inflammatory and renoprotective effects may be associated with maintaining mitochondrial energy and redox homeostasis and suppressing macrophage M1 polarization.

KEY WORDS

chrysophanol; sepsis-associated acute kidney injury; mitochondrial homeostasis; macrophages; lipopolysaccharide; M1 polarization

脓毒症是由感染引发的宿主免疫反应失调导致的危及生命的器官功能障碍临床综合征^[1], 肾脏是最易受累的器官之一^[2], 临床上约 60% 的患者会并发脓毒症相关急性肾损伤(sepsis-associated acute kidney injury, SA-AKI), 导致患者院内病死率显著升高^[3]。

目前, 临床上针对 SA-AKI 仍缺乏特异性治疗手段^[4]。失控的全身性炎症反应是 SA-AKI 的核心病理机制之一^[5]。在这一疾病发展进程中, 巨噬细胞作为先天免疫的关键效应细胞, 其功能极化状态至关重要^[6]。研究^[7-8]表明: 巨噬细胞可极化为经典活化型(M1 型)和

替代活化型(M2型)2种主要类型,并在脓毒症免疫调控中发挥不同甚至相反的功能,其中M1型巨噬细胞通过大量释放肿瘤坏死因子 α (tumor necrosis factor α , TNF- α)、白细胞介素(interleukin, IL)-6等促炎细胞因子加剧组织损伤,而M2型巨噬细胞则发挥抗炎及促进组织修复的作用。因此,干预巨噬细胞的极化方向,特别是抑制其向M1型极化,是治疗脓毒症及其相关器官损伤的潜在策略。

细胞代谢是巨噬细胞表型的重要决定因素^[9]。线粒体作为细胞能量代谢与多种信号转导的核心,其结构与功能的稳态对巨噬细胞的极化具有关键性作用^[10-11]。线粒体通过调控氧化磷酸化效率、活性氧生成及代谢中间产物,影响核因子 κ B(nuclear factor κ B, NF- κ B)等关键炎症信号通路的活化,最终决定巨噬细胞的极化表型^[12]。研究^[13]发现,巨噬细胞线粒体功能受到损害时,巨噬细胞M1型极化显著增强,表明维持线粒体稳态对巨噬细胞功能调控极为重要。大黄酚(chrysophanol, CHR)是从中药大黄中提取的一种蒽醌类单体成分,具有抗炎、抗氧化、抗凋亡及免疫调节等多种药理活性^[14-16]。本课题组前期研究^[17]发现:CHR可以抑制SA-AKI模型中巨噬细胞M1型极化,减轻炎症反应,延缓疾病进程,但CHR能否通过调控巨噬细胞线粒体稳态来影响其极化表型尚不清楚。本研究提出科学假说:CHR可能通过维持巨噬细胞线粒体稳态,抑制其向M1型表型极化,从而减轻SA-AKI程度。本研究综合应用体内/体外SA-AKI模型,旨在明确CHR的肾脏保护作用,验证该假说的科学性,以期对CHR治疗SA-AKI提供新的理论依据。

1 材料与方法

1.1 伦理声明

本研究获得石河子大学生物伦理委员会批准(审批号:A2024-274)。

1.2 实验动物

本研究采用6~8周龄无特定病原体(specific pathogen free, SPF)级雄性C57BL/6小鼠为实验动物,严格遵循美国国立卫生研究院(National Institutes of Health, NIH)发布的《实验动物使用与护理指南》(NIH publication #85-23, revised in 1985)相关准则。小鼠饲养条件包括自由摄食/饮水、每12 h光暗循环、垫料干燥且每2 d更换1次。

1.3 细胞系与细胞培养

人单核细胞系THP-1(目录号:SCSP-567)购自中

国科学院上海生物科学研究所细胞库,使用含10%胎牛血清和1%青霉素-链霉素双抗的RPMI-1640完全培养基,在37℃、5% CO₂恒温培养箱中培养。人肾小管上皮细胞HK-2(货号:CL-0109)购自武汉普诺赛生命科技有限公司,使用含10%胎牛血清和1%双抗的最低必须培养基(minimal essential medium, MEM)完全培养基,在37℃、5% CO₂恒温培养箱中培养。所有实验均使用处于对数生长期且状态良好的细胞。

1.4 体内SA-AKI模型构建与实验分组

1.4.1 动物实验分组与模型建立

将9只小鼠随机分为3组,即假手术组(Sham)、脓毒症模型组(cecal ligation and puncture, CLP)、CHR治疗组(CLP+CHR),每组3只。采用盲肠结扎穿孔法构建脓毒症模型小鼠:采用0.3%戊巴比妥钠溶液(30 mg/kg)经腹腔注射麻醉小鼠,麻醉成功后沿腹中线切开小鼠腹腔,游离盲肠,于盲肠根部结扎(结扎50%盲肠长度),并使用21 G针头贯通穿刺1次,挤出少量肠内容物后还纳腹腔,逐层缝合。Sham组小鼠实施开腹、暴露并游离盲肠等操作,不实施结扎与穿孔。

1.4.2 药物干预

CLP+CHR组在造模成功后立即经腹腔注射CHR(上海源叶生物科技有限公司,纯度 $\geq$ 98%,货号B21593)溶液(30 mg/kg)。Sham组与CLP组则在相同时间点注射等体积的生理盐水。所有小鼠在术后24 h麻醉处死,收集血液及肾组织样本进行后续分析。

1.5 体外细胞模型构建与实验设计

本研究设计了2种细胞实验策略来系统评估CHR的作用。

1.5.1 共培养模型

本研究采用巨噬细胞-肾小管上皮细胞的共培养模型评估CHR对SA-AKI的整体保护作用:将HK-2细胞接种于Transwell共培养系统的下室,待细胞贴壁后,采用10 μ g/mL的脂多糖(lipopolysaccharide, LPS)刺激24 h以模拟体内损伤;同时,将THP-1细胞采用100 ng/mL佛波酯(phorbol 12-myristate 13-acetate, PMA)诱导24 h分化为M0型巨噬细胞,再加入20 ng/mL的 γ -干扰素(interferon-gamma, IFN- γ)和100 ng/mL LPS刺激24 h极化为M1型巨噬细胞。将诱导成功的M1型巨噬细胞接种于Transwell上室(孔径0.4 μ m),与下室LPS诱导损伤的HK-2细胞共培养。共培养模型分组:M1-HK-2组, M1-LPS+HK-2组, M1+CHR-LPS+HK-2组。其中, M1+CHR-LPS+HK-2组在共培养期间向上室培养基中加入

10 $\mu\text{mol/L}$ CHR(以无血清 RPMI-1640 培养基配制)处理 24 h。

1.5.2 单层巨噬细胞模型

在明确 CHR 保护作用的基础上,利用单层巨噬细胞模型探究 CHR 对巨噬细胞 M1 型极化的影响及其内在机制。将 THP-1 细胞按照“步骤 1.5.1”方法诱导为 M1 型巨噬细胞后,直接设立 M1 组与 M1+CHR 组。M1+CHR 组加入 10 $\mu\text{mol/L}$ CHR 处理 24 h,随后进行巨噬细胞极化、线粒体功能及相关基因表达检测。

1.6 主要试剂与仪器

二氧化碳恒温培养箱(Forma Steri-Cult)购自美国 Thermo 公司;倒置显微镜(OLYMPUS TH4-200)购自日本 SANYO 公司;酶标仪(SpectraMax Plus³⁸⁴/190/Versamax/340PC³⁸⁴)、垂直电泳仪/转膜仪(货号:1645050)、凝胶成像分析系统(货号:1708256)均购自美国 Bio-Rad 公司。MEM 基础培养基(货号:PM150411)购自武汉普诺赛生命科技有限公司;胎牛血清(货号:C04001)、1640 基础培养基(货号:C3010)均购自上海逍鹏生物科技有限公司;LPS(货号:L2630)购自美国 Sigma 公司;IFN- γ (货号:P00028)购自北京索莱宝科技有限公司;细胞计数试剂盒-8(cell counting kit-8, CCK-8)试剂(货号:AC11L054)购自中国上海和元李记生物技术有限公司;Hoechst 33258 染色液(货号:BL2845A)购自中国 Biosharp 公司;TUNEL 细胞凋亡检测试剂盒(货号:C1086)、三磷酸腺苷(adenosine triphosphate, ATP)试剂盒(货号:S0026)、烟酰胺腺嘌呤二核苷酸磷酸氧化型/还原型(nicotinamide adenine dinucleotide phosphate oxidized/reduced, NADP⁺/NADPH)试剂盒(货号:S0179)、线粒体膜电位检测试剂盒 JC-1(货号:C2003S)均购自中国上海碧云天生物技术有限公司;酶联免疫吸附测定(enzyme-linked immunosorbent assay, ELISA)试剂盒(货号:EK282HS, EK206)购自杭州联科生物技术股份有限公司;血尿素氮(blood urea nitrogen, BUN)试剂盒(货号:C013-2-1)、肌酐(creatinine, Cr)试剂盒(货号:C011-2-1)均购自南京建成生物工程研究所有限公司;白细胞分化抗原 86(cluster differentiation 86, CD86)抗体(货号:BM4121)、线粒体转录因子 A(mitochondrial transcription factor A, TFAM)抗体(货号:PB0413)均购自中国武汉博士德生物工程有限公司;Bcl-2 相关 X 蛋白(Bcl-2 associated X protein, BAX)抗体(货号:bs-4605R)购自中国武汉三鹰生物技术有限公司;B

淋巴细胞瘤-2 蛋白(B-cell lymphoma-2, Bcl-2)抗体(货号:bsm-52304R)购自英国 Abcam 公司;过氧化物酶体增殖物激活受体 γ 辅激活因子 1 α (peroxisome proliferator-activated receptor gamma coactivator 1 α , PGC-1 α)抗体(货号:WL02123)购自沈阳万类生物科技有限公司;核呼吸因子 1(nuclear respiratory factor 1, NRF1)抗体(货号:CY7186)购自上海埃必威生物技术有限公司。

1.7 实验方法

1.7.1 肾脏组织病理分析与损伤评分

取小鼠肾组织样本,经 4% 多聚甲醛固定、石蜡包埋后,制成厚度 4 μm 的切片;采用苏木精-伊红(hematoxylin and eosin, HE)染色,在光镜下观察细胞的病理变化。肾小管损伤评分标准:0 分,无损伤(损伤面积 0%);1 分,损伤面积 1%~25%;2 分,损伤面积 26%~50%;3 分,损伤面积 51%~75%;4 分,损伤面积 76%~100%。

1.7.2 肾功能评估与炎症因子检测

采集小鼠血清样本,检测小鼠 BUN、Cr 水平,操作严格按照肾功能测定试剂盒说明书执行;收集小鼠血清与共培养模型中的细胞上清液样本,检测炎症因子 TNF- α 、IL-6 水平,操作严格按照 ELISA 试剂盒说明书执行。

1.7.3 细胞活力测定

采用 CCK-8 法,将 HK-2 细胞以 5×10^3 个/孔的密度接种于 96 孔板中,按照分组情况给予相应药物处理后,每孔加入 10 μL CCK-8 溶液,于 37 $^{\circ}\text{C}$ 下孵育 2 h,而后使用酶标仪测定 450 nm 波长处的吸光度值。

1.7.4 TUNEL 检测

肾组织石蜡切片经脱蜡、水化后,使用 TUNEL 细胞凋亡检测试剂盒进行检测:使用蛋白酶 K 修复组织抗原,加入 TUNEL 反应混合液避光孵育,再使用 Hoechst 33258 复染细胞核,最后使用抗荧光淬灭封片剂封片。

1.7.5 蛋白质印迹分析

采用放射免疫沉淀测定(radio immunoprecipitation assay, RIPA)裂解液提取肾脏组织或细胞总蛋白。经二辛可宁酸法(bicinchoninic acid assay, BCA)定量后,取等量蛋白进行凝胶电泳,并转至聚偏二氟乙烯(polyvinylidene fluoride, PVDF)膜,随后使用无蛋白快速封闭液封闭,膜与特定一抗[BAX(1:1 000)、Bcl-2(1:1 000)、PGC-1 α (1:1 000)、TFAM(1:1 000)、NRF1(1:1 000)、CD86(1:1 000),均按说明书稀释]在

4℃下孵育过夜。使用含吐温20的Tris缓冲盐溶液(Tris-buffered saline with Tween 20, TBST)洗涤后,与二抗在室温下孵育1 h,最后使用增强型化学发光(enhanced chemiluminescence, ECL)试剂显影,采用ImageJ软件分析目标条带的灰度值。

1.7.6 线粒体超微结构观察

取约1 mm×1 mm×1 mm的小鼠肾皮质组织,迅速投入预冷的3%戊二醛中固定,随后经1%锇酸处理后固定、丙酮梯度脱水、环氧树脂包埋,制备为超薄切片(厚度60~80 nm),再经醋酸铀酰和柠檬酸铅双重电子染色后,在透射电子显微镜下观察并采集线粒体图像。

1.7.7 线粒体功能检测

通过检测ATP含量、NADP⁺/NADPH与细胞模型中线粒体膜电位评估线粒体功能。采用ATP及NADP⁺/NADPH检测试剂盒,检测肾组织匀浆液或细胞裂解液中的ATP水平及NADP⁺/NADPH,具体步骤按照试剂盒说明书执行。采用JC-1荧光探针法检测线粒体膜电位。收集各组M1型巨噬细胞,与JC-1工作液在37℃下避光孵育30 min,使用缓冲液洗涤后,在荧光显微镜下观察并拍摄。

1.7.8 基因表达水平检测

采用实时荧光定量聚合酶链反应(real-time fluorescent quantitative polymerase chain reaction, qPCR)法检测基因表达水平:使用TRIzol法提取组织或细胞总RNA,反转录为互补DNA(complementary DNA, cDNA)后,以SYBR Green法进行PCR扩增。反应程序:95℃下30 s预变性,95℃10 s、60℃20 s,共40个循环。以甘油醛-3-磷酸脱氢酶(glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*)为内参,采用2^{-ΔΔCt}法计算*CD86*、*PGC-1α*、*NRF1*、*TFAM*等基因的mRNA相对表达量。引物序列见表1。

1.7.9 免疫荧光染色

为检测CHR对巨噬细胞极化的影响,将THP-1细胞诱导为M1型巨噬细胞,按照实验分组干预处理后继续培养24 h。洗涤细胞并使用4%多聚甲醛溶液固定15 min, PBS洗涤细胞3次,5% BSA封闭30 min,在湿盒中4℃下孵育抗体过夜,次日孵育荧光二抗2 h,再次洗涤细胞,滴加抗荧光淬灭剂封片后,在倒置荧光显微镜下观察拍照,采用ImageJ软件分析各组细胞荧光强度。

1.8 统计学处理

采用GraphPad Prism 10.0进行统计分析和绘图,计量资料采用均数±标准差表示,两组间比较采用独

立样本 t 检验,多组间比较采用单因素方差分析。 $P<0.05$ 为差异有统计学意义。

表1 引物名称及序列

Table 1 Names and sequences of primers

基因	引物序列(5'→3')
<i>CD86</i>	正向引物 CTGCTGGTGGTGACAAGCACATTT
	反向引物 ATGTCATGAGCAAAGGCGCAGAAC
<i>PGC-1α</i>	正向引物 TATGGAGTGACATAGAGTGTGCT
	反向引物 GTCGCTACACCACTTCAATCC
<i>NRF1</i>	正向引物 AGCACGGAGTGACCCAAAC
	反向引物 AGGATGTCCGAGTCATCATAAGA
<i>TFAM</i>	正向引物 AACACCCAGATGCAAACTTTCA
	反向引物 GACTTGGAGTTAGCTGCTCTTT
<i>GAPDH</i>	正向引物 ACAACAGCCTCAAGATCATCAGC
	反向引物 GCCATCACGCCACAGTTTCC

CD86: 分化抗原86; *PGC-1α*: 过氧化物酶体增殖物激活受体 γ 辅激活因子1 α ; *NRF1*: 核呼吸因子1; *TFAM*: 线粒体转录因子A; *GAPDH*: 甘油醛-3-磷酸脱氢酶。

2 结果

2.1 CHR减轻SA-AKI模型小鼠肾脏组织损伤并增强细胞活力

CHR对SA-AKI具有保护作用:1)在肾组织病理学变化方面,Sham组小鼠肾脏间质未见明确炎症反应;CLP组出现显著的肾小管上皮细胞肿胀、变性、管型形成甚至坏死脱落(图1A);经CHR干预后,肾组织上述病理损伤得到明显缓解。2)在肾小管损伤评分结果(图1B)方面,与CLP组相比,CLP+CHR组肾小管损伤评分降低,差异有统计学意义($P<0.05$)。体外实验采用CCK-8法检测HK-2细胞活力,结果显示LPS刺激可显著抑制细胞活力,CHR干预则能有效逆转这一效应(图1C),差异有统计学意义($P<0.01$ 或 $P<0.001$)。上述结果表明,体内/体外实验均证实CHR能有效缓解SA-AKI所致的肾脏组织与细胞损伤。

2.2 CHR缓解SA-AKI模型小鼠炎症反应并改善肾功能

ELISA结果显示:与Sham组相比,CLP组小鼠血清促炎细胞因子IL-6与TNF- α 水平均升高,而经CHR干预后IL-6与TNF- α 水平均降低,差异均有统计学意义($P<0.05$ 或 $P<0.001$,图2A、2B);在M1型

巨噬细胞与HK-2细胞的共培养模型中,LPS刺激显著升高上清液中IL-6与TNF- α 水平,而经CHR治疗后IL-6与TNF- α 水平均降低,差异均有统计学意义($P<0.01$ 或 $P<0.001$,图2C、2D);在肾功能方面,CLP组小鼠血清BUN和Cr水平均较Sham组升高,

而经CHR治疗后BUN和Cr水平均降低,差异均有统计学意义($P<0.05$ 或 $P<0.01$ 或 $P<0.001$,图2E、2F)。上述结果表明,CHR能够有效抑制SA-AKI模型小鼠的过度炎症反应,并改善由此导致的肾功能障碍。

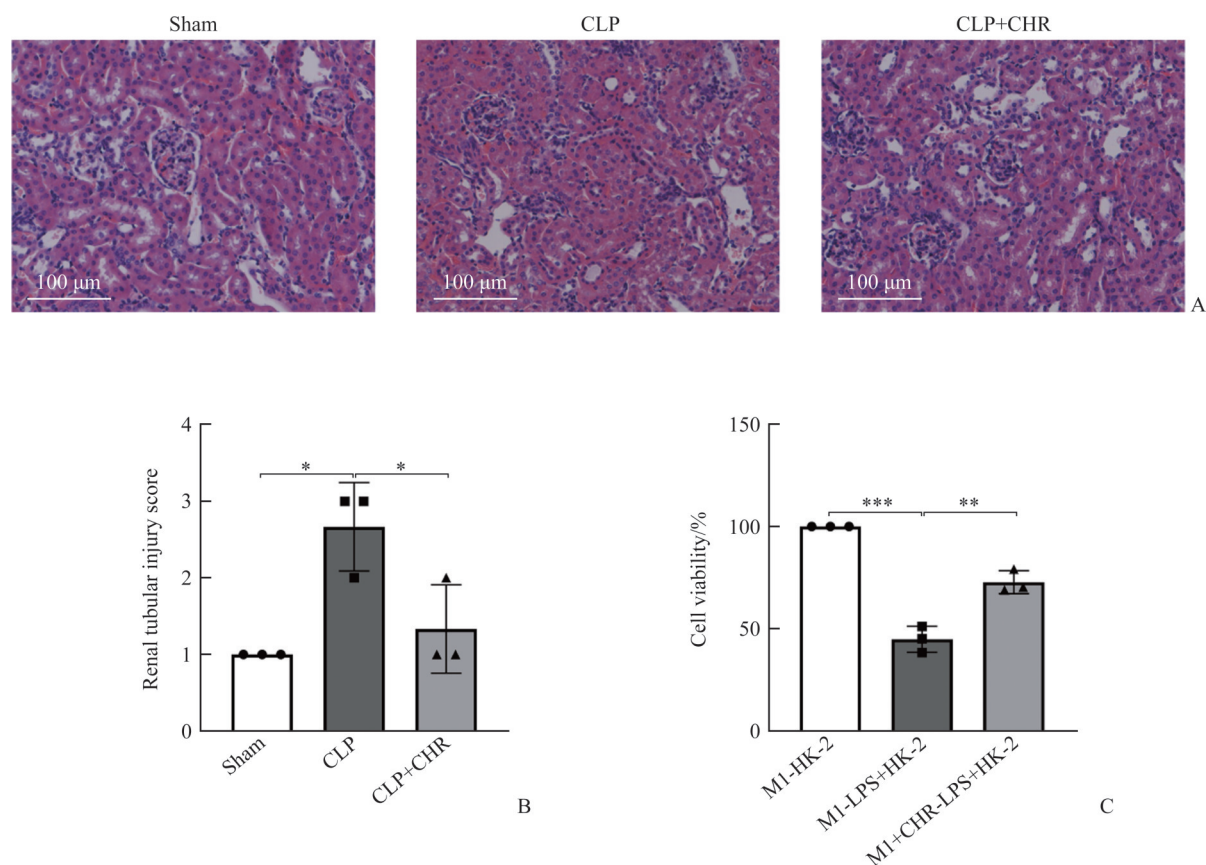


图1 CHR对SA-AKI模型小鼠肾脏结构以及共培养模型中HK-2细胞活力的保护作用

Figure 1 Protective effect of CHR on renal structure in SA-AKI model mice and viability of HK-2 cells in co-culture model

A: Representative HE staining images of kidney tissues of mice in the Sham, the CLP, and the CLP+CHR groups (with a scale of 100 μ m). B: Comparison of renal tubular injury score in the Sham, the CLP, and the CLP+CHR groups. C: Comparison of cell viability of LPS-induced HK-2 cells in the M1-HK-2, the M1-LPS+HK-2, and M1+CHR-LPS+HK-2 groups. Data are expressed as the mean $\pm$ standard deviation ($n=3$), the between-group comparisons are made with independent t -test, and multiple comparisons are made with one-way analysis of variance. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. CHR: Chrysophanol; SA-AKI: Sepsis-associated acute kidney injury; HE: Hematoxylin and eosin; CLP: Cecal ligation and puncture; CCK-8: Cell counting kit-8; LPS: Lipopolysaccharide; HK-2: Human renal tubular epithelial cells.

2.3 CHR抑制SA-AKI模型小鼠细胞凋亡

TUNEL染色评估肾组织中细胞凋亡结果显示:CLP组小鼠肾组织中TUNEL阳性细胞数较Sham组显著增加,而经CHR干预后细胞凋亡被显著抑制(图3A)。在体外共培养模型中,采用蛋白质印迹法检测凋亡相关蛋白表达(图3B~3E)的结果显示:与M1-HK-2组相比,M1-LPS+HK-2组细胞中促凋亡蛋白

BAX表达上调,而抗凋亡蛋白Bcl-2表达下调,差异均有统计学意义($P<0.05$ 或 $P<0.01$ 或 $P<0.001$);经CHR治疗后,BAX与Bcl-2蛋白表达比值(BAX to Bcl-2 ratio, BAX/Bcl-2)降低,差异有统计学意义($P<0.01$ 或 $P<0.001$)。上述结果表明,体内/体外实验均证实CHR能够有效抑制SA-AKI模型小鼠细胞凋亡。

2.4 CHR改善线粒体超微结构与功能并维持线粒体稳态

线粒体稳态分析结果显示:透射电镜观察(图4A)下,Sham组模型小鼠肾组织中线粒体结构完整,嵴清晰;CLP组线粒体出现严重肿胀、嵴断裂或消失及空泡化;经CHR干预则能显著改善上述线粒体超微结构损伤。在功能方面,CLP组模型小鼠肾组织ATP水平低于Sham组(图4B),而经CHR干预后肾脏组织与M1型巨噬细胞ATP水平均升高(图4C),差异均有统计学意义(均 $P<0.001$)。JC-1探针检测结果(图4D、4E)表明,CHR能够逆转LPS诱导的M1型巨噬细胞线粒体膜电位下降,差异有统计学意义($P<0.01$)。

蛋白质印迹法和qPCR(图5)结果显示:在SA-AKI模型小鼠肾组织以及M1型巨噬细胞中,线粒体生物合成关键基因 $PGC-1\alpha$ 、 $TFAM$ 、 $NRF1$ 的mRNA表达和蛋白质表达均下调,而经CHR干预则能显著上调基因 $PGC-1\alpha$ 、 $TFAM$ 、 $NRF1$ 的mRNA表达和蛋白质表达,差异均有统计学意义($P<0.05$ 或 $P<0.01$ 或 $P<0.001$)。NADP⁺/NADPH结果显示:CLP组模型小鼠肾组织及M1巨噬细胞的NADP⁺/NADPH均升高,经CHR干预可有效降低体内体外模型中NADP⁺/NADPH,差异均有统计学意义($P<0.05$ 或 $P<0.01$)。上述结果表明,CHR能够改善线粒体结构与功能,降低氧化应激水平并改善线粒体还原当量耗竭,促进线粒体生物合成,维持巨噬细胞的线粒体稳态。

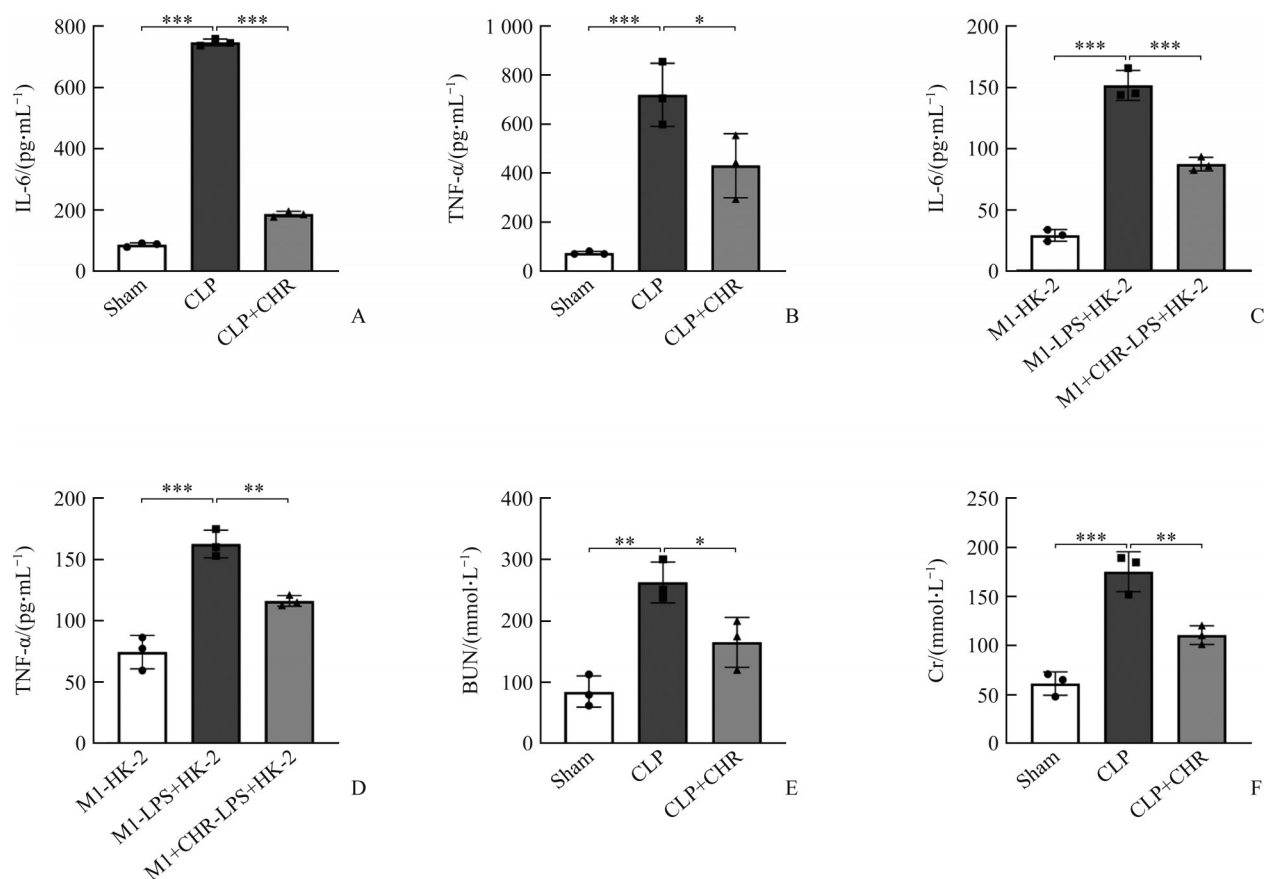


图2 CHR降低SA-AKI模型小鼠及共培养模型的炎症因子水平并改善肾功能

Figure 2 CHR reduces inflammatory cytokine levels and improves renal function of SA-AKI model mice and co-culture model

A and B: Comparison of the serum levels of IL-6 (A) and TNF- α (B) in mice of the Sham, the CLP, and the CLP+CHR groups. C and D: Comparison of levels of IL-6 (C) and TNF- α (D) in the supernatant of the M1-HK-2, the M1-LPS+HK-2, and M1+CHR-LPS+HK-2 groups. E and F: Comparison of the serum levels of BUN (E) and Cr (F) in mice of the Sham, the CLP, and the CLP+CHR groups. Data are expressed as the mean $\pm$ standard deviation ($n=3$), the between-group comparisons are made with independent t -test, and multiple comparisons are made with one-way analysis of variance. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. IL-6: Interleukin-6; TNF- α : Tumor necrosis factor-alpha; BUN: Blood urea nitrogen; Cr: Creatinine.

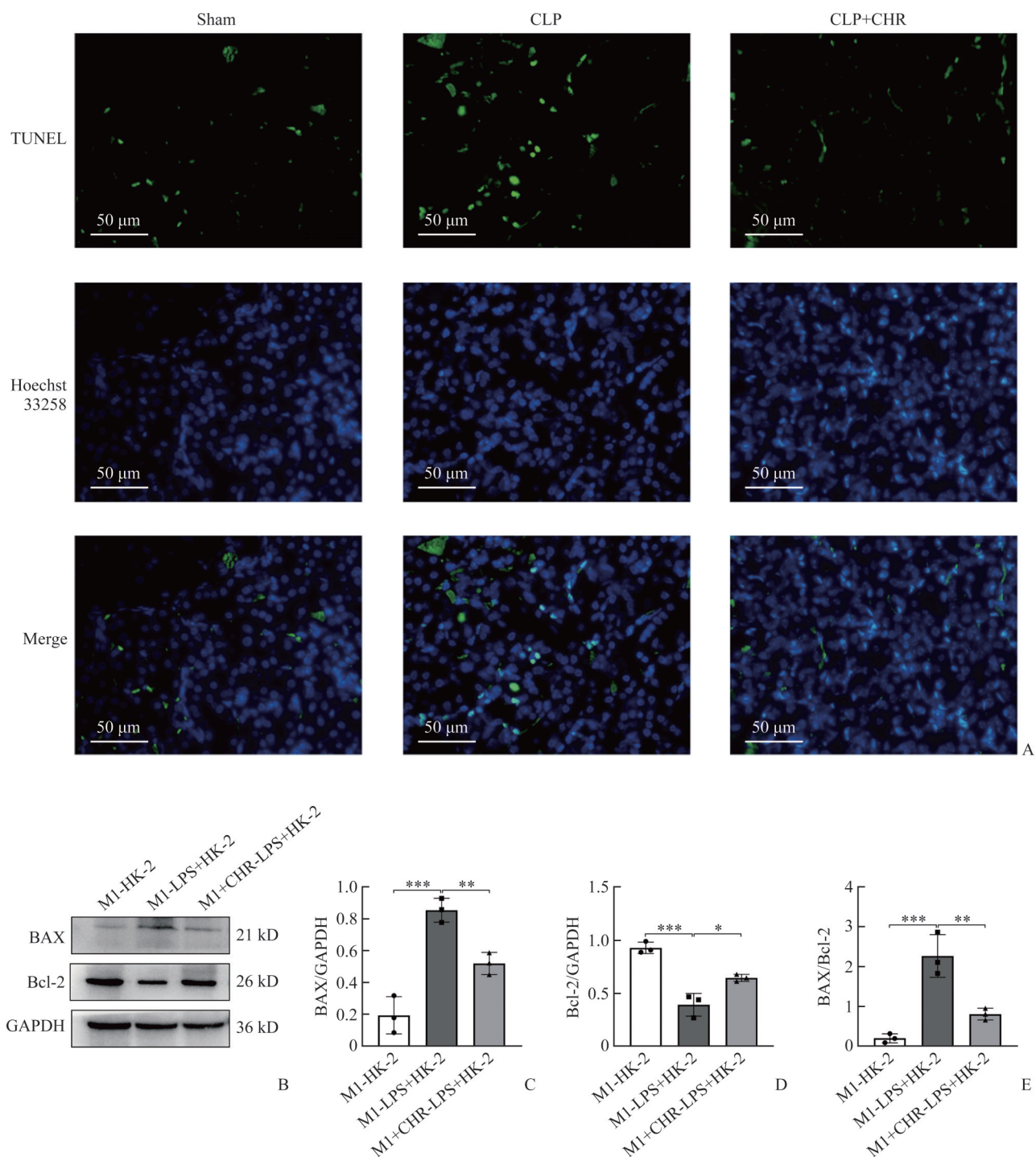


图3 CHR抑制SA-AKI模型小鼠与共培养模型中的肾小管上皮细胞凋亡

Figure 3 CHR inhibits apoptosis of renal tubular epithelial cells in SA-AKI model mice and co-culture model

A: Representative TUNEL staining images of kidney tissues of mice in the Sham, the CLP, and the CLP+CHR groups (with a scale of 50 μ m). B: Representative Western blotting bands of apoptosis-related proteins BAX and Bcl-2 in the M1-HK-2, the M1-LPS+HK-2, and M1+CHR-LPS+HK-2 groups. C-E: Comparison of the expressions of BAX (C), Bcl-2 (D) and BAX/Bcl-2 (E) in the M1-HK-2, the M1-LPS+HK-2, and M1+CHR-LPS+HK-2 groups. Data are expressed as the mean $\pm$ standard deviation ($n=3$), the between-group comparisons are made with independent t -test, and multiple comparisons are made with one-way analysis of variance. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; BAX: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma-2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

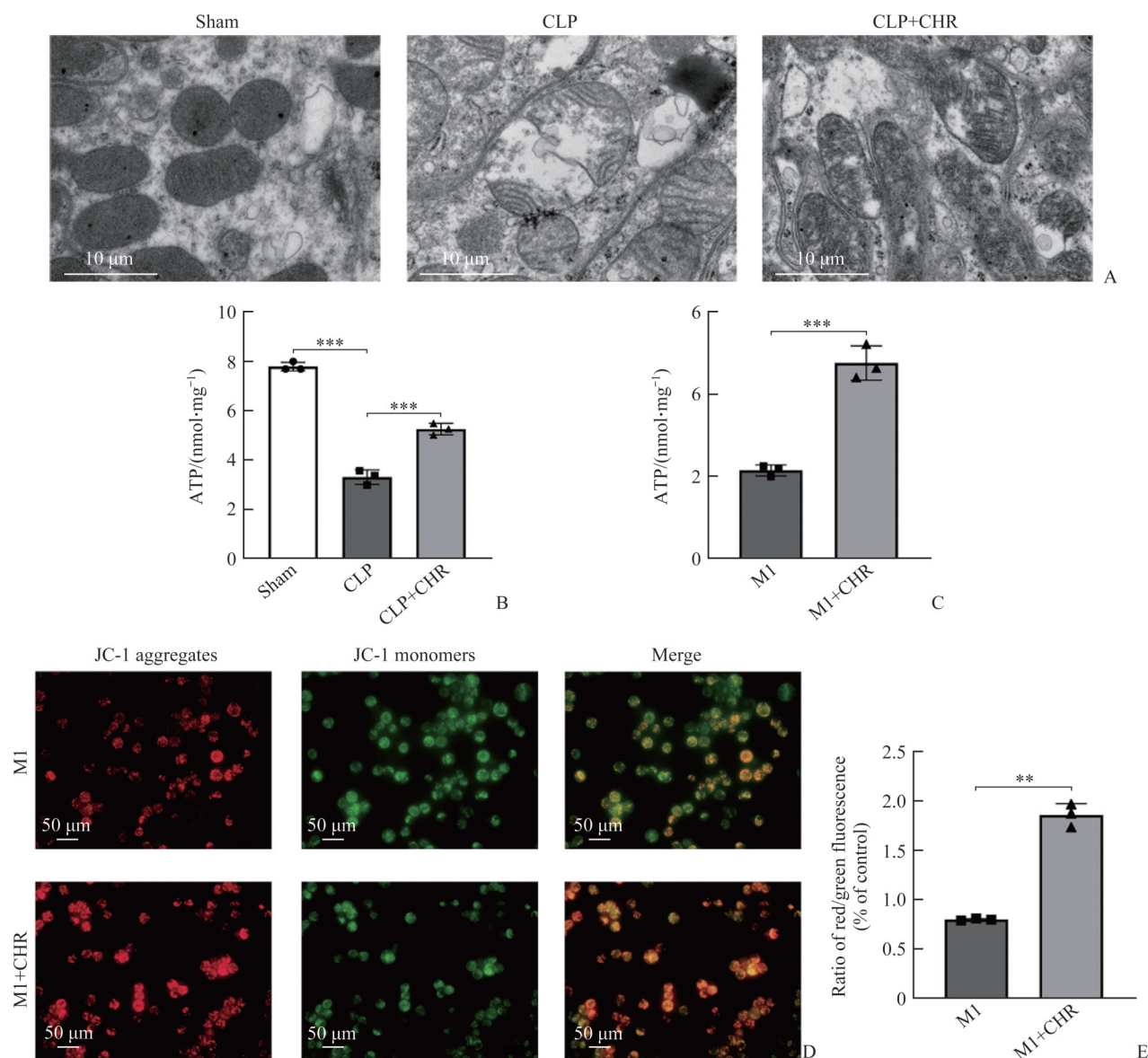


图4 CHR改善SA-AKI模型小鼠及M1型巨噬细胞的线粒体结构与功能

Figure 4 CHR ameliorates mitochondrial structure and function in SA-AKI model mice and M1 macrophages

A: Representative TEM images of mitochondria in kidney tissues of mice. B: Comparison of ATP in mice of the Sham, the CLP, and the CLP+CHR groups. C: Comparison of ATP between M1 and M1+CHR groups. D and E: Manifestation with a scale of 50 μ m (D) and comparison (E) of mitochondrial membrane potential in M1 macrophages detected by JC-1 staining. Data are expressed as the mean $\pm$ standard deviation ($n=3$), the between-group comparisons are made with independent t -test, and multiple comparisons are made with one-way analysis of variance. ** $P<0.01$, *** $P<0.001$. TEM: Transmission electron microscopy; ATP: Adenosine triphosphate; JC-1: 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide.

2.5 CHR抑制巨噬细胞向M1型表型极化

蛋白质印迹法与qPCR结果(图6A~6C)显示:与Sham组相比,CLP组模型小鼠肾组织中M1型巨噬细胞标志物CD86的蛋白表达与mRNA表达水平均升高,经CHR干预能抑制CD86表达,差异均有统计学意义($P<0.05$ 或 $P<0.01$ 或 $P<0.001$)。在体外实验中,qPCR结果(图6D)显示CHR干预能降低M1型巨噬细

胞中CD86 mRNA表达水平,差异有统计学意义($P<0.01$)。免疫荧光染色结果(图6E、6F)证实,CHR干预能减弱M1型巨噬细胞中CD86的荧光表达强度,差异有统计学意义($P<0.05$)。上述结果表明,CHR能够有效抑制SA-AKI模型中巨噬细胞向M1型表型的极化。

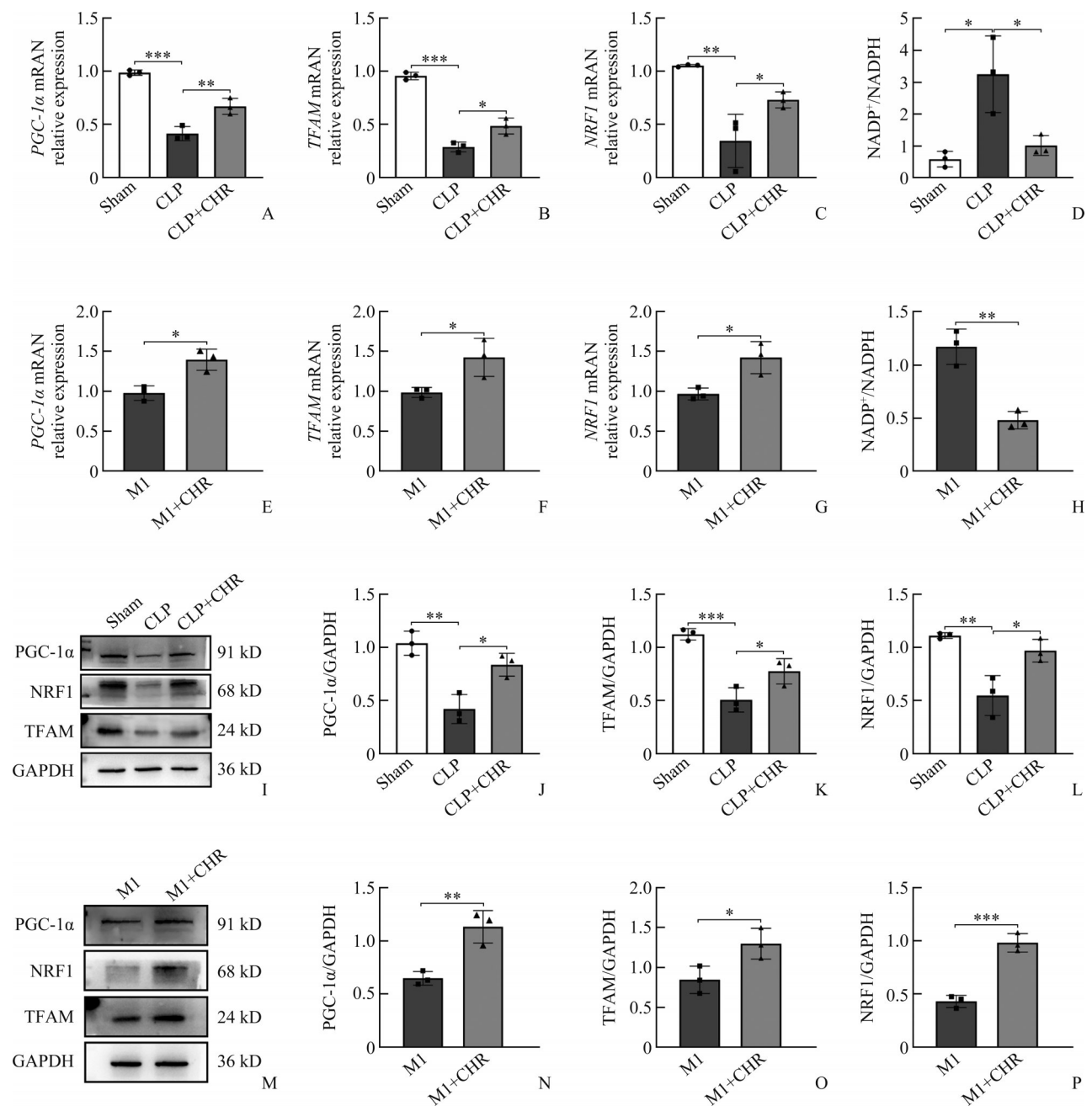


图5 CHR上调SA-AKI模型小鼠及M1型巨噬细胞中线粒体生物合成关键基因的表达

Figure 5 CHR upregulates the expression of key mitochondrial biogenesis genes in SA-AKI model mice and M1 macrophages

A–C: Relative mRNA expression levels of key mitochondrial biogenesis genes *PGC-1α* (A), *TFAM* (B), and *NRF1* (C) in SA-AKI model mice. D and H: Reduction of NADP⁺/NADPH in SA-AKI model mice (D) and M1 macrophages (H) by CHR intervention. E–G: Relative mRNA expression levels of key mitochondrial biogenesis genes *PGC-1α* (E), *TFAM* (F), and *NRF1* (G) in M1 macrophages. I–L: Western blotting result (I) and relative protein expression levels of key mitochondrial biogenesis genes *PGC-1α* (J), *TFAM* (K), and *NRF1* (L) in SA-AKI model mice. M–P: Western blotting result (M) and relative protein expression levels of key mitochondrial biogenesis genes *PGC-1α* (N), *TFAM* (O), and *NRF1* (P) in M1 macrophages. Data are expressed as the mean ± standard deviation ($n=3$), the between-group comparisons are made with independent t -test, and multiple comparisons are made with one-way analysis of variance. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. *PGC-1α*: Peroxisome proliferator-activated receptor gamma coactivator 1alpha; *TFAM*: Mitochondrial transcription factor A; *NRF1*: Nuclear respiratory factor 1; NADP⁺/NADPH: Nicotinamide adenine dinucleotide phosphate oxidized/reduced.

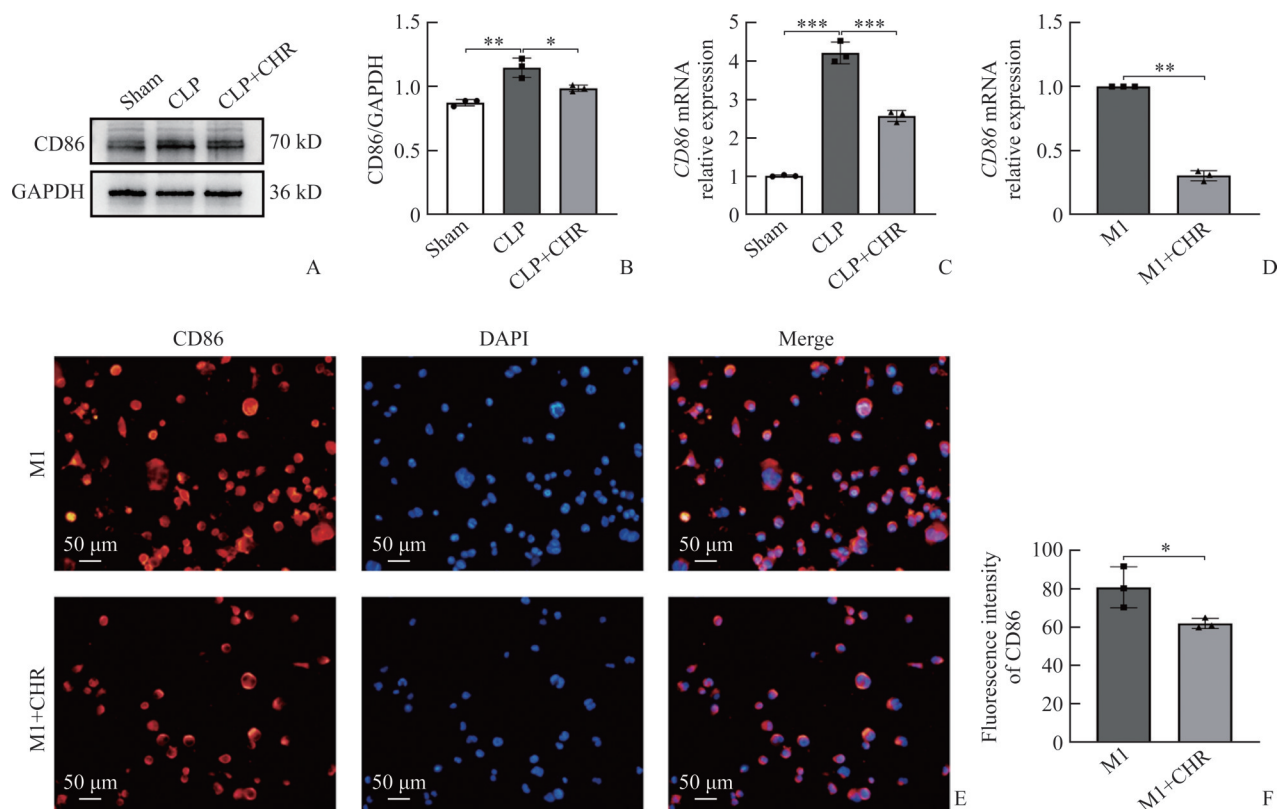


图6 CHR抑制巨噬细胞向M1型表型极化

Figure 6 CHR inhibits M1 polarization of macrophages

A: Representative Western blotting bands of CD86 protein in kidney tissues of mice. B and C: Relative protein expression (B) and mRNA expression (C) of CD86 in kidney tissues of mice. D: Relative *CD86* mRNA expression in M1 macrophages in vitro. E and F: Representative immunofluorescence images with a scale of 50 μ m (E) and quantitative fluorescence intensity analysis (F) of CD86 in M1 macrophages in vitro. Data are expressed as the mean $\pm$ standard deviation ($n=3$), the between-group comparisons are made with independent *t*-test, and multiple comparisons are made with one-way analysis of variance. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. CD86: Cluster differentiation 86.

3 讨论

SA-AKI病理机制极为复杂,其核心是失控的全身炎症反应以及由此产生的“细胞因子风暴”^[18]。巨噬细胞作为免疫系统的重要组成部分,其极化表型的异质性与靶器官受损、修复均密切相关^[19-20],在SA-AKI的发生和发展中发挥重要作用^[21-22]。研究^[23-24]表明:巨噬细胞M1型极化不仅会加重早期组织损伤,而且会通过释放大量促炎细胞因子驱动SA-AKI进展。线粒体功能稳态对巨噬细胞的极化具有决定性影响。线粒体不仅能维持细胞能量代谢,还通过调控NF- κ B等炎症信号通路来影响巨噬细胞的极化趋向和免疫应答强度^[25-27]。因此,通过重塑线粒体功能稳态来调控巨噬细胞的极化方向,可能对改善SA-AKI具有重要的临床意义。

CHR是一种从传统中药大黄中提取的天然蒽醌

类活性成分,既往研究^[28-29]已报道CHR具有广泛的药理活性,包括抗炎、抗氧化、抗肿瘤、神经保护以及减轻缺血再灌注损伤等作用。近年来研究^[30]发现:CHR可通过调节沉默调节蛋白6(sirtuin 6, Sirt6)/NF- κ B和核因子E2相关因子2(nuclear factor erythroid 2-related factor 2, Nrf2)/NF- κ B信号轴,有效减轻IL-1 β 诱导的软骨细胞炎症反应。在脓毒症模型中,CHR能够通过抑制炎症因子的释放,下调Toll样受体2(Toll-like receptor 2, TLR2)和Toll样受体4(Toll-like receptor 4, TLR4)的表达,以及阻断NF- κ B p65和细胞外调节蛋白激酶(extracellular regulated protein kinases, ERK)1/2通路的活化,对脓毒症急性肺损伤发挥显著的保护作用^[31]。本研究证实CHR对SA-AKI具有显著的肾脏保护作用:CHR不仅能改善模型小鼠肾功能、减轻组织病理损伤,还能有效抑制促炎细胞因子的释放,这种保护作用可能与CHR

对巨噬细胞线粒体稳态的维持有关;在线粒体结构方面,CHR修复了SA-AKI模型中受损的线粒体超微结构;在线粒体功能与能量代谢方面,CHR显著提高了线粒体膜电位和ATP水平。 $\text{NADP}^+/\text{NADPH}$ 是反映线粒体氧化还原稳态的核心指标之一, $\text{NADP}^+/\text{NADPH}$ 失衡提示线粒体抗氧化防御功能下降。本研究结果表明,CHR能显著降低M1型巨噬细胞中 $\text{NADP}^+/\text{NADPH}$ 的异常升高水平,降低SA-AKI模型小鼠的氧化应激水平,维持巨噬细胞线粒体功能稳态。上述实验结果共同提示CHR可能通过维持巨噬细胞线粒体的功能与结构稳态,降低促炎细胞因子水平,减轻SA-AKI的炎症反应和肾组织损伤。

PGC-1 α 是线粒体生物合成与功能表达的核心调控因子,其表达上调通常伴随线粒体新生与功能增强^[32-33]。研究^[34-35]表明:巨噬细胞极化表型高度依赖细胞的代谢重塑,促炎的M1型极化往往伴随着线粒体氧化磷酸化受损与氧化应激;PGC-1 α 活化能够改善线粒体代谢网络受损,抑制M1型极化表型并促进细胞恢复免疫稳态。本研究明确了CHR对线粒体生物合成基因PGC-1 α 及其下游NRF1、TFAM表达的影响。qPCR与蛋白质印迹法结果显示,CHR能显著上调巨噬细胞中基因PGC-1 α 、NRF1及TFAM的mRNA表达和蛋白表达,促进SA-AKI巨噬细胞线粒体生物合成。上述结果表明,CHR可能通过上调基因PGC-1 α 表达来改善巨噬细胞线粒体功能稳态与能量代谢,抑制巨噬细胞向M1型极化。本课题组前期研究^[17]表明,NF- κ B信号通路是CHR调控巨噬细胞极化、改善SA-AKI的主要通路。相关研究表明,PGC-1 α 介导的线粒体代谢与NF- κ B介导的炎症反应存在密切关联。一方面,PGC-1 α 上调可降低活性氧积累水平或直接抑制p65亚基的磷酸化与核转位,从而抑制NF- κ B的转录活性^[36];另一方面,被抑制的NF- κ B信号也能解除对PGC-1 α 等代谢基因的转录抑制^[37]。本研究推测:CHR可能通过激活PGC-1 α 以恢复巨噬细胞的线粒体稳态与能量代谢,这一代谢重塑过程会进一步调控下游NF- κ B等促炎信号通路,最终导致巨噬细胞M1型极化受到抑制,炎症因子释放减少,肾脏组织损伤得以缓解。

本研究存在的局限性:1)目前尚无明确证据表明CHR与PGC-1 α 等靶点之间存在直接的物理结合;2)CHR是否通过上调PGC-1 α 抑制NF- κ B信号活化以改善SA-AKI尚未明确,仍需通过基因敲除等分子生物学手段加以验证;3)CHR对M2型巨噬细胞极化的潜在影响以及CHR在SA-AKI后期组织修复阶段的作用

是未来值得深入研究的方向。

综上所述,本研究证实CHR对肾脏具有保护作用,揭示了CHR通过上调PGC-1 α 等关键因子、改善巨噬细胞线粒体稳态进而抑制M1型极化的潜在机制,这为深入理解CHR的药理作用提供了新的实验依据,也为SA-AKI的药物治疗策略拓展了新的思路。

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