四川大学学报（医学版） 2024，55（5）：1175－1185
J Sichuan Univ（Med Sci） doi: 10.12182/20240960204

在线全文

异长叶烯通过减少肠上皮细胞凋亡改善小鼠克罗恩病样结肠炎*

段 婷[1]，耿志军[2,3]，杨晶晶[4]，殷丽霞[5]，孙明熙[3]，王舜印[3]，
张小凤[2,3]，李 静[3,5]，胡建国[3,5]，陆国玉[1△]

1. 蚌埠医科大学第一附属医院 急诊内科（蚌埠 233000）；2. 蚌埠医科大学第一附属医院 中心实验室（蚌埠 233000）；
3. 炎症相关性疾病基础与转化研究安徽省重点实验室（蚌埠 233000）；4. 蚌埠医科大学第一附属医院 胃肠外科（蚌埠 233000）；
5. 蚌埠医科大学第一附属医院 检验科（蚌埠 233000）

【摘要】 目的 探究异长叶烯（isolongifolene, ISO）对肠上皮细胞凋亡和2, 4, 6-三硝基苯磺酸（TNBS）诱导的小鼠克罗恩病（Crohn's disease, CD）样结肠炎的作用及分子机制。**方法** 动物实验：将小鼠随机分为Wild type（WT）组、2,4,6-三硝基苯磺酸（2,4,6-trinitrobenzenesulfonic acid, TNBS）组和TNBS+ISO组，每组8只。TNBS组及TNBS+ISO组小鼠经直肠灌注TNBS构建结肠炎模型，TNBS+ISO组小鼠在造模后予ISO灌胃（10 mg/kg）干预，其余两组予等量生理盐水灌胃，第7天处死小鼠。检测小鼠体质量变化、疾病活动评分（disease activity index, DAI）、结肠长度，进行结肠组织跨上皮电阻（TEER）检测，根据HE染色计算结肠炎症程度评分，RT-PCR和ELISA法检测肠黏膜炎症因子〔肿瘤坏死因子-α（TNF-α）、干扰素-γ（IFN-γ）、白细胞介素（IL）-1β和IL-6〕水平，TUNEL染色检测小鼠结肠组织细胞凋亡，Western blot和免疫荧光检测其凋亡蛋白（cleaved caspase-3/caspase-3和Bax）、抗凋亡蛋白（Bcl-2）和紧密连接蛋白（ZO-1和claudin-1）表达。细胞实验：TNF-α诱导肠上皮细胞Caco-2凋亡模型，进行ISO治疗，再加入AMPK抑制剂Compound C干预。TUNEL染色、Western blot和免疫荧光检测同前。基因功能注释（Gene Ontology, GO）富集预测ISO的生物学功能。然后用小鼠和Caco-2细胞进行机制验证：Western blot检测各组小鼠结肠组织及Caco-2细胞中p-AMPK/AMPK和p-PGC1α表达水平，TUNEL染色检测细胞凋亡。**结果** 动物实验结果显示，ISO能缓解实验性结肠炎和肠屏障功能障碍，表现为小鼠体质量降低（$P<0.05$）、结肠长度缩短（$P<0.05$），DAI评分（$P<0.05$）、炎症程度评分（$P<0.05$）、TEER值（$P<0.05$）改善，结肠组织中促炎因子（TNF-α、IFN-γ、IL-1β和IL-6）（$P<0.05$）、紧密连接蛋白〔ZO-1（$P<0.05$）和claudin-1（$P<0.05$）〕的表达改善。细胞实验中，在TNF-α诱导的肠上皮细胞模型中也发现ISO能保护肠屏障受损。ISO减少肠上皮细胞的凋亡率（$P<0.05$）、cleaved caspase-3/caspase-3（$P<0.05$）和Bax（$P<0.05$）的表达，且上调Bcl-2（$P<0.05$）的水平。GO富集预测分析显示，ISO改善CD样肠炎可能与负向调控细胞凋亡有关。机制验证发现，p-AMPK和p-PGC1α在ISO治疗后的小鼠结肠组织和Caco-2细胞表达明显上调（$P<0.05$），而Compound C则升高了ISO治疗的Caco-2细胞的凋亡率（$P<0.05$）以及降低ZO-1、claudin-1的相对表达量（$P<0.05$）。**结论** ISO至少部分通过激活AMPK/PGC1α信号通路减少肠上皮细胞凋亡，从而缓解TNBS诱导的小鼠肠屏障功能障碍和CD样结肠炎。

【关键词】 克罗恩病 异长叶烯 肠上皮细胞凋亡 肠屏障 AMPK/PGC1α

Isongifolene Improves Crohn's Disease-Like Colitis in Mice by Reducing Apoptosis of Intestinal Epithelial Cells

DUAN Ting[1], *GENG Zhijun*[2,3], *YANG Jingjing*[4], *YIN Lixia*[5], *SUN Mingxi*[3], *WANG Shunyin*[3], *ZHANG Xiaofeng*[2,3], *LI Jing*[3,5], *HU Jianguo*[3,5], *LU Guoyu*[1△]. 1. *Department of Emergency, The First Affiliated Hospital of Bengbu Medical University*, Bengbu 233000, China; 2. *Central Laboratory, The First Affiliated Hospital of Bengbu Medical University*, Bengbu 233000, China; 3. *Anhui Province Key Laboratory of Basic and Translational Research of Inflammation-Related Diseases*, Bengbu 233000, China; 4. *Department of Gastrointestinal Surgery, The First Affiliated Hospital of Bengbu Medical University*, Bengbu 233000, China; 5. *Clinical Laboratory, The First Affiliated Hospital of Bengbu Medical University*, Bengbu 233000, China

△ Corresponding author, E-mail: 13033013637@163.com

【Abstract】 Objective To investigate the effect and molecular mechanism of isolongifolene (ISO) on the apoptosis of intestinal epithelial cells and 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced Crohn's disease (CD)-like colitis in mice. **Methods** In the animal experiments, mice were randomly assigned to the wild type (WT) group, TNBS group and TNBS+ISO group, with 8 mice in each group. Colitis models of mice were established in the TNBS group and the TNBS+ISO group by rectal injection of TNBS. After modeling, the mice in the TNBS+ISO group were given ISO intervention via intragastric gavage (10 mg/kg), and the other two groups were given the same amount of normal saline

* 安徽省高校优秀科研创新团队项目（No. 2023AH010067）和蚌埠医学院重大科技项目孵育计划（No. 2020byfy003）资助

△ 通信作者，E-mail: 13033013637@163.com

出版日期：2024-09-20

via intragastric gavage. The mice were sacrificed on the 7th day. The changes in body mass, disease activity scores (DAI), and the colon length of mice were measured, and transepithelial electrical resistance (TEER) of the colon tissues was determined. The score of colon inflammation was calculated according to HE staining. The levels of intestinal mucosal inflammatory factors, including tumor necrosis factor alpha (TNF-α), interferon (IFN)-γ, interleukin (IL)-1β, and IL-6, were measured by RT-PCR and ELISA. The apoptosis of colon tissue cells was determined by TUNEL assay. The expressions of apoptotic proteins (cleaved caspase-3/caspase-3 and Bax), an anti-apoptotic protein (Bcl-2), and tight junction proteins (ZO-1 and claudin-1) were detected by Western blot and immunofluorescence. In the cell experiment, TNF-α was used to induce intestinal epithelial cell Caco-2 apoptosis model, which was treated with ISO. Then, intervention with the AMPK inhibitor Compound C was given. TUNEL assay, Western blot assay, and immunofluorescence assay were performed to measure apoptosis and the expression of apoptosis proteins in the Caco-2 cells. Gene Ontology (GO) enrichment analysis was performed to predict the biological function of ISO. Then, the mechanism involved was verified by examination of the mice and Caco-2 cells. Western blot was performed to determine the expression levels of p-AMPK/AMPK and p-PGC1α in the colon tissues from the mice of different groups and Caco-2 cells. The apoptosis of the cells was determined by TUNEL assay. **Results** According to the results of the animal experiment, ISO could alleviate experimental colitis and intestinal barrier dysfunction, leading to improvements in body mass loss, colon length shortening, DAI score, inflammatory rating, and TEER values (all $P<0.05$) in mice. Furthermore, ISO decreased the expression of pro-inflammatory factors TNF-α, IFN-γ, IL-1β, and IL-6 and increased the expression of the tight junction proteins ZO-1 and claudin-1 (all $P<0.05$). In the cell experiment, in a TNF-α-induced intestinal epithelial cell model, ISO was also found to protect intestinal barrier against damage. ISO reduced the proportion of apoptotic intestinal epithelial cells, reduced the expression of cleaved-caspase-3/caspase-3 and Bax, and upregulated the level of Bcl-2 (all $P<0.05$). GO enrichment predictive analysis showed that the role of ISO in improving CD-like enteritis might be associated with the negative regulation of apoptosis. Verification of the mechanism showed that the expression of p-AMPK and p-PGC1α in the mice colon tissue was significantly upregulated after ISO intervention ($P<0.05$). In contrast, the AMPK inhibitor Compound C increased the apoptosis rate of ISO-treated Caco-2 cells and decreased the relative expression levels of ZO-1 and claudin-1 ($P<0.05$). **Conclusion** ISO reduces intestinal epithelial cell apoptosis at least in part by activating AMPK/PGC1α signaling pathway, thereby alleviating TNBS-induced intestinal barrier dysfunction and CD-like colitis in mice.

【Key words】 Crohn's disease Isolongifolene Intestinal epithelial cell apoptosis Intestinal barrier AMPK/PGC1α

克罗恩病(Crohn's disease, CD)是一种慢性炎性肉芽肿性疾病[1],以反复腹痛、腹泻和体质量下降为主要临床表现[2],其发病率在我国呈逐步上升趋势[3]。虽然生物制剂的应用在一定程度上延缓了本病的进展,但也存在诸如免疫原性、需肠外给药和药物副作用等局限性。因此,探寻更为安全有效的药物是目前CD治疗的重要方向。肠屏障是由肠上皮细胞和紧密连接蛋白等组成的防御机制[4],其主要功能是阻止肠道内的抗原、菌群等某些有害因子进入肠黏膜。当肠道受到不良饮食、感染、应激等多因素刺激,肠上皮细胞受损出现过度凋亡,进而破坏肠屏障完整性,诱发肠道的炎症和损伤[5-6]。近年来,多项研究均已证实,抑制肠上皮细胞凋亡和保护肠屏障可改善CD肠炎[7-9]。因此靶向细胞凋亡、保护肠屏障的新型药物可为CD的新药开发提供思路,天然植物提取物以不易耐药和副作用小等众多优势而受到炎症性肠病领域内诸多研究者的关注[10],异长叶烯(isolongifolene, ISO)是从天然植物九里香中分离得到的三环倍半萜烯类化合物,研究报道其通过减少小鼠肝细胞[11]和神经细胞[12]的凋亡,发挥护肝及保护神经的作用,也被报道可缓解阿霉素所致的肾脏损伤[13]。鉴于抗肠上皮细胞凋亡是目前CD治疗的重要靶点,而ISO在多种疾病中被报道可拮抗细胞凋亡从而缓解疾病进展,且当前尚未有相关研究揭示ISO在炎症性肠病中对细胞凋亡的作用。本研究拟使用2, 4, 6-三硝基苯磺酸(TNBS)诱导的CD样结肠炎小鼠作为研究对象,探究ISO对CD样结肠炎的作用,结合网络药理学预测分析、结肠炎模型和TNF-α诱导的Caco-2细胞凋亡模型探索ISO对肠上皮细胞和肠屏障的作用及机制,以期为CD药物治疗策略提供新参考。

1 材料与方法

1.1 材料

1.1.1 实验动物和细胞

6 ~ 8周龄,*C57bl/6j*品系健康雄性野生型小鼠购于江苏集萃药康生物科技股份有限公司,小鼠体质量约为

(22±3) g。在无特定病原菌条件下正常喂养。本研究所用Caco-2细胞购于中国科学院细胞库。本研究经蚌埠医学院第一附属医院伦理委员会审核批准(伦动科批字[2021]第226号)。

1.1.2 *实验药物和试剂*

ISO购于陶术生物有限公司; TNBS、肿瘤坏死因子-α(TNF-α)和牛血清白蛋白购自Sigma公司; Compound C(CC)购于MCE; cDNA反转录试剂盒购自TaKaRa公司; HE染色试剂盒购自索莱宝科技有限公司; ELISA检测试剂盒购自博士德生物工程有限公司; anti-C-Cas3 (anti-cleaved-caspase3), anti-Bax, anti-Bcl-2, anti-AMPK (anti-AMP-activated proteinkinase), anti-PGC1α (anti-peroxisome proliferator-activated receptor-γ coactivator-1α), anti-p-AMPK, anti-β-actin, anti-ZO-1, anti-claudin-1和Goat Anti-Rabbit lgG H&L(FITC)抗体购自Abcam; anti-p-PGC1α购自R&D System; 辣根酶标记山羊抗兔/鼠IgG(H+L)抗体购自中杉金桥生物技术有限公司; TUNEL细胞凋亡检测试剂盒购于赛维尔生物科技有限公司; RIPA裂解液、细胞膜蛋白与细胞浆蛋白提取试剂盒及超敏ECL化学发光试剂盒购自上海碧云天生物技术有限公司。

1.2　方法

1.2.1 *构建动物模型、干预措施及组织取检*

24只*C57bl/6j*小鼠随机分为WT组、TNBS组和TNBS+ISO组, 每组8只; TNBS组和TNBS+ISO组小鼠建立结肠炎模型: 腹腔注射戊巴比妥钠(40 mg/kg)溶液麻醉小鼠, 5%TNBS和无水乙醇按1∶1体积比混合后注入100 μL于小鼠直肠, 并将小鼠倒立4 min。上述步骤完成后, TNBS+ISO组每天进行ISO灌胃操作(10 mg/kg, 0.1 mL, 1次/d, 连续6 d), 其余两组小鼠予以100 μL生理盐水灌胃。于实验第7天处死小鼠, 取检小鼠结肠组织并平均分成两个部分用于开展后续实验研究。

1.2.2 *构建细胞模型和细胞实验分组*

在含10%胎牛血清(FBS)的MEM培养基(37 ℃、体积分数5%CO_2)中培养Caco-2细胞, 待细胞生长至对数期后接种至6孔板, 实验分为对照组(control组)、模型组(TNF-α组, TNF-α孵育24 h, 100 μg/mL)、TNF-α+ISO组(ISO与TNF-α共孵育24 h, 20 μmol/L)。

此外, 在机制验证的阻断实验中(2.8.2小节), 将细胞分成6组: control组、TNF-α组、TNF-α+ISO组、TNF-α+ISO+CC组、ISO+CC组和CC组, 其中前3组的处理方式同上, 而TNF-α+ISO+CC组是100 μg/mL的TNF-α、20 μmol/L的ISO和10 μmol/L的CC[14]共同处理24 h, ISO+CC组用20 μmol/L的ISO、10 μmol/L的CC共孵育24 h, CC组则用CC孵育24 h, 收集细胞用于后续检测。其中, 对p-AMPK、AMPK和p-PGC1α蛋白的Western blot检测只使用control组、TNF-α组、TNF-α+ISO组、TNF-α+ISO+CC组这4组细胞; 对ZO-1、claudin-1、C-Cas3、Cas3、Bax、Bc1-2蛋白的Western blot检测和TUNEL染色使用6组细胞。

1.3　检测方法

1.3.1 *小鼠肠炎疾病活动度评分、体质量及结肠长度测量*

实验前后对小鼠体质量进行测量并记录; 并于取检当天对各组小鼠进行肠炎疾病活动度评分(disease activity index, DAI)并记录, 评分范围0 ~ 4分, DAI评分越高即疾病活动度越高[15]; 并于取检结肠组织后对结肠长度进行测量。

1.3.2 *小鼠肠炎组织学评估*

将取检的小鼠结肠组织制成蜡块后切片(4 μm厚度), HE染色后进行炎症程度评分。以Spencer推荐的肠炎组织学评分量表为评分依据, 分值范围为0 ~ 4分, 评分越高即结肠炎症反应越重[16]。

1.3.3 *跨上皮电阻(TEER)检测*

获取小鼠结肠组织后先予以PBS洗净, 随后置于Krebs缓冲液中完全浸润组织, 将完成上述处理后的肠管组织切割成块状后放入样本夹, 再将样本夹放入Ussing chamber系统, 往肠管组织两侧腔室倒入Krebs缓冲液, 最后往两侧分别添加葡萄糖(10 mmol/L)、甘露醇(10 mmol/L), 调节电压及电流, 测量总电子发射比。

1.3.4 *RT-PCR检测*

采用Trizol法提取总RNA, 随后利用cDNA反转录试剂盒进行反转录, 再设置程序进行扩增: 变性(95 ℃, 5 s), 退火(60 ℃, 34 s), 重复40个循环。用于定量RT-qPCR的引物序列为: *TNF-α*, F: 5'-CAGGCGGTGCCTATGTCTC-3', R: 5′-CGATCACCCCGAAGTTCAGTAG-3′; *IL-1β*, F: 5′-GCAACTGTTCCTGAACTCAACT-3′, R: 5′-ATCTTTTGGGGTCCGTCAACT-3′; *IL-6*, F: 5′-CTGCAAGAGACTTCCATCCAG-3′, R: 5′-AGTGGTATAGACAGGTCTGTTGG-3′; *IFN-γ*, F: 5'-GCCACGGCACAGTCATTGA-3'; R: 5'-TGCTGATGGCCTGATTGTCTT-3'; β-actin, F: 5′-CATGTACGTTGCTATCCAGGC-3′, R: 5′-CTCCTTAATGTCACGCACGAT-3′。最后以β-actin为内参, 使用$2^{-\Delta\Delta Ct}$法使mRNA表达标准化, 进行相对定量分析。

1.3.5 *ELISA检测*

刮取小鼠结肠黏膜组织进行称质量, 加入生理盐水进行匀浆, 离心完成后轻柔吸取上清液。依据ELISA试剂盒说明书步骤进行TNF-α、IFN-γ、IL-1β和IL-6表达水平

的检测。

1.3.6 TUNEL染色

小鼠结肠石蜡切片以及Caco-2细胞爬片依据试剂盒说明书步骤进行染色。评估细胞凋亡：显微镜下随机选择5个不重复视野并统计细胞凋亡率。细胞凋亡率(%)为TUNEL阳性细胞数量与DAPI阳性细胞数量的百分比。

1.3.7 Western blot检测

分别收集小鼠结肠组织与Caco-2细胞，依据细胞膜蛋白与细胞浆蛋白提取试剂盒说明书所述的实验步骤提取结肠组织及Caco-2细胞的膜蛋白，制胶后经电泳、转膜、封闭(5%脱脂牛奶，1 h)、一抗孵育(anti-ZO-1，1∶1000；anti-claudin-1，1∶1 000；anti-β-actin、anti-Bax、anti-Bcl-2、anti-C-Cas3、anti-AMPK、anti-PGC1α、anti-p-AMPK、anti-p-PGC1α，1∶2000)和二抗孵育〔辣根酶标记山羊抗兔/鼠IgG(H+L)，1∶3000〕、ECL化学发光，凝胶成像系统采集图片，Image J软件测定灰度值，以目的条带灰度值与内参条带灰度值的比值为目的蛋白的相对表达量。

1.3.8 免疫荧光检测

①小鼠石蜡切片免疫荧光染色：小鼠结肠组织经切片后烤片，再经脱蜡水化、抗原修复、室温封闭(5 %牛血清蛋白)，孵育一抗(anti-ZO-1，1∶200；anti-claudin-1，1∶400，4 ℃)和二抗〔Goat Anti-Rabbit lgG H&L(FITC)，1∶3000〕、DAPI着色细胞核，于激光共聚焦显微镜下采集图像。②细胞爬片免疫荧光染色：取干预后的细胞爬片，多聚甲醛固定、Triton破膜和封闭，经一抗(anti-ZO-1，1∶200；anti-claudin-1，1∶400，4 ℃)、二抗〔Goat Anti-Rabbit lgG H&L(FITC)，1∶3000〕和DAPI孵育后，激光共聚焦显微镜获取图像。

1.4 生物信息学预测分析

利用Swiss Target Prediction、Pharmmapper、SuperPred和SEA数据库筛选ISO可能作用靶点，利用GeneCards、PharmGKB、DisGeNet、DrugBank及TTD数据库筛选CD相关疾病靶点。使用Venny 2.1在线工具获取二者的交集靶点，将所得的交集靶点导入David数据库进行GO富集分析，微生信绘制图形。

1.5 统计学方法

运用SPSS 25.0进行统计分析。计量资料以$\bar{x}\pm s$表示，t检验进行两组间比较；多组间比较采用单因素方差分析和Student-Newman-Keuls检验；α=0.05。

2 结果

2.1 ISO减轻TNBS小鼠的结肠炎症状

如图1所示，TNBS+ISO组的小鼠体质量下降幅度(P<0.05，图1A)和DAI评分(P<0.05，图1B)低于TNBS组，而结肠长度大于TNBS组(P<0.05，图1C、1D)。HE染色显示，TNBS+ISO组炎症评分较TNBS组降低(P<0.05)，且小鼠结肠组织巨噬细胞、中性粒细胞等炎症细胞浸润较TNBS组显著减少(图1E、1F)。

2.2 ISO降低TNBS诱导结肠炎小鼠肠黏膜中炎症因子的表达

PCR和ELISA检测显示，TNBS+ISO组小鼠TNF-α、IL-1β、IL-6和IFN-γ表达水平较TNBS组降低(P<0.05，表1)。

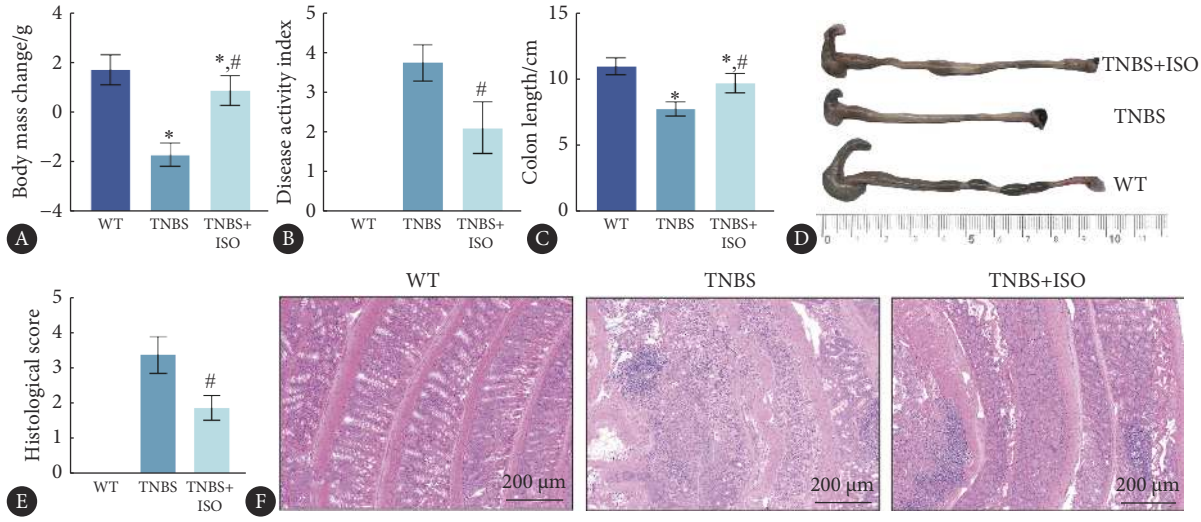

图 1 ISO对TNBS小鼠肠炎症状的影响

Fig 1 Effect of ISO on enteritis symptoms in TNBS mice

A, Body mass change in the mice (n=8); B, changes in diseaseactivity index (DAI) scores (n=8); C-D, changes in colon length (n=8); E, histopathological score of the colon (n=8); F, HE stain results. * P<0.05, vs. WT; # P<0.05, vs. TNBS.

表 1　ISO干预对小鼠结肠组织中炎症因子表达水平的影响

Table 1　Effect of ISO intervention on the expression of inflammatory factors in the colon tissues of mice

Index		WT	TNBS	TNBS+ISO
RT-PCR (relative mRNA expression)	*TNF-α*	1.00±0.14	5.93±0.39*	3.75±0.39*,#
	IL-1β	1.00±0.13	7.88±0.48*	1.78±0.20*,#
	IL-6	1.00±0.09	12.34±0.79*	5.05±0.43*,#
	IFN-γ	1.00±0.13	6.72±0.48*	2.64±0.40*,#
ELISA/(pg/mg protein)	TNF-α	11.08±1.67	60.22±3.44*	30.34±2.37*,#
	IL-1β	15.97±0.93	51.01±2.86*	30.80±1.99*,#
	IL-6	18.70±1.54	42.84±2.21*	29.63±2.26*,#
	IFN-γ	14.85±1.34	38.92±2.50*	20.29±2.62*,#

* P<0.05, vs. WT; # P<0.05, vs. TNBS. n=8.

2.3　ISO改善TNBS小鼠的肠道屏障功能

电阻抗检测显示, TNBS+ISO组小鼠TEER值较TNBS组升高(P<0.05, 图2A)。Western blot结果显示, ISO干预后显著地缓解了TNBS诱导的结肠组织中ZO-1和claudin-1表达水平的降低(P<0.05, 图2B ~ 2D)。免疫荧光显示, 在TNBS组中ZO-1和claudin-1在结肠组织中出现移位和表达水平下降的现象, 而TNBS+ISO组ZO-1和claudin-1表达水平增高, 且表达于上皮细胞的表面(图2E)。

2.4　ISO抑制TNBS小鼠结肠组织中肠上皮细胞的凋亡

图3中, TUNEL检测发现TNBS+ISO组小鼠的肠上皮细胞凋亡率较TNBS组下降(P<0.05)。Western blot检测发现, TNBS+ISO组小鼠结肠黏膜中凋亡蛋白(C-Cas3/Cas3、Bax)表达量相较于TNBS组降低, 而抗凋亡蛋白(Bcl-2)的表达量则升高(P<0.05)。

2.5　ISO缓解TNF-α诱导Caco-2细胞屏障功能障碍

见图4。免疫荧光和Western blot发现, TNF-α+ISO组Caco-2细胞与TNF-α组相比, ZO-1和claudin-1的表达水平增加(P<0.05)。

2.6　ISO减轻TNF-α诱导Caco-2细胞的凋亡

如图5、表2所示, TUNEL染色显示, TNF-α组Caco-2细胞的凋亡率显著增加, 而ISO干预则抑制了细胞凋亡

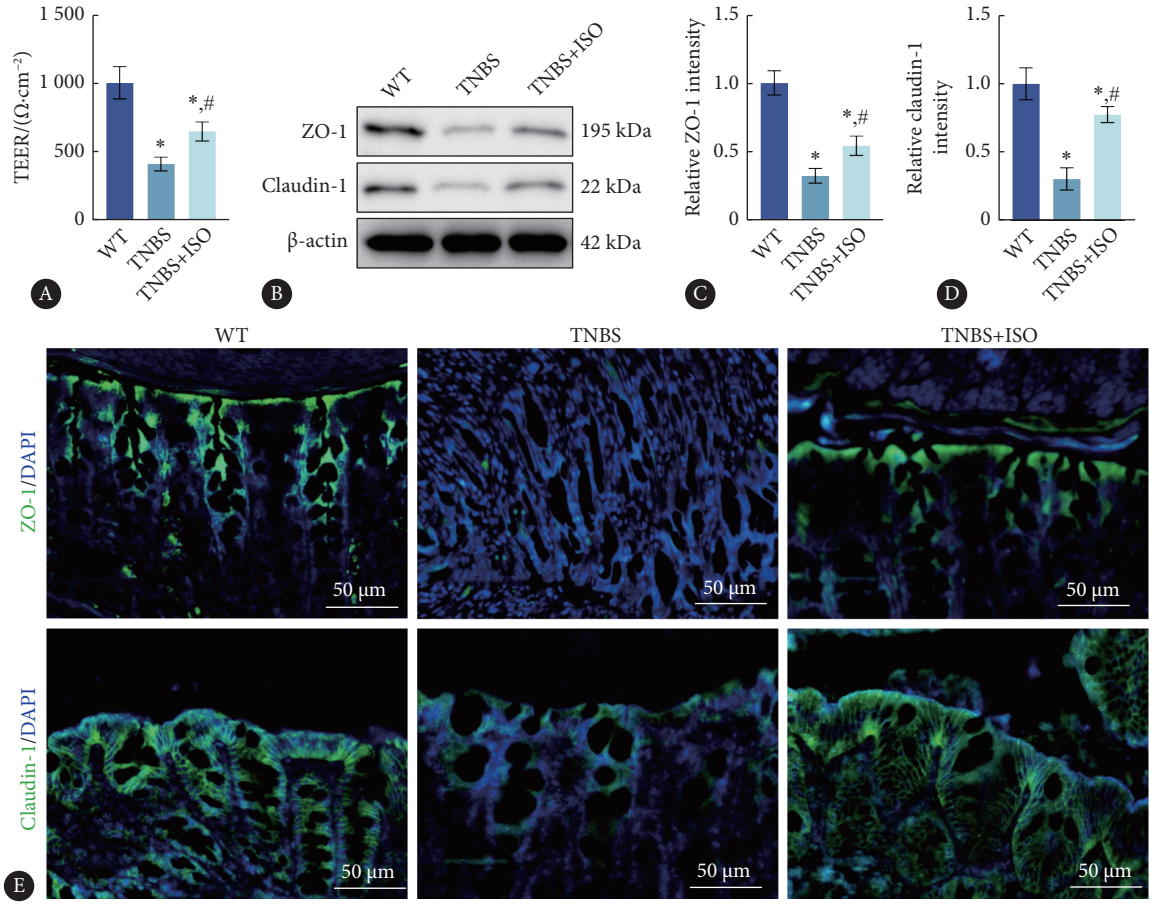

图 2　ISO治疗改善TNBS模型小鼠肠屏障损伤

Fig 2　ISO treatment improves intestinal barrier injury in TNBS model mice

A, Tissue transepithelial electrical resistance (TEER) value of mice (n=8); B-D, Western blot of claudin-1 and ZO-1 in colon mucosa (n=8); E, the expression of ZO-1 and claudin-1 in the colon tissue of mice was determined by immunofluorescence. * P<0.05, vs. WT group; # P<0.05, vs. TNBS group.

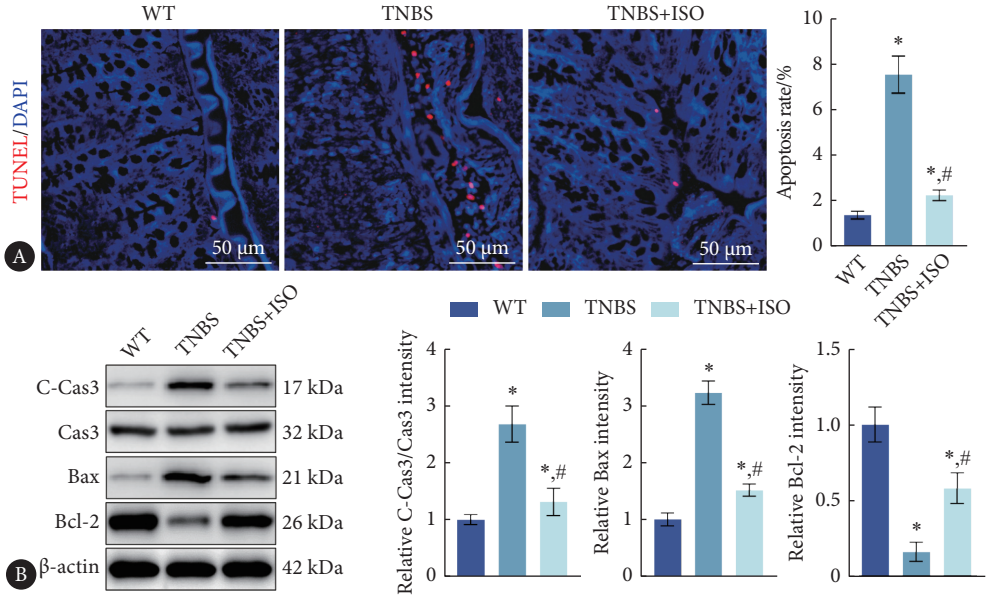

图 3 ISO治疗减少TNBS模型小鼠结肠组织中肠上皮细胞凋亡

Fig 3 ISO treatment reduces the apoptosis of intestinal epithelial cells in the colonic tissue of TNBS model mice

A, TUNEL assay was performed to detect apoptosis of colonic tissue in the mice; B, the expression levels of apoptosis proteins (C-Cas3, Cas3, and Bax) and anti-apoptosis protein (Bcl-2) in the colonic mucosa of the mice (n=8). C-Cas3: cleaved caspase-3; Cas3: caspase-3. * P<0.05, vs. WT; # P<0.05, vs. TNBS.

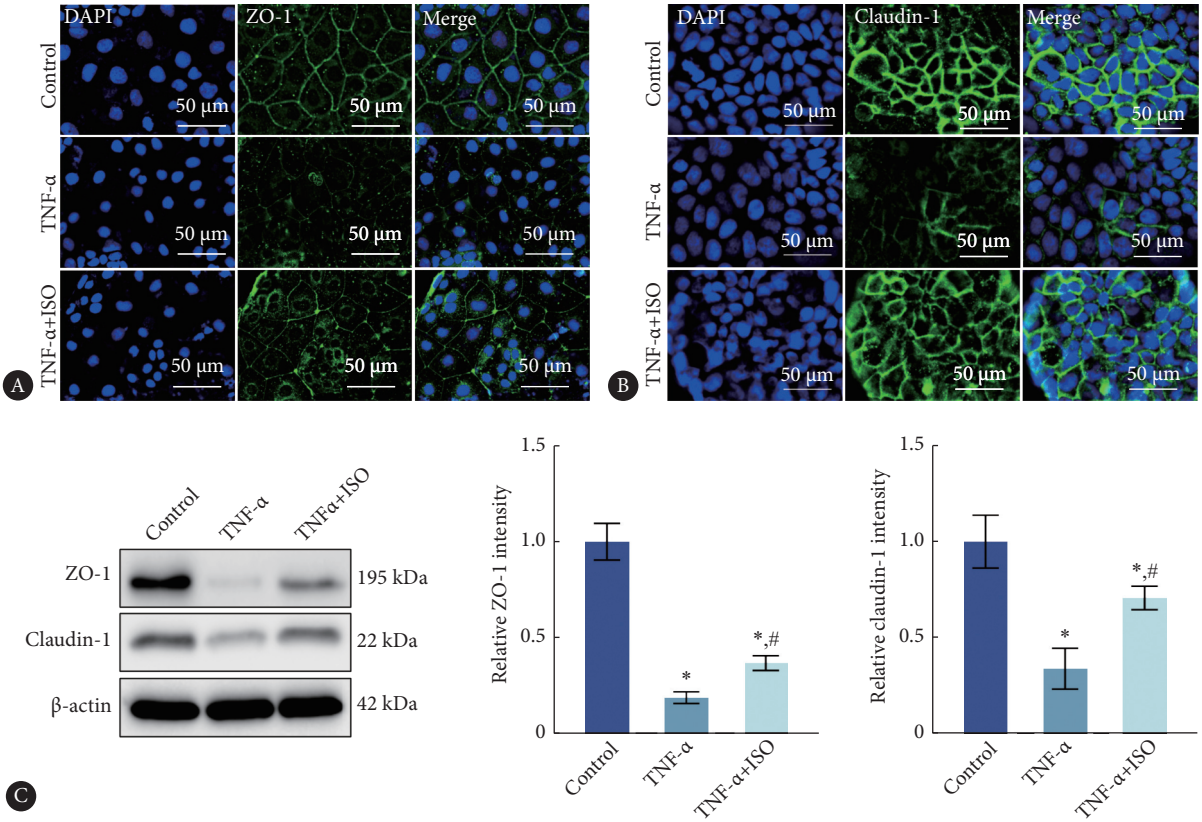

图 4 ISO对Caco-2细胞紧密连接蛋白的作用

Fig 4 Effect of ISO on tight junction proteins in Caco-2 cells

A-B, The expression of ZO-1 and claudin-1 in Caco-2 cells was determined by immunofluorescence; C, Western blot of ZO-1 and claudin-1 in Caco-2 cells (n=3). * P<0.05, vs. control; # P<0.05, vs. TNF-α.

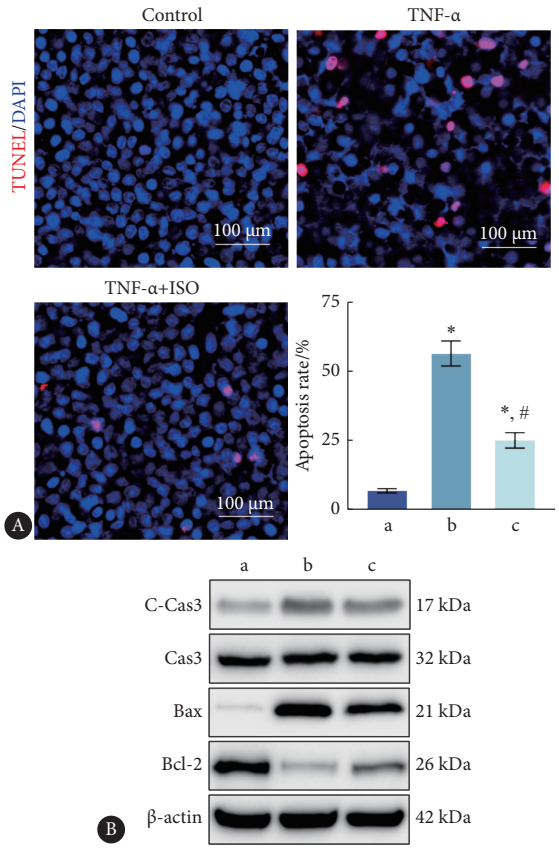

图 5　ISO干预对Caco-2细胞凋亡的影响

Fig 5　Effect of ISO intervention on the apoptosis of Caco-2 cells

A, TUNEL assay was conducted to assess the apoptosis of Caco-2 cells (n=3); B, the expression levels of apoptosis proteins (C-Cas3, Cas3, and Bax) and anti-apoptosis protein (Bcl-2) in Caco-2 cells. * P<0.05, vs. control; # P<0.05, vs. TNF-α. a: control; b: TNF-α; c: TNF-α+ISO; C-Cas3: cleaved caspase-3; Cas3: caspase-3.

表 2　ISO对Caco-2细胞凋亡蛋白的影响（$\bar{x}\pm s$）

Table 2　Effect of ISO on Caco-2 cell apoptosis protein ($\bar{x}\pm s$)

Group	C-Cas3/Cas3	Bax	Bcl-2
Control	1.00±0.18	1.00±0.11	1.00±0.14
TNF-α	2.00±0.21*	5.40±0.24*	0.24±0.06*
TNF-α+ISO	1.55±0.08*,#	2.76±0.32*,#	0.46±0.09*,#

* P<0.05, vs. control; # P<0.05, vs. TNF-α. n=3. C-Cas3: cleaved caspase-3; Cas3: caspase-3.

(P<0.05)。Western blot结果显示, TNF-α+ISO组相比于TNF-α组, Bcl-2表达上调, 而C-Cas3/Cas3和Bax的表达水平则下降(P<0.05)。

2.7　GO富集分析预测ISO的生物学功能与抑制细胞凋亡有关

Venny图显示, ISO和CD的交集作用靶点共有147个(图6A), GO富集预测分析显示, ISO在改善CD样肠炎可能与负向调控细胞凋亡有关(图6B)。

2.8　机制验证

2.8.1　ISO对肠上皮细胞凋亡的调控作用可能与AMPK/PGC1α有关

见图7、表3。Western blot结果显示, ISO干预后小鼠结肠组织(P<0.05)及Caco-2细胞(P<0.05)中p-AMPK/AMPK和p-PGC1α表达水平升高。

2.8.2　ISO通过激活AMPK/PGC1α信号通路缓解肠上皮细胞凋亡

图8A示, 与TNF-α+ISO组相比, 经CC干预后, Western blot结果显示: p-AMPK、p-PGC1α的蛋白表达水

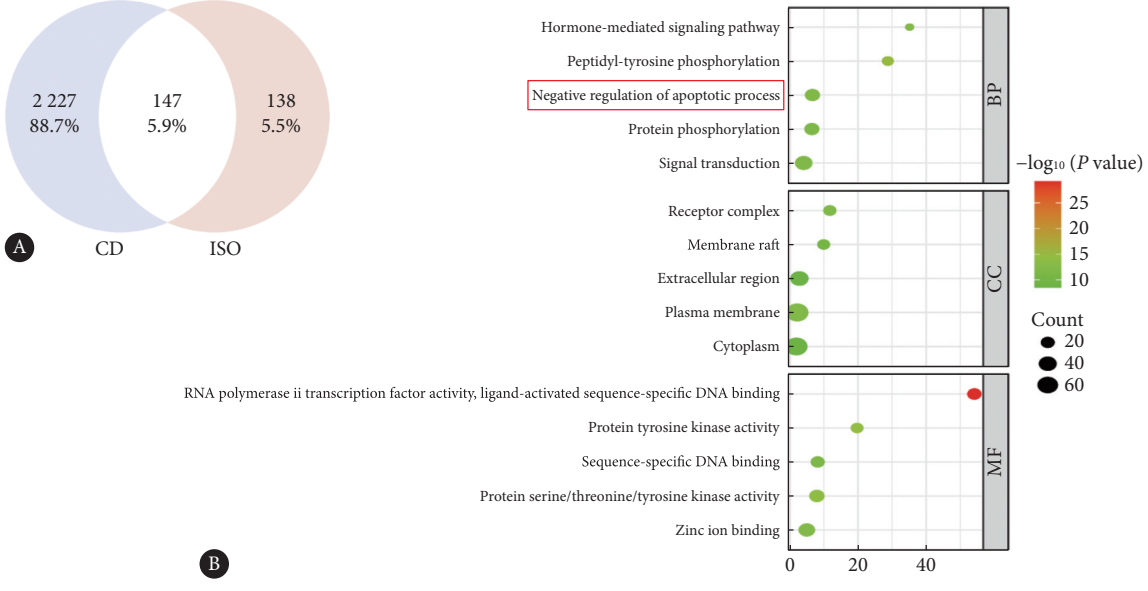

图 6　生物信息学预测ISO在CD中的功能与作用

Fig 6　Predictive analysis of the possible biological function and effect of ISO in CD was made by GO enrichment

A, Venn diagram; B, GO enrichment. BP: biological process; CC: cellular component; MF: molecular function.

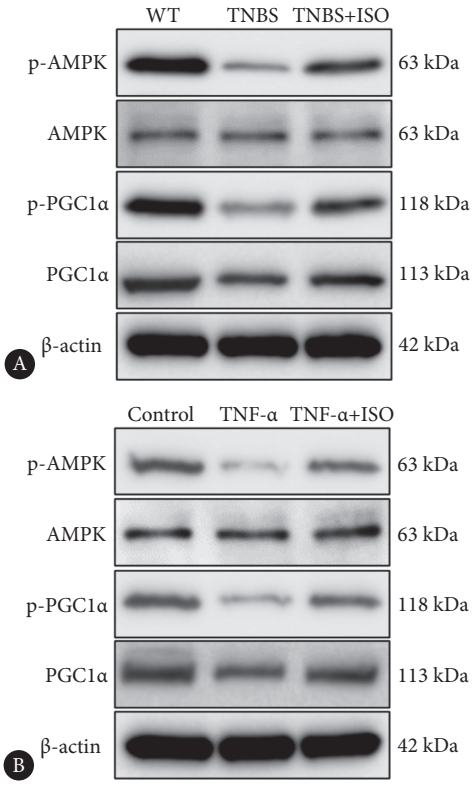

图 7 Western blot检测ISO对AMPK/PGC1α信号通路的作用

Fig 7 Western blot was conducted to assess the effect of ISO intervention on the AMPK/PGC1α pathway *in vivo* and *in vitro*

A, Mice; B, Caco-2 cells.

表 3 ISO对小鼠和Caco-2细胞AMPK/PGC1α信号通路的影响（$\bar{x}\pm s$）

Table 3 Effects of ISO on AMPK/PGC1α signaling pathway ($\bar{x}\pm s$)

Group	p-AMPK/AMPK	p-PGC1α
WT[a]	1.00±0.12	1.00±0.10
TNBS[a]	0.29±0.04*	0.30±0.05*
TNBS+ISO[a]	0.58±0.06*,#	0.50±0.05*,#
Control[b]	1.00±0.14	1.00±0.15
TNF-α[b]	0.25±0.03*	0.38±0.05*
TNF-α+ISO[b]	0.69±0.05*,#	0.74±0.04*,#

[a] Mice (n=8); [b] Caco-2 cells (n=3). * P<0.05, vs. WT or control; # P<0.05, vs. TNBS or TNF-α.

平下降(P<0.05)，ZO-1、claudin-1、Bcl-2表达均下调，而C-Cas3/Cas3和Bax表达水平上调(P<0.05)。TUNEL结果显示(图8B)，相较于TNF-α+ISO组，CC干预后的Caco-2细胞凋亡率增多(P<0.05)。

3 讨论

鉴于CD肠屏障受损往往在导致肠道病变的同时引发全身其他组织的炎症反应，故以保护肠屏障为靶标的干预策略在CD治疗中占据重要地位。本研究的结果表明ISO至少部分通过激活AMPK/PGC1α信号抑制肠上皮细胞凋亡，进而保护TNBS诱导CD样小鼠的肠屏障和改善肠炎。

肠屏障是指人体肠道中的一层物理和化学屏障，起到保护肠道免受有害物质和微生物侵害的作用[17]。肠屏障受损是CD发病的关键环节，其中又以机械屏障的损伤最为重要，位于肠上皮细胞顶端的紧密连接是机械屏障的组成部分之一，其在连接细胞间隙、调节肠黏膜通透性等方面发挥极其关键作用[18]。本研究对结肠组织进行TEER和紧密连接蛋白检测，结果证实ISO对实验性结肠炎的小鼠肠屏障具有保护作用。肠上皮细胞由吸收细胞和杯状细胞构成，作为覆盖在肠黏膜表面的单层细胞，在肠道免疫防御中担任重要角色[19]，其过度凋亡可导致肠屏障功能损伤和引发肠道炎症[20]，因此基于靶向抑制肠上皮细胞凋亡的治疗策略可助益缓解肠道炎症。随着现代药理学和分子生物学技术的高速发展，当前研究发现诸多天然小分子化合物如青蒿素[21]、芍药苷[22]-芦荟多糖[23-24]等具有治疗CD的潜力，某些单体分子譬如雷公藤多苷已被报道应用于临床上治疗CD[25]。ISO是九里香中提取的三环倍半萜烯类化合物，据报道ISO可通过抑制肝细胞凋亡缓解肝脏因缺血再灌注诱发的损伤[11]；ISO还可通过减少大鼠神经细胞凋亡，改善帕金森病症状[12]。基于以上研究背景，为探索ISO在CD中的作用，本研究通过体内实验证实ISO可缓解小鼠CD样结肠炎症状和减少结肠黏膜炎症因子表达水平。为了进一步明确ISO是通过何种途径缓解结肠炎，本研究利用生物信息学预测分析结果显示ISO抗肠炎的作用可能与负向调控细胞凋亡有关，而体内研究发现ISO抑制了肠上皮细胞的凋亡。为了更进一步证实ISO可以直接抑制炎症诱导的肠上皮细胞凋亡，本研究建立了TNF-α诱导的Caco-2细胞凋亡模型。通过免疫印迹和免疫荧光等技术，证实了ISO可以直接改善肠上皮细胞的凋亡。以上数据表明ISO通过抑制肠上皮细胞凋亡，进而保护肠屏障和改善肠炎，但是，相关机制不明。

AMPK是细胞内一种感受能量状态的蛋白激酶，PGC1α则为转录共激活因子，AMPK/PGC1α信号可以调控细胞的能量代谢并对细胞凋亡过程产生影响。在炎症性肠病中亦有研究报道激活AMPK/PGC1α信号可以保护肠屏障[26-27]，而AMPK/PGC1α信号通路的激活可以抑制心肌细胞[28]、肝细胞[29]、海马神经细胞[30]凋亡过程。笔者推测ISO在CD中发挥的抗凋亡从而靶向增强肠屏障作用可能与AMPK/PGC1α信号相关，据本研究Western blot结果显示，ISO可使肠上皮细胞中p-AMPK/AMPK、p-PGC1α的表达升高。在此基础上，AMPK抑制剂CC则逆转了ISO对肠上皮细胞凋亡的抑制作用，这些结果共同证

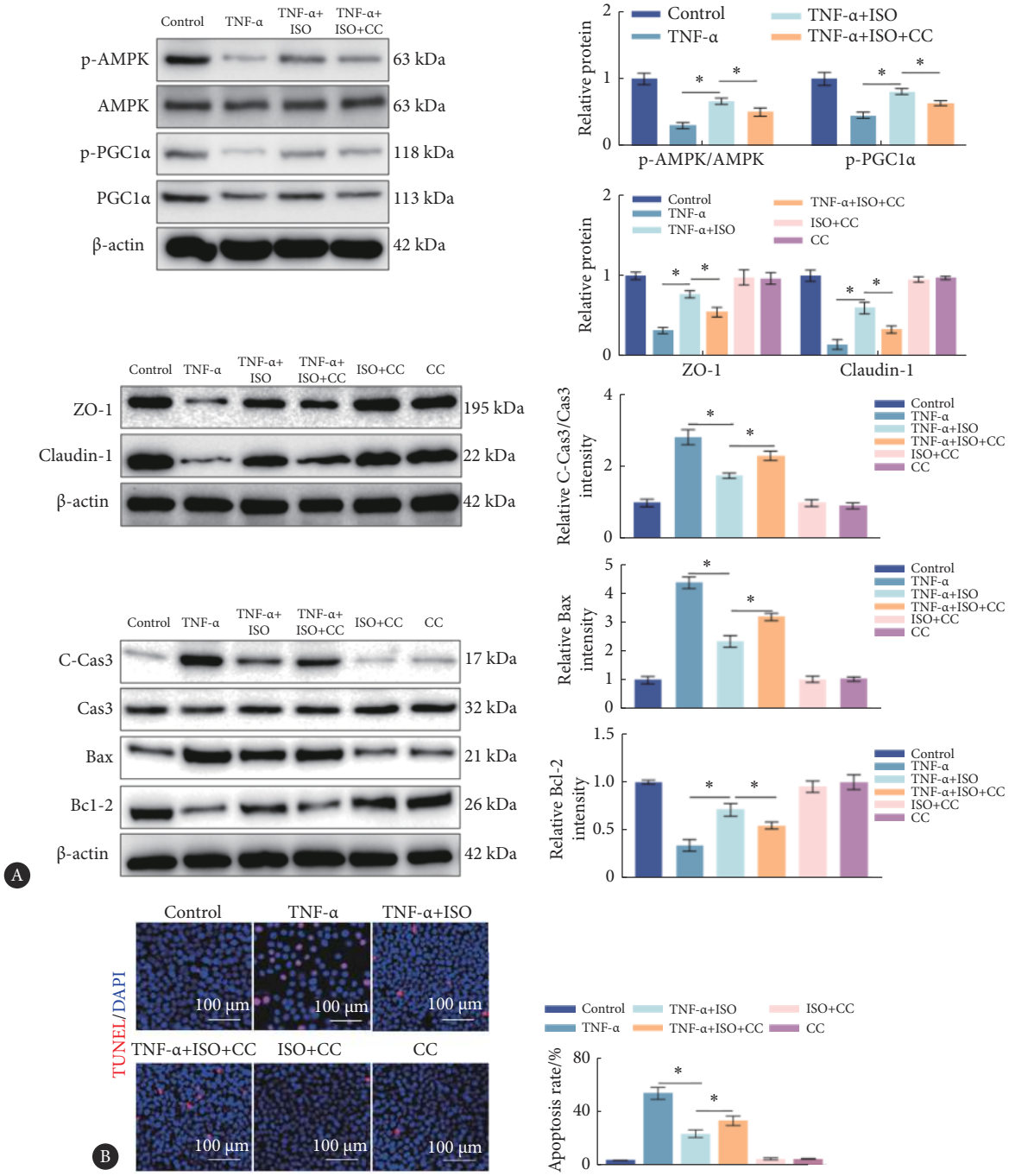

图 8　ISO干预后阻断AMPK/PGC1α信号通路对Caco-2的细胞凋亡及肠屏障的作用

Fig 8　The effect of ISO on Caco-2 cell apoptosis and intestinal barrier by blocking AMPK/PGC1α signaling pathway

A, Western blot was conducted to determine the expression of p-AMPK, p-PGC1α, ZO-1, claudin-1, Bcl-2, Cas3, C-Cas3, and Bax proteins in Caco-2 cells (n=3); B, Caco-2 cell apoptosis was determined by TUNEL assay (n=3). * P<0.05.

实ISO通过激活AMPK/PGC1α信号实现对肠上皮细胞凋亡的负向调控作用。

本研究发现ISO可通过激活AMPK/PGC1α信号从而减少肠上皮细胞的凋亡，在当前研究基础上进一步拓展了ISO在CD中发挥作用的生物学功能及背后的信号机制。再者，本研究表明ISO可缓解小鼠CD样结肠炎，为其作为潜在的CD治疗的药物提供了初步的理论支撑，为CD的临床治疗提供参考。

此外，关于ISO改善肠炎的作用途径，本研究仅讨论了ISO调控肠上皮细胞凋亡的作用，未能再进一步地对ISO在CD中除抑制肠上皮细胞凋亡以外是否参与调节肠道免疫、调节肠道菌群等其它作用展开研究；而ISO在CD中除AMPK/PGC1α信号通路以外其他可能调控肠上皮细胞凋亡的分子机制亦有待进一步补充。最后，在实

验方法上，本研究仅基于公共数据库进行预测分析ISO在CD中的生物功能，未能对小鼠组织进行RNA-seq，在后续的研究中课题组将结合RNA-seq分析结果进行深入探索，以期更加全面完整地探索ISO缓解CD调控机制。

综上所述，本研究显示ISO至少部分通过激活AMPK/PGC1α信号通路从而负向调控肠上皮细胞凋亡，最终增强TNBS诱导小鼠的肠屏障功能和缓解CD样结肠炎，本研究为寻找靶向肠上皮细胞凋亡途径治疗CD提供了新的参考。

* * *

作者贡献声明 段婷负责论文构思和初稿写作，耿志军负责研究项目管理，杨晶晶负责数据审编，殷丽霞和王舜印负责软件，孙明熙负责研究方法，王舜印负责软件，张小凤负责可视化，李静负责正式分析，胡建国负责提供资源和监督指导，陆国玉负责经费获取和审读与编辑写作。所有作者已经同意将文章提交给本刊，且对将要发表的版本进行最终定稿，并同意对工作的所有方面负责。

Author Contribution DUAN Ting is responsible for conceptualization and writing--original draft. GENG Zhijun is responsible for project administration. YANG Jingjing is responsible for data curation. YIN Lixia and WANG Shunyin are responsible for software. SUN Mingxi is responsible for methodology. ZHANG Xiaofeng is responsible for visualization. LI Jing is responsible for formal analysis. HU Jianguo is responsible for resources and supervision. LU Guoyu is responsible for funding acquisition and writing--review and editing. All authors consented to the submission of the article to the Journal. All authors approved the final version to be published and agreed to take responsibility for all aspects of the work.

利益冲突 所有作者均声明不存在利益冲突

Declaration of Conflicting Interests All authors declare no competing interests.

参 考 文 献

[1] TORRES J, MEHANDRU S, COLOMBEL J F, *et al*. Crohn's disease. Lancet, 2017, 389(10080): 1741–1755. doi: 10.1016/S0140-6736(16)31711-1.

[2] VEAUTHIER B, HORNECKER J R. Crohn's disease: diagnosis and management. Am Fam Physician, 2018, 98(11): 661–669.

[3] 陈鑫明, 刘洋, 何运胜, 等. 克罗恩病发病影响因素的研究进展. 现代消化及介入诊疗, 2023, 28(7): 912–918. doi: 10.3969/j.issn.1672-2159.2023.07.026.
CHEN X M, LIU Y, HE Y S, et, al. Research progress of influencing factors of Crohn's disease pathogenesis. Modn Int Diagn Treat Gastroenterol, 2023, 28(7): 912–918. doi: 10.3969/j.issn.1672-2159.2023.07.026.

[4] RODA G, NG S C, KOTZE P G, *et al*. Crohn's disease. Nat Rev Dis Primers, 2020, 6(1): 22. doi: 10.1038/s41572-020-0156-2.

[5] CAMILLERI M, MADSEN K, SPILLER R, *et al*. Intestinal barrier function in health and gastrointestinal disease. Neurogastroenterol Motil, 2012, 24(6): 503–512. doi: 10.1111/j.1365-2982.2012.01921.x.

[6] TURNER J R. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol, 2009, 9(11): 799–809. doi: 10.1038/nri2653.

[7] GÜNTHER C, NEUMANN H, NEURATH M F, *et al*. Apoptosis, necrosis and necroptosis: cell death regulation in the intestinal epithelium. Gut, 2013, 62(7): 1062–1071. doi: 10.1136/gutjnl-2011-301364.

[8] VERNIA F, VALVANO M, LONGO S, *et al*. Vitamin D in inflammatory bowel diseases. Mechanisms of action and therapeutic implications. Nutrients, 2022, 14(2): 269. doi: 10.3390/nu14020269.

[9] ZHUANG X, CHEN B, HUANG S, *et al*. Hypermethylation of miR-145 promoter-mediated SOX9-CLDN8 pathway regulates intestinal mucosal barrier in Crohn's disease. EBioMedicine, 2022, 76: 103846. doi: 10.1016/j.ebiom.2022.103846.

[10] ZHOU L, ZHU L, WU X, *et al*. Decreased TMIGD1 aggravates colitis and intestinal barrier dysfunction via the BANF1-NF-κB pathway in Crohn's disease. BMC Med, 2023, 21(1): 287. doi: 10.1186/s12916-023-02989-2.

[11] YANG L, LUO H, TAN D, *et al*. A recent update on the use of Chinese medicine in the treatment of inflammatory bowel disease. Phytomedicine, 2021, 92: 153709. doi: 10.1016/j.phymed.2021.153709.

[12] LI J, LI J, FANG H, *et al*. Isolongifolene alleviates liver ischemia/reperfusion injury by regulating AMPK-PGC1α signaling pathway-mediated inflammation, apoptosis, and oxidative stress. Int Immunopharmacol, 2022, 113(Pt A): 109185. doi: 10.1016/j.intimp.2022.109185.

[13] BALAKRISHNAN R, VIJAYRAJA D, MOHANKUMAR T, *et al*. Isolongifolene mitigates rotenone-induced dopamine depletion and motor deficits through anti-oxidative and anti-apoptotic effects in a rat model of Parkinson's disease. J Chem Neuroanat, 2021, 112: 101890. doi: 10.1016/j.jchemneu.2020.101890.

[14] AMARASIRI S S, ATTANAYAKE A P, ARAWWAWALA L, *et al*. Nephroprotective activity of *Vetiveria zizanioides (L.) Nash* supplement in doxorubicin-induced nephrotoxicity model of Wistar rats. J Food Biochem, 2021, 45(9): e13901. doi: 10.1111/jfbc.13901.

[15] PENG L, LI Z R, GREEN R S, *et al*. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. J Nutr, 2009, 139(9): 1619–1625. doi: 10.3945/jn.109.104638.

[16] ZHANG J, CEN L, ZHANG X, *et al*. MPST deficiency promotes intestinal epithelial cell apoptosis and aggravates inflammatory bowel disease via AKT. Redox Biol, 2022, 56: 102469. doi: 10.1016/j.redox.2022.102469.

[17] SPENCER D M, VELDMAN G M, BANERJEE S, *et al*. Distinct inflammatory mechanisms mediate early versus late colitis in mice. Gastroenterology, 2002, 122(1): 94–105. doi: 10.1053/gast.2002.30308.

[18] MINTON K. Intestinal barrier protection. Nat Rev Immunol, 2022, 22(3): 144–145. doi: 10.1038/s41577-022-00685-5.

[19] MEHANDRU S, COLOMBEL J F. The intestinal barrier, an arbitrator turned provocateur in IBD. Nat Rev Gastroenterol Hepatol, 2021, 18(2):

83–84. doi: 10.1038/s41575-020-00399-w.

[20] OKUMURA R, TAKEDA K. Roles of intestinal epithelial cells in the maintenance of gut homeostasis. Exp Mol Med, 2017, 49(5): e338. doi: 10.1038/emm.2017.20.

[21] PATANKAR J V, BECKER C. Cell death in the gut epithelium and implications for chronic inflammation. Nat Rev Gastroenterol Hepatol, 2020, 17(9): 543–556. doi: 10.1038/s41575-020-0326-4.

[22] HUAI M, ZENG J, GE W. Artemisinin ameliorates intestinal inflammation by skewing macrophages to the M2 phenotype and inhibiting epithelial-mesenchymal transition. Int Immunopharmacol, 2021, 91: 107284. doi: 10.1016/j.intimp.2020.107284.

[23] LUO X, WANG X, HUANG S, *et al*. Paeoniflorin ameliorates experimental colitis by inhibiting gram-positive bacteria-dependent MDP-NOD2 pathway. Int Immunopharmacol, 2021, 90: 107224. doi: 10.1016/j.intimp.2020.107224.

[24] ZHANG D, ZHOU X, ZHANG K, *et al*. Glucomannan from Aloe vera gel maintains intestinal barrier integrity via mitigating anoikis mediated by Nrf2-mitochondria axis. Int J Biol Macromol, 2023, 235: 123803. doi: 10.1016/j.ijbiomac.2023.123803.

[25] 付正伟, 王丽霞, 葛海燕. 芦荟多糖调控Th17/Treg细胞平衡缓解肠炎的实验研究. 中华普通外科学文献(电子版), 2019, 13(6): 435–440. doi: 10.3877/cma.j.issn.1674-0793.2019.06.004.

FU Z W, WANG L X, GE H Y. Experimental study of the aloe polysaccharides relieving enteritis by regulating Th17/Treg cell balance. Chin Arch Gen Surg, 2019, 13(6): 435–440. doi: 10.3877/cma.j.issn.1674-0793.2019.06.004.

[26] TRIANTAFYLLIDI A, XANTHOS T, PAPALOIS A, *et al*. Herbal and plant therapy in patients with inflammatory bowel disease. Ann Gastroenterol, 2015, 28(2): 210–220.

[27] HERZIG S, SHAW R J. AMPK: guardian of metabolism and mitochondrial homeostasis. Nat Rev Mol Cell Biol, 2018, 19(2): 121–135. doi: 10.1038/nrm.2017.95.

[28] SUN X, YANG Q, ROGERS C J, *et al*. AMPK improves gut epithelial differentiation and barrier function via regulating Cdx2 expression. Cell Death Differ, 2017, 24(5): 819–831. doi: 10.1038/cdd.2017.14.

[29] TIAN Q, XU Z, SUN Q, *et al*. Broccoli-derived glucoraphanin activates AMPK/PGC1α/NRF2 pathway and ameliorates dextran-sulphate-sodium-induced colitis in mice. Antioxidants (Basel), 2022, 11(12): 2404. doi: 10.3390/antiox11122404.

[30] ZHANG L, WANG Y N, JU J M, *et al*. Mzb1 protects against myocardial infarction injury in mice via modulating mitochondrial function and alleviating inflammation. Acta Pharmacol Sin, 2021, 42(5): 691–700. doi: 10.1038/s41401-020-0489-0.

（2024 – 04 – 08收稿, 2024 – 08 – 26修回）

编辑　吕　熙