·基础研究·

没食子酸对卡介苗感染巨噬细胞的免疫调控

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摘　要：【目的】探讨没食子酸（GA）在卡介苗（BCG）感染巨噬细胞中的宿主免疫调控作用及其分子机制，为结核分枝杆菌的宿主导向抗结核研究提供实验依据。【方法】采用刃天青显色法测定 GA 对 BCG 的最低抑菌浓度（MIC），结合细菌生长曲线、MTT 比色法评价 GA 对 BCG 增殖及侵袭能力的影响。构建 BCG 感染 RAW264.7 巨噬细胞模型，设置感染对照组（Ctrl）、异烟肼（INH）组及 GA 低、中、高剂量组。采用流式细胞术联合细菌铺板计数检测胞内 BCG 存活；AnnexinV-APC/7-AAD 双染法检测细胞凋亡，qRT-PCR 检测凋亡相关基因 *Caspase-3*、*Bcl-2* mRNA 表达；流式细胞术检测巨噬细胞极化表型；DHE 探针检测胞内活性氧（ROS）；Griess 法检测上清一氧化氮（NO）；ELISA 检测上清 TNF-α、IFN-γ、IL-6、IL-10 细胞因子水平。【结果】GA 对 BCG 的 MIC 为 1 250 μmol/L，可浓度依赖性抑制 BCG 增殖与侵袭（$P<0.05$）；选择 20、40、80 μmol/L 的药物安全浓度开展细胞实验，GA 预处理 36 h 可显著降低胞内 BCG 菌载量（$P<0.05$）。GA 双向调控巨噬细胞凋亡，感染早期抑制凋亡、后期促进凋亡；感染早期诱导 M1 极化，感染后期促进 M2 极化（$P<0.05$）；同时降低胞内 ROS 与 NO 水平，下调促炎因子分泌、上调抗炎因子 IL-10 分泌（$P<0.05$）。【结论】GA 体外可抑制 BCG 增殖侵袭；并通过双向调控巨噬细胞凋亡与极化、发挥抗氧化抗炎效应，改善宿主免疫微环境，具备宿主导向抗分枝杆菌的潜在价值。

关键词：没食子酸；卡介苗；巨噬细胞；免疫功能；抗分枝杆菌作用
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Immunoregulatory Effects of Gallic Acid on BCG-infected Macrophages

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Abstract：【Objective】To explore the host immunomodulatory effect and its molecular mechanism of gallic acid（GA）in *Bacillus Calmette-Guérin*（BCG）infected macrophages，and to provide experimental evidence for host-directed anti-tuberculosis research against *Mycobacterium tuberculosis*（Mtb）.【Methods】The minimum inhibitory concentration（MIC）of GA against BCG was determined by resazurin colorimetric assay. Bacterial growth-curve test and MTT colorimetric assay were used to evaluate the effects of GA on BCG proliferation and invasion. A BCG-infected RAW264.7 macrophage model was established，including infection control group（Ctrl），isoniazid（INH）group，and low-，medium- and high-dose GA

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groups. Flow cytometry combined with colony-plate counting was performed to detect intracellular BCG survival. Cell apoptosis was measured by Annexin V-APC/7-AAD double staining, and qRT-PCR was used to detect the mRNA expression of apoptosis-related genes *Caspase-3* and *Bcl-2*. Macrophage polarization phenotypes were analyzed by flow cytometry. Intracellular reactive oxygen species (ROS) were detected using DHE fluorescent probe. The Griess assay was applied to determine supernatant nitric oxide (NO) concentration. ELISA was used to detect the levels of TNF-α, IFN-γ, IL-6 and IL-10 in cell culture supernatant.【Results】The MIC of GA against BCG was 1 250 μmol/L. GA inhibited BCG proliferation and invasion in a concentration-dependent manner ($P<0.05$). Safe concentrations of 20, 40 and 80 μmol/L were selected for subsequent cellular experiments. Thirty-six-hour GA pretreatment markedly reduced the intracellular BCG bacterial load ($P<0.05$). GA exerted bidirectional regulation on macrophage apoptosis: it inhibited apoptosis at the early-stage infection and promoted apoptosis at the late-stage infection. GA induced M1-type polarization in early infection and facilitated M2-type polarization in late infection ($P<0.05$). Meanwhile, GA decreased intracellular ROS and NO levels, down-regulated the secretion of pro-inflammatory cytokines and up-regulated the secretion of anti-inflammatory cytokine IL-10 ($P<0.05$).【Conclusion】GA inhibits BCG proliferation and invasion in vitro. It remodels host immune microenvironment via bidirectional regulation of macrophage apoptosis and polarization as well as anti-oxidative and anti-inflammatory effects, showing potential value for host-directed anti-mycobacterial therapy.

Key words: gallic acid; Bacillus Calmette-Guérin; macrophages; immune function; anti-mycobacterial activity

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结核病是由结核分枝杆菌(*Mycobacterium tuberculosis*, Mtb)引发的慢性传染病,对全球公共卫生构成严重威胁。世界卫生组织《2024年全球结核病报告》指出,全球结核病防控进展缓慢,距离“2030年前终结结核病”的目标仍存在显著差距[1-2]。我国属于结核病高负担国家,部分地区疫情防控形势依旧严峻[3-4]。目前,以异烟肼(isoniazid, INH)、利福平为核心的一线抗结核化疗方案受耐药问题的严重制约,耐多药/利福平耐药结核病(multidrug-resistant/rifampicin-resistant tuberculosis, MDR/RR-TB)的流行显著提升了治疗失败率与病死率,给公共卫生安全造成沉重负担[1,5-9]。传统抗生素主要依靠直接杀菌发挥疗效,但Mtb可通过*pepQ*、*Rv1979c*等耐药基因突变逃逸药物杀伤[10-12]。因此,开发基于宿主免疫调控的新型治疗策略已成为结核病研究的热点方向。宿主导向治疗(host-directed therapy, HDT)可通过靶向宿主免疫通路、重塑免疫微环境,增强巨噬细胞清除胞内病原菌的能力并减轻炎症损伤,是极具应用潜力的结核辅助治疗手段。

巨噬细胞是抗分枝杆菌固有免疫的核心效应细胞,也是Mtb主要的胞内寄生宿主,可通过吞噬作用与细胞因子分泌启动抗菌免疫应答[13-14]。Mtb通过表面病原相关分子模式激活巨噬细胞模式识别受体,诱导促炎因子分泌和免疫细胞募集,同时通过抑制吞噬体-溶酶体融合、诱导M2型极化等策略逃逸免疫清除[15-18]。没食子酸(gallic acid, GA)是广泛存在于多种藏药中的天然多酚化合物,具有抗氧化、抗炎及抑菌活性[19-22]。已有研究证实GA可抑制分枝杆菌增殖,并通过调控NF-κB、MAPK信号通路调节巨噬细胞极化[23-30]。但GA能否通过调控巨噬细胞凋亡、极化及氧化应激水平发挥抗分枝杆菌作用,其具体免疫调控机制仍缺乏系统验证,相关宿主机制研究仍存在空白。

卡介苗(*Bacillus Calmette-Guérin*, BCG)与Mtb基因组高度同源、安全性高,是研究分枝杆菌感染的经典体外模型[31-32]。基于此,本研究构建BCG感染巨噬细胞模型,选取感染早期(4 h)与胞内增殖炎症期(24 h)这2个关键时间点,系统探究GA的直接抑菌活性及对巨噬细胞免疫功能的调控效应,旨在阐明GA抗分枝杆菌的分子机制,为GA应用于宿主导向抗结核辅助治疗提供实验依据。

1 材料与方法

1.1 细菌菌株和生长条件

BCG由本实验室保存,将增强型绿色荧光蛋白(enhanced green fluorescent protein, EGFP)的质粒

通过电转至BCG构建BCG-GFP。BCG培养于体积分数10%油酸白蛋白葡萄糖过氧化氢酶补充剂增菌液的7H9液体培养基中，置于37 ℃恒温摇床振荡培养，经2~3周连续培养后可见培养基浑浊。待细菌生长状况良好后，每周液体传代1次。选择对数生长期的菌株进行后续实验。

1.2　细胞培养

将RAW264.7细胞置于完全培养基（含有10%FBS的DMEM高糖基础培养基）中，在37 ℃、相对湿度为95%以上、5%CO_2培养箱中培养，当细胞密度达到70%~80%时进行传代。

1.3　刃天青微量显色法检测MIC

采用刃天青微量显色法测定最低抑菌浓度（minimum inhibitory concentration，MIC）。将BCG稀释至1×10^7 CFU/mL，设置药物梯度实验组、阴性组、阳性对照组，37 ℃培养7 d后加入刃天青显色。以培养基保持蓝色、细菌增殖被抑制的最低药物浓度作为MIC。MIC仅反映抑菌效应，菌落形成单位（colony-forming units，CFU）计数用于评价杀菌效果。

1.4　BCG生长曲线检测

BCG菌液分组加入含有GA、INH的7H9液体培养基，控制每组菌液起始OD_{600}值为0.1，37 ℃环境培养，每3天取样离心洗涤后重悬，用紫外分光光度计测OD_{600}至27 d。

1.5　BCG对巨噬细胞侵袭能力检测

BCG按MIC系列浓度GA预处理24 h后洗涤去除药物再感染巨噬细胞，检测OD_{600}并计算菌量，以MOI=10感染细胞4 h，弃上清，MTT试验检测孔内细胞相对存活率。

1.6　MTT试验

Raw 264.7细胞密度调整为1×10^5个/mL后，按100 μL/孔接种于96孔板，37 ℃、5%CO_2培养24 h。分别加入不同浓度的GA（1，5，10，20，40，80，160和320 μmol/L）和INH（5，10，25，50，75，100，125和150 μmol/L），查阅相关文献[33-34]并结合本实验MIC的结果，参考结核病患者INH体内血药浓度目标值以及INH对BCG的最低抑菌浓度，后续体外抗BCG实验选取10 μmol/L INH作为阳性对照浓度，且该浓度对RAW264.7细胞无明显毒性。设正常对照孔，继续培养24 h。每孔加入10 μL MTT溶液（5 mg/mL），37 ℃孵育4 h后弃上清，加入二甲基亚砜（dimethyl sulfoxide，DMSO）150 μL溶解甲瓒结晶，酶标仪检测490 nm波长处吸光度以计算细胞活性。

1.7　BCG感染巨噬细胞模型的构建

将培养至对数生长期的BCG收集浓缩于无菌离心管中，用PBS洗涤菌体2次后，用体积分数10%甘油的7H9液体培养基重悬，加入20~30个直径2 mm的已灭菌的玻璃珠，涡旋振荡15 min后收集菌液，500×g离心力离心2 min，上层菌液即为单细菌。

将细胞稀释至5×10^5个/mL，接种于6孔板，使细胞均匀分布于孔底，置于37 ℃、5%CO_2的恒温培养箱孵育12 h，待细胞贴壁稳定、融合度达到70%左右时，进行BCG感染处理。按照MOI=10用BCG单细菌对孔板内细胞进行感染，感染4 h后弃去培养基，PBS清洗去除胞外菌，加入含200 μg/mL庆大霉素的完全培养基孵育2 h以杀灭残留细菌。再次清洗后，换用含20 μg/mL庆大霉素的完全培养基继续培养至24 h，全程维持各实验组药物处理。

1.8　流式细胞术检测胞内细菌生存率

实验分为感染对照组（Ctrl组）、异烟肼组（INH组）、没食子酸低剂量组（GA-L组）、没食子酸中剂量组（GA-M组）和没食子酸高剂量组（GA-H组），选用INH的给药浓度是10 μmol/L，GA的给药浓度分别是20、40、80 μmol/L作为低、中、高浓度进行后续实验。除感染对照组外，其余各组于感染前36 h进行药物预处理，感染后维持对应给药浓度。

弃去孔内培养基后，向细胞培养板中加入体积分数0.5% Triton X-100或无菌纯水以释放胞内细菌，取裂解液用7H9肉汤培养基分别进行100倍及1 000倍稀释，各吸取100 μL稀释液以无菌玻璃珠滚动辅助铺板，使菌液均匀涂布于7H11琼脂培养基表面，将培养平板用封口膜密封后，置于37 ℃培养箱中培养3周；收集孔内细胞，用预冷PBS清洗2次，用500 μL PBS将细胞重悬于流式检测管中，用流式细胞仪进行检测，用GFP通道检测胞内BCG的存活率。

1.9　流式细胞术检测巨噬细胞凋亡与极化

分别于BCG感染后4 h、24 h收集细胞，4 ℃、500× g离心5 min，预冷PBS洗涤2次，分装样本分别用于凋亡与极化检测。

细胞凋亡检测：取细胞以500 μL 1×Binding Buffer重悬，设置膜联蛋白V-别藻蓝蛋白（annexin V conjugated with allophycocyanin，Annexin V-

APC)和7-氨基放线菌素D(7-aminoactinomycin D, 7-AAD)单染管及空白未染色管用于电压调节与荧光补偿。样本管依次加入5 μL Annexin V-APC、10 μL 7-AAD,涡旋混匀,室温避光孵育5 min后上机检测。采用FlowJo软件进行荧光补偿校正与凋亡定量分析。

巨噬细胞极化检测:取细胞样本加入0.5 μL CD_{86}抗体,冰上孵育30 min;预冷PBS洗涤2次,250 μL流式固定缓冲液4 ℃固定30 min,破膜缓冲液洗涤后加入0.5 μL CD_{206}抗体,冰上孵育30 min。PBS洗涤后以500 μL预冷PBS重悬,4 ℃避光保存,并于2 h内完成流式上机检测。数据采用FlowJo软件分析。

1.10 qRT-PCR检测巨噬细胞mRNA表达

感染后4 h及24 h收集细胞,将细胞用裂解液裂解后经无水乙醇沉淀、离心柱纯化提取总RNA,洗脱后测浓度。取1 μg RNA逆转录为cDNA,反应体系含5×缓冲液4 μL、酶混合物1 μL及无酶水,程序为50 ℃ 15 min、85 ℃ 5 s。qRT-PCR配制20 μL体系,含SYBR Green、引物及cDNA模板,以GAPDH为内参。反应程序:95 ℃预变性3 min,95 ℃ 10 s/60 ℃ 30 s循环40次,熔解曲线分析。采用$2^{-\Delta\Delta Ct}$法计算目标基因相对表达量,数据以均值±标准差表示。

1.11 二氢乙锭荧光探针染色法检测胞内活性氧

二氢乙锭(dihydroethidium, DHE)用DMSO制成5 mmol/L母液。弃去孔内培养基,预温PBS轻柔清洗细胞两次。每孔加入5 μmol/L的DHE工作液500 μL,37 ℃、5%CO_2避光孵育30 min。孵育后PBS清洗3次,加入新鲜培养基,倒置荧光显微镜观察并成像;或收集细胞,预冷PBS重悬后转移至流式管,4 ℃避光保存,2 h内完成流式检测。实验全程严格避光操作,数据以荧光强度均值表示。

1.12 细胞培养上清中一氧化氮浓度的检测

收集各组细胞培养上清液,4 ℃,500×g离心5 min,以去除上清中细胞碎片、蛋白质及微小颗粒等。用完全培养基将1 mol/L浓度的标准品梯度稀释至作为标准曲线。在96孔板中按照每孔加入50 μL加样培养上清样本和标准品,各孔分别加入50 μL已经恢复至室温的Griess Reagent Ⅰ和Griess Reagent Ⅱ,轻微晃动混匀后去除孔内气泡。在酶标仪上540 nm波长测量各孔吸光度值,根据标准品曲线计算出样本中一氧化氮(nitric oxide, NO)的浓度。

1.13 ELISA

采用ELISA法检测细胞培养上清中细胞因子的水平,严格根据试剂盒说明书要求进行操作,用未加样本和酶标剂的空白孔调零,加入终止剂后15 min内,在λ=450 nm处读取标准品及样本的吸光度数值,使用标准品吸光度绘制标准曲线,统计并分析样本细胞因子水平。

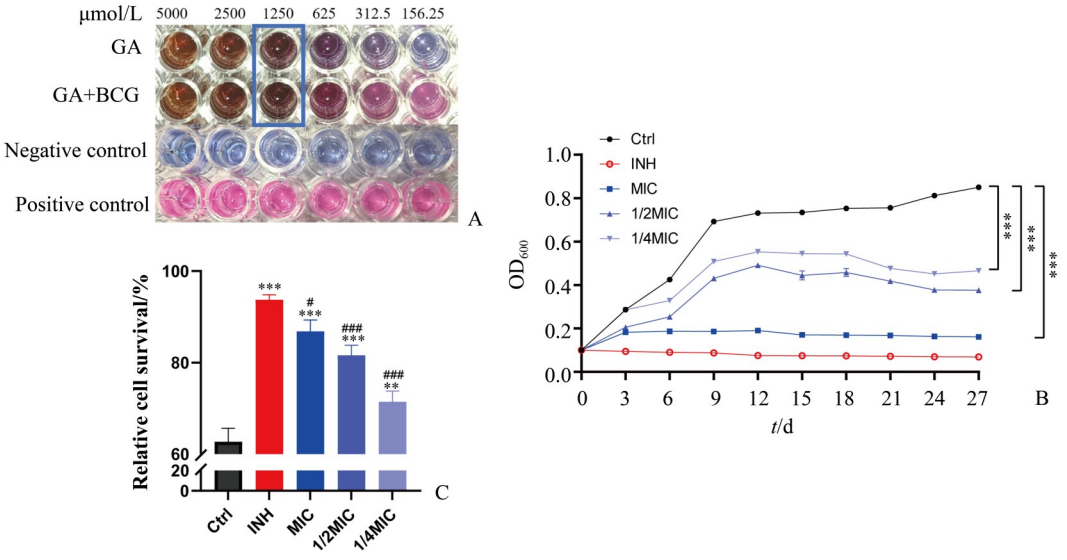
1.14 统计分析

采用GraphPad Prism 9.5.0软件进行统计学分析。符合正态分布的定量数据以均值±标准差($\bar{x}\pm s$)表示;两组比较采用t检验,多组比较行单/双因素方差分析,以Brown-Forsythe检验进行方差齐性检验,本研究全部计量指标均满足方差齐条件,两两比较采用Tukey法校正,控制多重比较的Ⅰ类错误。ELISA、qRT-PCR等计量指标均采用上述统计策略,关键结果于图注标注检验统计量F与P值。显著性标记:*为与Ctrl组比较,#为与INH组比较;$P<0.05$标记为*/#,$P<0.01$标记为**/##,$P<0.001$标记为***/###,ns代表差异无统计学意义。每组设置3次独立生物学重复。本研究为体外实验,未做事前样本量估算,n=3条件下检验效能有限,后续体内实验需完成样本量估算以降低Ⅱ类错误。

2 结 果

2.1 没食子酸对BCG的体外抑菌作用

该研究采用刃天青微量显色法测定GA对BCG的MIC,并评估其抑菌活性及作用机制。实验证实GA对BCG的抑菌活性,并确定其MIC为1 250 μmol/L(图1A)。进一步分析发现(图1B),通过梯度浓度(MIC、1/2 MIC、1/4 MIC)处理BCG,监测生长曲线发现:GA呈浓度依赖性抑制BCG增殖,MIC组呈现完全生长抑制,抑菌强度随浓度降低呈阶梯式减弱($P<0.001$)。侵袭实验表明(图1C),GA处理24 h后洗涤去除药物再感染巨噬细胞,各浓度组均显著抑制BCG对巨噬细胞的侵袭能力($P<0.05$),其中MIC组抑制作用最强,且抑制效果与浓度正相关,异烟肼对照同样有效,巨噬细胞存活率升高,进一步支持实验体系的有效性。该研究明确了GA在体外能够有效抑制BCG的活性,抑制BCG生长及侵袭能力且具有浓度依赖性,为结核病治疗提供了潜在药物活性证据。



A: Antibacterial effect of GA on BCG in vitro MIC value of GA for BCG. B: Growth curves of BCG under different concentrations of GA treatment. C: The effect of GA treated BCG on the viability of macrophages, indicating the effect of GA on the ability of BCG to invade macrophages. Compared with the Ctrl group, $^{**}P<0.01$, $^{***}P<0.001$; compared with the INH group, $^{\#}P<0.05$, $^{\#\#\#}P<0.001$, $n=3$.

图1　不同剂量检测GA对BCG的最低抑菌浓度与抑菌活性

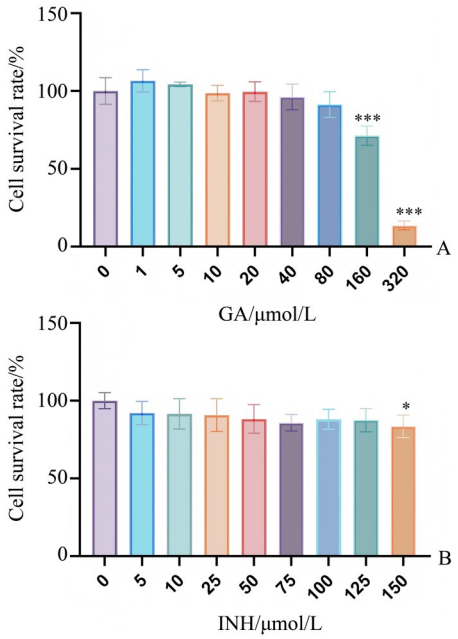
Fig. 1　Determination of the minimum inhibitory concentration and antibacterial activity of GA against BCG at different doses

2.2　没食子酸对Raw264.7细胞增殖的影响

MTT比色法筛选GA和INH的安全给药浓度。结果所示，80 μmol/L及以下的GA对Raw264.7细胞无毒性作用（$P>0.05$；图2A）；与正常对照组相比，125 μmol/L及以下的INH对Raw264.7细胞活性无显著影响（$P>0.05$；图2B）。本文选用20、40和80 μmol/L的GA分别作为低、中、高浓度进行后续试验。

2.3　没食子酸对BCG感染巨噬细胞胞内细菌生存率的影响

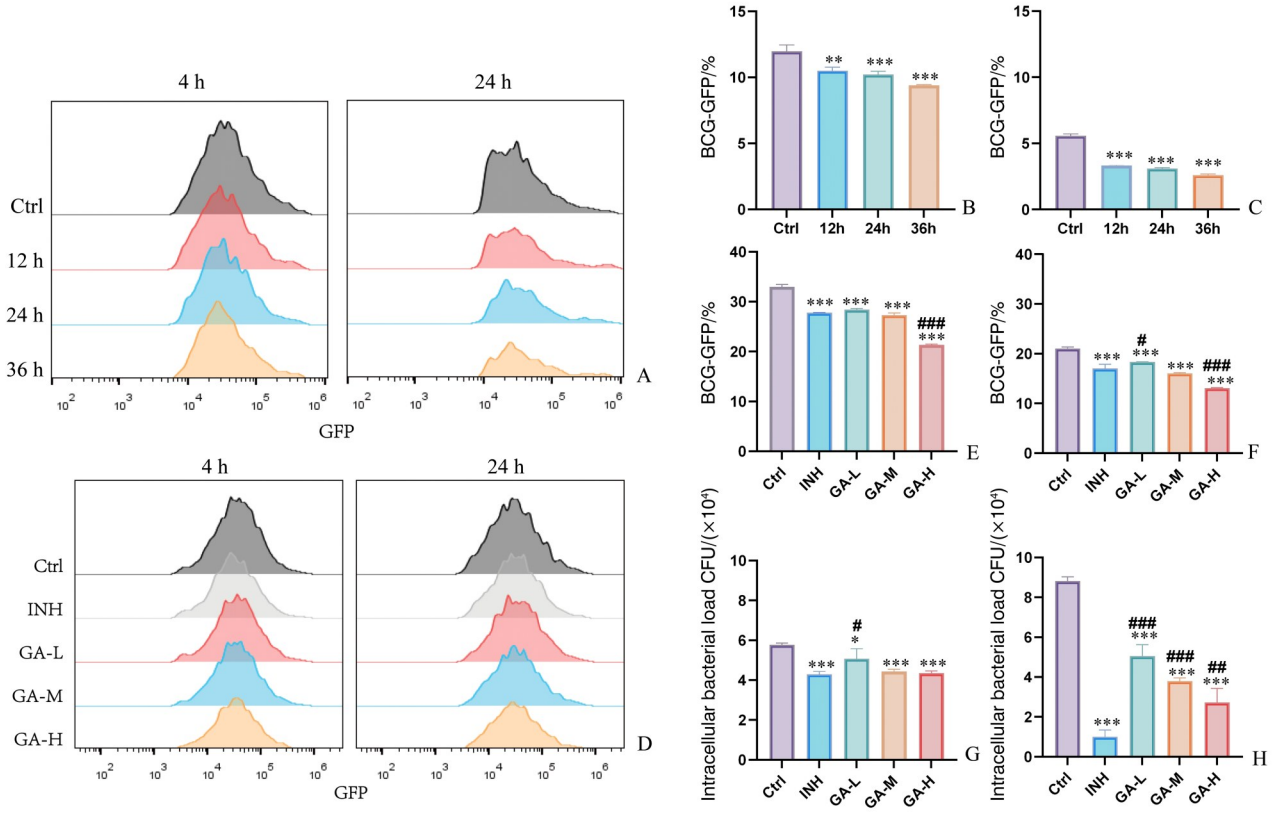
为探究GA对BCG感染巨噬细胞的调控效应，采用流式细胞术评估胞内细菌存活情况。以80 μmol/L GA预处理巨噬细胞后构建BCG感染模型，分别检测感染4 h、24 h胞内细菌存活水平。结果表明，GA预处理可显著降低胞内BCG存活率（$P<0.05$），其中36 h预处理抑制效果最优（图3A-C），因此后续实验选用感染前36 h预处理方案。在感染4 h及24 h，各GA给药组胞内BCG存活率均较感染对照组显著下降（$P<0.05$；图3D-F），效应呈浓度依赖性。为排除死亡细菌碎片对流式检测的干扰，进一步开展CFU平板计数，结果与流式检测一致（图3G-H）；GA处理组胞内BCG活菌载量显著低于感染对照组（$P<0.05$），同样存在浓度效应，提示GA可增强巨噬细胞清除胞内活菌的能力。



Effect of GA and INH on the proliferation of Raw264.7 cells. A: MTT assays were used to detect the proliferation ability of Raw264.7 cells with GA treatment; B: MTT assays were used to detect the proliferation ability of Raw264.7 cells with INH treatment. Compared with the ctrl group, $^{*}P<0.05$, $^{***}P<0.001$, n=3.

图2　筛选不同剂量的GA和INH的安全给药浓度

Fig. 2　Identify the safe dosage ranges for different doses of GA and INH



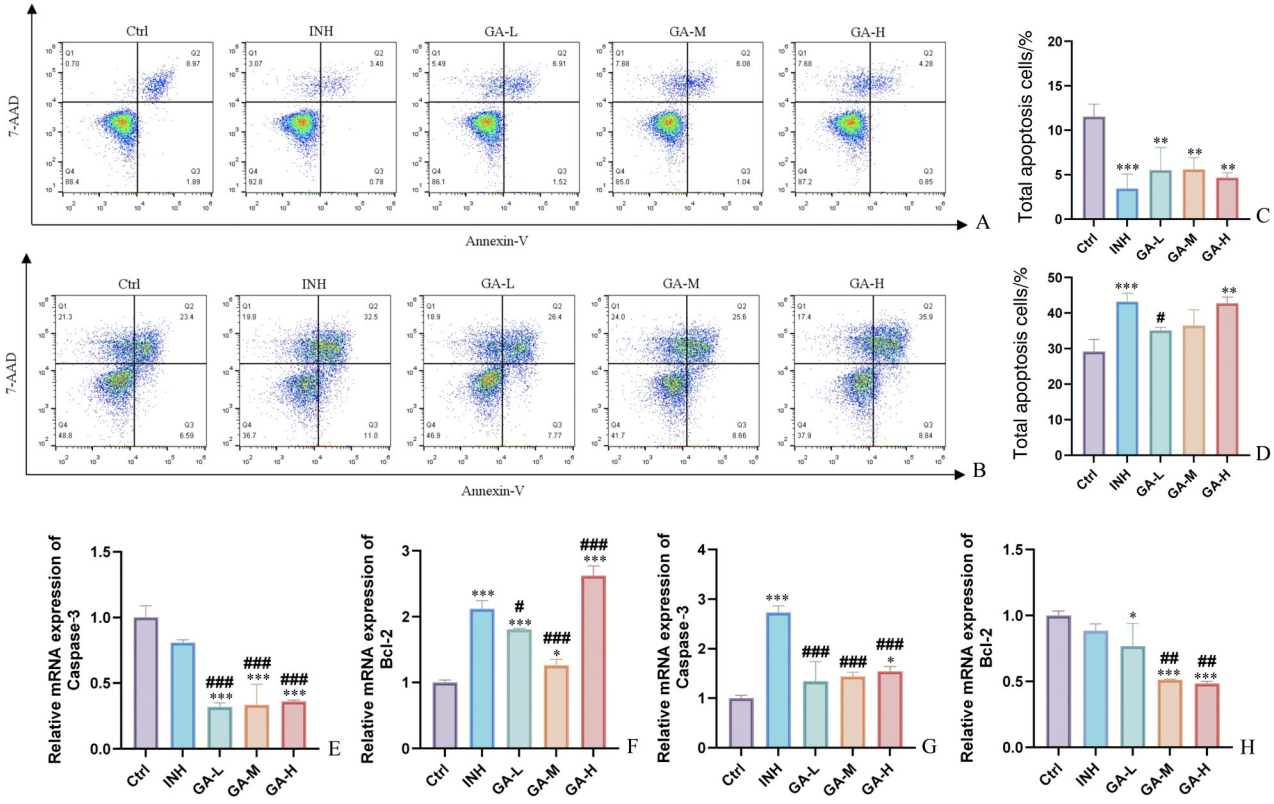
Effect of GA on intracellular bacterial survival in BCG-infected macrophages. A-C: The impact of varying durations of GA pretreatment on the survival of intracellular bacteria in BCG-infected macrophages is illustrated; D-F: Effect of different concentrations of GA on intracellular bacterial survival in BCG-infected macrophages detected by flow cytometry; A, D: Representative results of intracellular bacterial survival of BCG-infected macrophages detected by flow cytometry, the GFP positive peak is shown in the figure; B, E: Statistical results of intracellular bacterial survival of macrophages after 4 h of BCG infection (F=37.17, P<0.001); C, F: Statistical results of intracellular bacterial survival of macrophages after 24 h of BCG infection (F=748.0, P<0.001); G: statistical results of CFU of intracellular bacteria after 4 h of BCG infection (F=7.195, P=0.005); H: statistical results of CFU of intracellular bacteria after 24 h of BCG infection (F=59.14, P<0.001). 12 h, 24 h and 36 h represent the duration of GA pretreatment on macrophages; 4 h and 24 h represent the incubation time after BCG infection. Compared with the Ctrl group, $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001; Compared with the INH group, $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, n=3.

图 3 GA抑制BCG感染巨噬细胞胞内细菌生存率

Fig. 3 GA inhibits the survival of intracellular bacteria in BCG-infected macrophages

2.4 没食子酸对BCG感染巨噬细胞凋亡的影响

GA发挥胞内抑菌效应的作用浓度远低于其BCG的MIC,提示GA主要通过调控巨噬细胞宿主功能清除胞内细菌,而非直接杀菌。本研究进一步检测GA对感染巨噬细胞凋亡、极化、活性氧(reactive oxygen species,ROS)、NO及细胞因子的影响,解析其作用机制。流式细胞术检测巨噬细胞凋亡结果显示(图4A-D):感染4 h,GA、INH组巨噬细胞凋亡率较Ctrl组显著降低(P<0.05);感染24 h,各给药组凋亡率显著升高(P<0.05),GA各组凋亡率低于INH组,且随GA浓度升高凋亡率逐步接近INH组。提示感染早期GA可抑制凋亡以维持巨噬细胞存活,感染后期则促进凋亡以利于清除胞内病原体。qRT-PCR检测凋亡相关基因*Bcl-2*、*Caspase-3* mRNA表达(图4E-H):感染4 h,与Ctrl组相比,GA组促凋亡基因*Caspase-3* mRNA表达显著下调(P<0.05),抗凋亡基因*Bcl-2* mRNA表达上调;感染24 h,GA组*Bcl-2* mRNA表达降低(P<0.05),*Caspase-3* mRNA表达显著升高(P<0.05),与流式凋亡检测结果一致,说明GA可通过动态调控Bcl-2/Caspase-3表达,分阶段调节巨噬细胞功能状态。



Effect of GA on apoptosis in BCG-infected macrophages. A: Representative results of flow cytometric detection of macrophage apoptosis after 4 h of BCG infection (F=11.33, P<0.001); B: Representative results of flow cytometric detection of macrophage apoptosis after 24 h of BCG infection(F=7.679, P=0.004); C: Statistics of total apoptosis rate of cells in A; D: Statistics of total apoptosis rate of cells in B; E-F: Relative expression levels of Caspase-3 (F=46.30, P<0.001) and Bcl-2(F=140.5, P<0.001) mRNA after 4 h of BCG infection; G-H: Relative expression levels of Caspase-3 (F=33.97, P<0.001) and Bcl-2 (F=22.93, P<0.001) mRNA after 24 h of BCG infection. Compared with the Ctrl group, *P<0.05, **P<0.01, ***P<0.001; Compared with the INH group, $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, n=3.

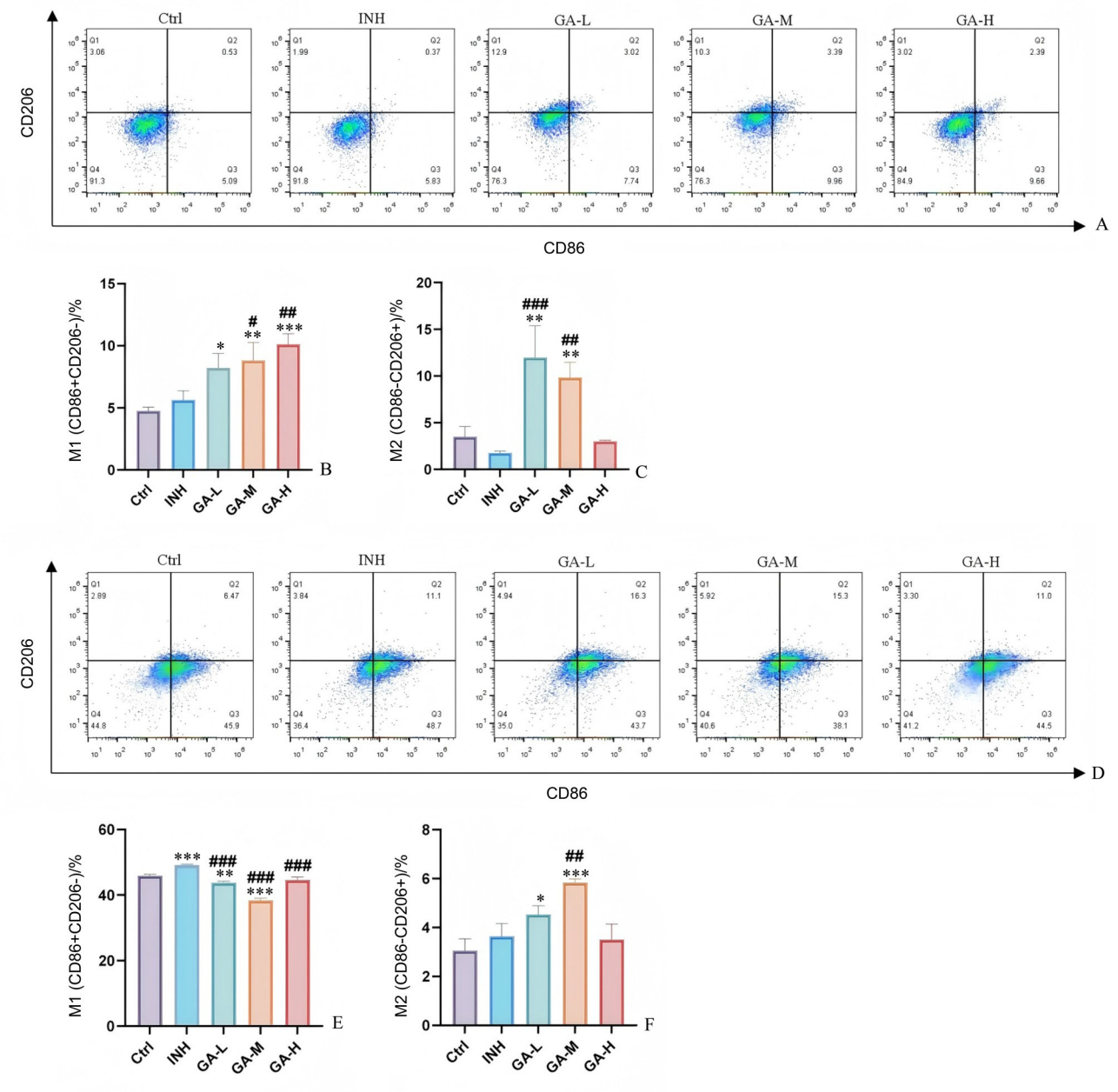
图4　GA抑制BCG感染巨噬细胞凋亡

Fig. 4　GA inhibits apoptosis in macrophages infected with BCG

2.5　没食子酸对BCG感染巨噬细胞极化的影响

为探究GA对BCG感染巨噬细胞极化表型的调控作用，本研究选取膜表面标志物CD_{86}、CD_{206}分别表征巨噬细胞M1、M2极化状态。其中，iNOS作为胞内标志物无法与GFP标记的BCG共定位检测，且BCG刺激后CD_{86}表达上调较CD_{80}更为稳定，因此选用CD_{86}评价M1型极化；CD_{206}为分枝杆菌感染模型中经典的M2型膜标志物。鉴于巨噬细胞M1-M2极化呈连续动态变化，仅依靠表面标志物存在一定局限性，后续可联合iNOS、Arg-1等胞内指标进一步完善验证。本研究通过流式细胞术检测BCG感染4 h、24 h巨噬细胞CD_{86}与CD_{206}的表达水平，并统计分析各组细胞极化差异。

研究结果显示（图5A-C），与Ctrl组相比，在BCG感染4 h时，GA处理组及INH组能够显著促进巨噬细胞向M1型极化（P<0.05），呈现浓度依赖效应；GA低剂量组和中剂量组较感染对照组M2型显著增加（P<0.05），但随GA浓度的增加，M2型巨噬细胞呈减少趋势。在BCG感染24 h时，GA则表现出与感染4 h时不同的调控作用（图5D-F），与Ctrl组相比，在BCG感染24 h时，GA处理组呈现显著的M1型极化抑制（P<0.05），同时GA低剂量组和中剂量组M2型比例显著增加（P<0.05），形成由促炎向促修复表型转换的特征。然而有趣的是GA高剂量组M2型巨噬细胞虽较Ctrl组增加，但无显著差异，其原因有待进行研究阐明。综上所述，GA在BCG感染早期和晚期均能影响巨噬细胞的极化，通过调控巨噬细胞在BCG感染不同阶段的免疫功能，有助于维持机体免疫平衡，进一步协同促进胞内BCG细菌的清除，显著发挥巨噬细胞抗分枝杆菌的作用。



Effect of GA on polarization in BCG-infected macrophages. A: Representative results of M1 and M2 polarization of macrophages after 4 h of BCG infection detected by flow cytometry; B: Statistical results of M1 polarization levels after 4 h of BCG infection (F=15.56, P<0.001); C: Statistical results of M2 polarization levels after 4 h of BCG infection (F=20.20, P<0.001); D: Representative results of M1 and M2 polarization of macrophages after 24 h of BCG infection detected by flow cytometry; E: Statistical results of M1 polarization levels after 24 h of BCG infection (F=130.2, P<0.001); F: Statistical results of M2 polarization levels after 24 h of BCG infection (F=17.19, P<0.001). Compared with the Ctrl group, *P<0.05, **P<0.01, ***P<0.001; Compared with the INH group, $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, n=3.

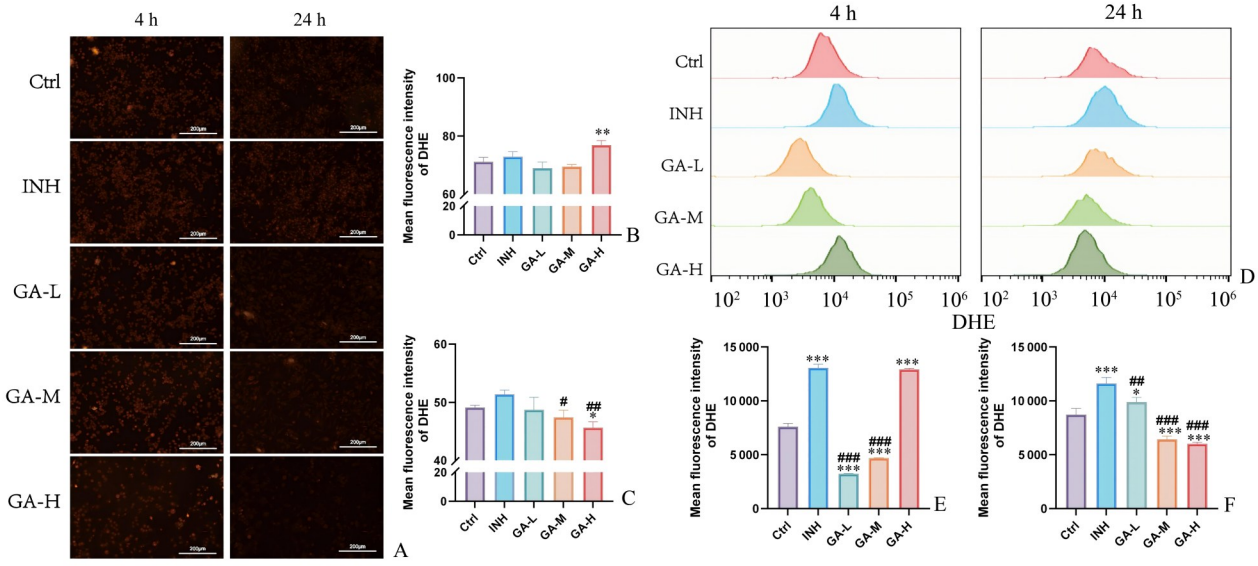
图5 GA影响BCG感染巨噬细胞极化

Fig. 5 GA influences the polarization of BCG-infected macrophages

2.6 没食子酸对BCG感染巨噬细胞中ROS含量的影响

本研究检测评估GA对BCG感染的巨噬细胞胞内ROS动态变化。首先通过DHE荧光探针标记进行显微成像分析(图6A-C),在BCG感染早期,低、中剂量GA处理组巨噬细胞荧光强度较Ctrl组呈现弱阳性信号,提示其具有潜在抗氧化趋势;而GA高剂量组DHE荧光强度明显增加(P<0.05),表明ROS生成增强。至感染后期,各剂量GA组荧光信号显著减弱,呈现剂量依赖性降低特征。为进一

步验证巨噬细胞胞内ROS的水平，用流式细胞术进行定量分析（图6D-E）。感染4 h时，GA低、中剂量组的ROS水平较对照组显著降低（$P<0.05$），而异烟肼组和GA高剂量组的DHE荧光强度均显著升高（$P<0.05$）；感染24 h后，GA处理组ROS水平呈现明显剂量效应关系的下降，GA中、高剂量组较对照组荧光强度显著下降（$P<0.05$）；异烟肼组仍维持显著升高趋势（$P<0.05$）。流式细胞术定量结果与荧光显微镜成像结果趋势相一致，高浓度GA在感染早期可增强ROS生成，而中低浓度则降低ROS水平，后期通过有效清除过量ROS减轻氧化损伤。



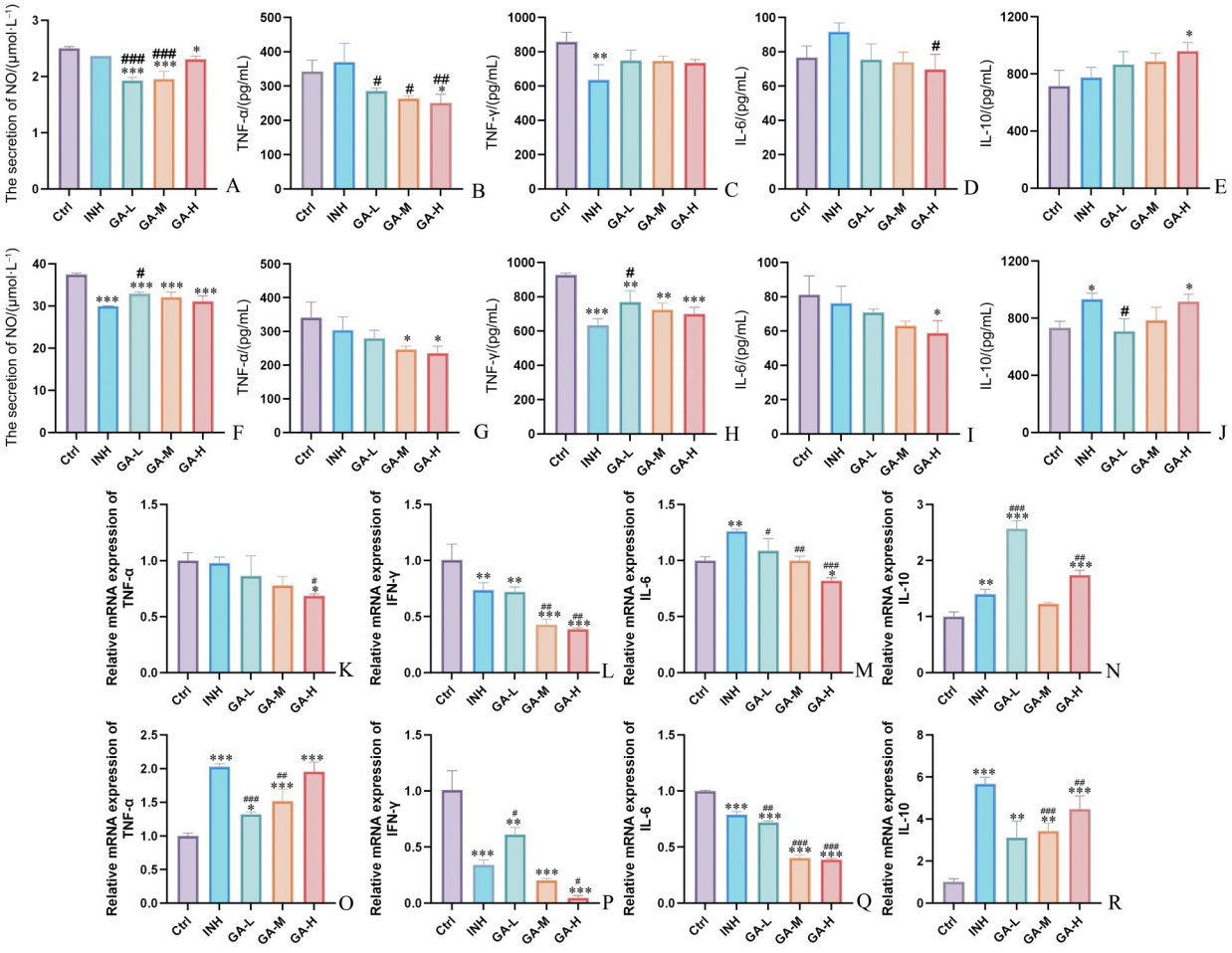
Effect of GA on ROS levels in BCG-infected macrophages. A: DHE fluorescent probe staining for ROS content in cells detected by fluorescence microscopy imaging; B: statistical results of mean fluorescence intensity the ROS level of macrophages after 4 h of BCG infection(F=1 512, P<0.001); C: statistical results of mean fluorescence intensity the ROS level of macrophages after 24 h of BCG infection (F=92.47, P<0.001); D: DHE fluorescent probe staining was used to detect the ROS content in cells by flow cytometry; E: statistical results of mean fluorescence intensity the ROS level of macrophages after 4 h of BCG infection (F=12.89, P<0.001); F: statistical results of mean fluorescence intensity the ROS level of macrophages after 24 h of BCG infection (F=8.984, P=0.002). Compared with the Ctrl group, *P<0.05, **P<0.01, ***P<0.001; Compared with the INH group, $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, n=3.

图6　GA对BCG感染的巨噬细胞胞内活性氧动态变化

Fig. 6　Dynamic changes of intracellular reactive oxygen species in BCG infected macrophages by GA

2.7　没食子酸对BCG感染巨噬细胞NO分泌和细胞因子分泌的影响

为探究GA的免疫调控效应，检测巨噬细胞上清NO、细胞因子水平，并采用qRT-PCR检测细胞因子mRNA表达（图7A-F）。与Ctrl组相比，各GA处理组NO分泌水平显著下降；感染4 h即可抑制NO释放（$P<0.05$），24 h仍维持抑制效应，且抑制效果随GA浓度升高而增强。ELISA结果显示，BCG感染4 h时，GA可显著下调促炎因子TNF-α、IL-6分泌，上调抗炎因子IL-10（$P<0.05$），减轻过度炎症损伤；GA对IFN-γ的抑制效应弱于异烟肼组，可部分保留IFN-γ介导的抗菌活性。如图7G-J所示，感染24 h，与Ctrl组相比，GA组持续抑制TNF-α、IL-6分泌，抑制效应呈浓度依赖性（$P<0.05$）；给药组细胞培养上清中IL-10水平较Ctrl组均升高，其中高剂量组差异有统计学意义（$P<0.05$）。24 h时GA低剂量组对IFN-γ的抑制作用增强，与INH组比较差异显著（$P<0.05$），相关机制有待进一步研究。qRT-PCR检测结果与蛋白分泌趋势基本一致：感染4 h，GA组*TNF-α*、*IFN-γ*、*IL-6* mRNA表达下调，*IL-10* mRNA表达上调，与ELISA结果相吻合。感染24 h，可能受细胞凋亡影响，上清*TNF-α*的mRNA表达高于Ctrl组、低于INH组（$P<0.05$），提示GA对TNF-α的抑制主要发生于转录水平。



Effect of GA on NO secretion and cytokine TNF-α, IFN-γ, IL-6 and IL-10 secretion in BCG-infected macrophages. A-E: Secreted by macrophages after 4 h of BCG infection (TNF-α: F=8.144, P=0.003; IL-10: F=4.390, P=0.03); F-J: Secreted by macrophages after 24 h of BCG infection (TNF-α: F=5.959, P=0.01; IL-10: F=7.265, P=0.005); K-R: Represent the expression levels of TNF-α, IFN-γ, IL-6, and IL-10 mRNA in macrophages detected by qRT-PCR; K-N: Show the results of macrophages after 4 h of BCG infection (TNF-α: F=5.759, P=0.01; IL-10: F=135.3, P<0.001); O-R: Show the results of macrophages after 24 h of BCG infection (TNF-α: F=50.79, P<0.001; IL-10: F=35.31, P<0.001). Compared with the Ctrl group, *P<0.05, **P<0.01, ***P<0.001; Compared with the INH group, $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, n=3.

图7 GA对BCG感染巨噬细胞NO分泌和细胞因子分泌的影响

Fig. 7 The effects of GA on NO and cytokine secretion in macrophages infected with BCG

3 讨 论

3.1 主要研究发现

本研究探究了GA干预下巨噬细胞清除胞内BCG的杀菌效应。当结核分枝杆菌侵入人体后,主要驻留于肺部,并被肺泡巨噬细胞等免疫细胞吞噬,继而导致免疫细胞活化,并触发机体的免疫调控作用[35]。然而,Mtb利用其特有的生物特性介导巨噬细胞内的免疫逃逸,在宿主体内增殖并长期潜伏,增加了治疗难度和复发风险[36]。已有多项体外基础研究证实,GA具有抗结核分枝杆菌的生物活性,中草药来源的GA对结核分枝杆菌存在抑菌、杀菌效应,并且具有浓度依赖性,可与一线药物异烟肼发挥协同作用,但相关作用机制仍有待阐明[37-39]。GA对多种微生物具备广谱抑菌活性:针对多重耐药大肠杆菌,GA下调*acrA*、*acrB*、*tolC*等外排泵基因表达,抑制细菌生物膜形成,提升感染动物模型存活率[40];对于金黄色葡萄球菌,GA结合RecA蛋白干扰复合物组装,增强喹诺酮类药物杀菌效果并降低耐药风险[41];针对耐甲氧西林金黄色葡萄球菌,GA下调*sarA*基因,抑制多糖胞间黏附素

合成，破坏生物膜结构，削弱细菌侵袭能力[42]。本研究发现GA可抑制BCG生长及其对巨噬细胞的侵袭能力。但本研究测得GA针对BCG的MIC高于文献中结核分枝杆菌报道值[23]，考虑与BCG为减毒牛分枝杆菌，且本实验菌株携带EGFP质粒及潮霉素抗性相关[43]。巨噬细胞是抗分枝杆菌感染的核心宿主细胞，本研究证实GA能够有效降低巨噬细胞胞内BCG载量，提示GA可通过调控巨噬细胞抗菌功能，抑制胞内分枝杆菌存活。

3.2　GA调控巨噬细胞凋亡的分子机制

巨噬细胞凋亡在限制分枝杆菌感染中发挥关键作用，结核分枝杆菌可通过抑制巨噬细胞凋亡实现免疫逃逸[44-45]，能否诱导感染巨噬细胞发生程序性死亡是评价抗结核宿主导向策略的重要指标。已有研究报道，NK细胞来源外泌体miR-1249-3P可下调SKI家族转录辅抑制因子-1，诱导感染巨噬细胞凋亡以抑制分枝杆菌胞内增殖[46]；GA可经由线粒体通路诱导肿瘤细胞凋亡，涉及细胞色素C释放与Caspase-9/3活化[47]。结合本研究4 h与24 h凋亡、氧化应激相关基因检测结果，可对GA双向调控凋亡的转换机制做出阐释：感染早期BCG触发氧化应激爆发，GA可能激活Nrf2通路清除过量ROS，维持线粒体膜完整性，上调Bcl-2、抑制Caspase-3表达，减少巨噬细胞急性凋亡损伤，保留吞噬杀菌功能；感染至24 h，胞内BCG大量增殖并释放毒力因子，线粒体氧化损伤持续累积，Nrf2抗氧化代偿达到阈值，氧化还原稳态失衡成为凋亡调控发生转换的关键节点。此时GA不再抑制凋亡，适度上调Caspase-3，诱导高载菌巨噬细胞程序性凋亡，避免细胞坏死造成的细菌扩散，促进胞内BCG清除。由此可见，GA对巨噬细胞凋亡的双向调控是其介导宿主免疫保护的重要途径。

3.3　GA调控巨噬细胞极化、氧化应激与炎症应答

巨噬细胞具有高度可塑性，M1主要介导炎症应答与病原体清除，M2型参与抗炎修复，二者动态平衡决定感染转归[48-49]。值得注意的是，抗结核药物治疗可促进M2型极化，有助于缓解过度炎症并改善免疫功能[50-51]。研究显示，GA可经p38MAPK/STAT6信号通路以浓度依赖方式促进M2型极化，上调IL-10、Arg1和TGF-β1等抗炎介质表达，并抑制M1型极化，从而在炎症模型中发挥抗炎作用[52-53]。本研究结果显示，感染早期GA促进M1极化以强化巨噬细胞杀菌能力；感染24 h后GA推动细胞向M2方向极化，但高浓度组M2极化未见统计学差异，提示GA对M2极化的诱导作用有限，不存在严格浓度依赖关系。该动态调控模式有利于平衡抗菌免疫与组织修复，协同清除胞内BCG。研究表明，在Toll样受体（toll-like receptor，TLR）敲除模型中，耻垢分枝杆菌通过招募MyD88并沉默MxA，激活NF-κB信号，促进TNF-α和IL-6表达，从而增强免疫控制能力[54-55]。研究结果显示，GA能够降低BCG感染巨噬细胞胞内ROS水平，减少NO分泌，抑制BCG诱导的过度炎症反应。作为多酚类物质，GA的抗炎抗氧化特性已得到证实，其通常通过抑制MAPK、NF-κB通路减少炎症介质释放[56-58]。但本研究仅检测下游细胞因子水平，未检测p-NF-κB、p-p38、p-JNK、p-ERK等磷酸化蛋白，缺少通路活化的直接证据，GA调控BCG感染巨噬细胞的上游通路机制尚属于基于表型的推论，尚未形成完整证据链。后续可结合通路磷酸化蛋白检测、抑制剂干预实验进一步验证。已有文献同样佐证GA可抑制多种感染模型的炎症因子与趋化因子分泌，参与TLR4/MyD88/TRIF等通路调控，发挥免疫调节效应[59-61]。提示GA可通过抑制感染巨噬细胞过度炎症应答，减轻结核相关组织损伤，具备宿主导向抗分枝杆菌治疗的潜在价值。

3.4　局限性与展望

本研究证实GA体外对BCG具有抑菌活性，并通过调控巨噬细胞凋亡、极化、氧化应激及炎症应答改善胞内细菌清除效果，缓解感染相关损伤。但本研究仍存在明显局限：本研究仅采用体外永生化巨噬细胞-BCG感染模型，无法复刻体内复杂免疫微环境，细胞系与原代肺泡巨噬细胞生物学表型仍存在差异；体外给药不能模拟GA体内吸收、代谢及组织分布；仅设置4 h、24 h这2个时间点，难以模拟结核慢性感染进程；同时BCG毒力弱于致病性Mtb。因此本研究结论尚需动物体内感染模型进一步验证。本研究初步系统阐释藏药活性成分GA抗BCG感染的多维度宿主免疫调节作用，为GA开发作为抗结核候选治疗药物提供体外实验依据，也为民族药用植物资源开发利用提供参考。

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