·基础研究·

京尼平对脂多糖介导的血管通透性增加的影响及其可能机制

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【摘 要】 **目的** 探讨京尼平对脂多糖(LPS)介导的血管通透性增加的影响及可能机制。**方法** 体外实验:将人脐静脉内皮细胞(HUVECs)分为4组,对照组仅接受0.1%的二甲基亚砜(DMSO)处理24.5 h(DMSO);模型组接受同等浓度DMSO预处理30 min后与500 ng/ml LPS共孵育24 h(DMSO+LPS);治疗组接受京尼平50 μmol预处理30 min后与500 ng/ml LPS共孵育(京尼平+LPS);抑制剂组接受10 μmol EX527及京尼平50 μmol预处理30 min后与500 ng/ml LPS共孵育(京尼平+EX527+LPS)。检测各组细胞内皮通透性Pa值(Transwell法)、天冬氨酸特异性半胱氨酸蛋白酶3(caspase-3)活性(酶标仪检测荧光强度)、Sirtuin-1蛋白(SIRT1)表达(Western blotting)、线粒体膜电位变化(JC-1探针法)及细胞凋亡情况(TUNEL染色法)。体内实验:另将雌性大鼠24只按随机数表法分为4组,对照组大鼠注射0.5 ml DMSO 30 min后注射生理盐水0.5 ml(DMSO+生理盐水);模型组大鼠注射0.5 ml DMSO 30 min后注射LPS 10 mg/kg(DMSO+LPS);治疗组大鼠注射京尼平5 mg/kg 30 min后注射LPS 10 mg/kg(京尼平+LPS);抑制剂组大鼠接受5 mg/kg京尼平及5 mg/kg EX527 30 min后注射LPS 10 mg/kg(京尼平+EX527+LPS)。生理盐水或LPS注射60 min后检测大鼠血管通透性ΔI(FITC-白蛋白荧光法)及SIRT1表达(Western blotting)。应用SPSS 20.0软件进行统计分析。根据数据类型,组间比较采用单因素方差分析、LSD两两比较或Tamhane's *T2*检验;组内比较采用单因素重复测量的方差分析。**结果** 体外实验:与对照组[Pa:1.00±0.04;caspase-3活性:100.0±4.4;JC-1绿色/红色荧光比例:(100.0±4.8)%;细胞凋亡率:(2.3±1.4)%;SIRT1:(100.0±8.9)%]比较,模型组[1.68±0.09、216.0±23.5、(343.0±28.3)%、(40.8±8.9)%、(61.0±8.5)%],治疗组[1.35±0.07、165.0±15.0、(220.0±13.3)%、(28.2±6.6)%、(86.7±7.6)%]及抑制剂组[1.60±0.10、202.0±16.5、(309.0±18.5)%、(38.0±9.5)%、(64.3±4.8)%]细胞Pa值、caspase-3活性、JC-1绿色/红色荧光比例及细胞凋亡率显著增加,SIRT1水平显著降低;与模型组比较,治疗组Pa、caspase-3、绿色/红色荧光比例及细胞凋亡率显著下降,SIRT1表达显著升高;与治疗组比较,抑制剂组Pa、caspase-3、JC-1绿色/红色荧光比例及细胞凋亡率显著增加,SIRT1表达显著降低,差异均有统计学意义($P<0.05$)。体内实验:模型组、治疗组及抑制剂组大鼠在LPS注射30 min及60 min后,与处理前及对照组比较血压明显下降($P<0.05$),但3组大鼠间血压比较差异无统计学意义($P>0.05$)。与对照组[SIRT1:(100.0±4.0)%,ΔI:(0.12±0.03)]比较,模型组[(49.3±8.3)%、0.54±0.07],治疗组[(87.3±4.7)%、0.32±0.05]及抑制剂组[(55.3±4.9)%、0.53±0.06]SIRT1表达量显著降低,ΔI显著增加;与模型组比较,治疗组SIRT1表达显著上升,ΔI显著减弱;与治疗组比较,抑制剂组SIRT1表达显著降低,ΔI显著增强,差异均有统计学意义($P<0.05$)。**结论** 京尼平通过抑制线粒体凋亡信号激活最终抑制LPS介导的血管通透性增加,其作用可能与上调SIRT1有关。

【关键词】 京尼平;脂多糖;血管通透性;线粒体
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Effect of genipin on lipopolysaccharide-mediated vascular hyperpermeability and its underlying mechanism *in vitro* and *in vivo*

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【**Abstract**】 **Objective** To investigate the effect of genipin on lipopolysaccharide (LPS)-mediated hyperpermeability and its possible mechanism. **Methods** *In vitro* experiment: human umbilical vein endothelial cells (HUVECs) were divided into 4 groups, that is, control group (DMSO, 0.1% DMSO for 24.5 h), model group (DMSO+LPS, same concentration of DMSO pretreatment for 0.5 h followed by 500 ng/ml LPS for 24 h), treatment group (genipin+LPS, 50 μmol pretreatment with genipin for 0.5 h followed by same

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LPS treatment), and inhibitor group (genipin + EX527 + LPS, 0.5 h pretreatment of 10 μmol EX527 and 50 μmol genipin and then same dose of LPS). The Pa value (representing endothelial permeability) was tested by Transwell chamber test, activity of cysteine-containing aspartate-specific proteases 3 (caspase-3) was measured by fluorescence intensity with microplate reader, Sirtuin-1 (SIRT1) level was detected by Western blotting, the change of mitochondrial membrane potential was measured by MitoProbe™ JC-1 assay kit, and apoptosis rate was tested by TUNEL. *In vivo* experiment: 24 female rats were randomly divided into 4 groups. The rats of control, model, treatment and inhibitor groups were respectively injected with 0.5 ml DMSO pretreated and 0.5 ml normal saline (DMSO + normal saline), 0.5 ml DMSO pretreatment followed by 10 mg/kg LPS treatment (DMSO + LPS), 5 mg/kg of genipin pretreatment and then LPS treatment (genipin + LPS), and pretreatment of 5 mg/kg genipin and 5 mg/kg EX527 and LPS treatment (genipin + EX527 + LPS). The pretreatment time lasted for 30 min in all the groups. The vascular permeability ΔI was measured by FITC-albumin fluorescence assay, and expression level of SIRT1 was detected with Western blotting in 60 min after LPS or normal saline injection. SPSS statistics 20.0 was used for statistical analysis. According to the data type, single factor analysis of variance, LSD multiple comparison or Tamhane's *T2* test was used for intergroup comparison, and single factor repeated measurement analysis of variance was used for intragroup comparison. **Results** *In vitro* experiment: compared with control group [Pa: 1.00±0.04; caspase-3 activity: 100.0±4.4; JC-1 green/red fluorescence ratio: (100.0±4.8)%; apoptosis rate: (2.3±1.4)%; SIRT1: (100.0±8.9)%], Pa value, caspase-3 activity, JC-1 green/red fluorescence ratio and apoptosis rate were significantly increased in the model group [1.68±0.09, 216.0±23.5, (343.0±28.3)%, (40.8±8.9)%], treatment group [1.35±0.07, 165.0±15.0, (220.0±13.3)%, (28.2±6.6)%] and inhibitor group [1.60±0.10, 202.0±16.5, (309.0±18.5)%, (38.0±9.5)%], and the expression level of SIRT1 level was decreased[(61.0±8.5)%, (86.7±7.6)%, and (64.3±4.8)% respectively]. Compared with the model group, the former indices in treatment group were reduced obviously, while SIRT1 level was increased ($P<0.05$). Compared with the treatment group, the Pa, caspase-3, JC-1 green/red ratio and apoptosis rate were elevated significantly in the inhibitor group, while the expression of SIRT1 was decreased significantly ($P<0.05$). *In vivo* experiment: the blood pressure of rats in the model, treatment and inhibitor groups were decreased significantly ($P<0.05$) after LPS injection for 30 and 60 min when compared with the values before LPS pretreatment and those of the control group, but there was no significant difference in blood pressure among the 3 groups ($P<0.05$). Compared with the control group [SIRT1: (100.0±4.0)%, ΔI: (0.12±0.03)], the expression level of SIRT1 was decreased while ΔI was increased in the model group [(49.3±8.3)%, 0.54±0.07], the treatment group [(87.3±4.7)%, 0.32±0.05], and the inhibitor group [(55.3±4.9)%, (0.53±0.06)]. When compared with the model group, the expression of SIRT1 was increased, and ΔI was decreased significantly in the treatment group, but the level was significantly decreased and ΔI was significantly increased in the inhibitor group. Statistical significances were seen in above indicators ($P<0.05$). **Conclusion** Genipine inhibits LPS-mediated hyperpermeability by inhibiting the activation of mitochondrial apoptotic signal, and this effect may be related to the up-regulation of SIRT1.

【Key words】 genipin; lipopolysaccharide; vascular permeability; mitochondria

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脓毒症是临床常见危重症,而血管通透性增加导致的全身浮肿是脓毒症的主要症状,也是临床工作中亟待解决的问题之一[1]。血管内皮细胞激活是导致血管通透性增加的主要机制[2,3]。研究证实京尼平在调节血管内皮细胞功能、改善心血管疾病方面具有显著作用[4,5],但其对于脓毒症血管通透性增加的作用尚未见报道。本课题组前期证实线粒体凋亡信号通过激活天冬氨酸特异性半胱氨酸蛋白酶 3(cysteine-containing aspartate-specific proteases 3,caspase-3)裂解内皮细胞紧密链接 β-catenin 蛋白,介导内皮通透性增加,而抑制内皮细胞凋亡信号激活可以减轻血管通透性增加[6]。且研究证实沉默信息调节因子 1(silent information regulator 1,SIRT1)参与调节脂多糖介导的内皮屏障功能[7],但机制并未阐述清楚。因此本研究通过体内及体外脂多糖(lipopolysaccharide,LPS)处理模拟脓毒症血管通透性增加,观察京尼平对血管通透性的影响并研究其相关机制。

1 材料与方法

1.1 材料

FITC-白蛋白、京尼平及 EX527 购于美国 Sigma 公司;TUNEL 检测试剂盒、线粒体膜电位 JC-1 试剂盒、caspase-3 活性检测试剂盒及线粒体分离试剂盒购于美国 Biovision 公司;Sirtuin-1 蛋白(SIRT1)单克隆抗体、3-磷酸甘油醛脱氢酶(glyceraldehyde-3-phosphatede hydrogenase,GAPDH)及辣根过氧化物酶(HRP)标记的二抗购于美国 ABclonal 公司。人脐静脉内皮细胞(human umbilical vein endothelial

cells,HUVECs)购于广州赛库生物公司。

1.2 分组及处理

1.2.1 体外实验 参照文献[7],HUVECs分为4组:对照组细胞仅接受0.1%二甲基亚砜(DMSO)处理24.5 h;模型组细胞接受0.1% DMSO预处理30 min后与500 ng/ml LPS共孵育24 h;治疗组细胞接受京尼平50 μmol预处理30 min后同模型组;抑制剂组细胞接受10 μmol EX527及京尼平50 μmol预处理30 min后同模型组。

1.2.2 体内实验 雌性SD大鼠24只,采用随机数表法分为4组($n=6$):对照组大鼠经尾静脉注射0.5 ml DMSO 30 min后注射生理盐水0.5 ml,60 min后进行相关指标检测;模型组大鼠经尾静脉注射0.5 ml DMSO 30 min后注射LPS 10 mg/kg(溶于0.5 ml生理盐水),60 min后进行相关指标检测;治疗组大鼠尾静脉注射京尼平5 mg/kg 30 min后同模型组;抑制剂组大鼠经尾静脉注射EX527 5 mg/kg及京尼平5 mg/kg 30 min后同模型组。

1.3 体外指标检测

1.3.1 内皮细胞通透性检测 参照文献[7],HUVECs接种于24孔Transwell上层小室上,待细胞长至90%融合后,无血清DMEM/F12培养基继续培养12 h,使细胞同步生长并处于静止期。根据实验方案处理,24 h后检测各组荧光A值(测定波长520 nm,激发波长492 nm)。内皮通透性Pa=(各组A值)/(对照组A值)。

1.3.2 caspase-3活性检测 细胞充分裂解后离心(4℃,12 000转/min,20 min),取上清。与试剂盒中反应液在37℃下孵育2 h,酶标仪检测荧光强度(波长405 nm),BCA法检测蛋白浓度,以荧光强度与蛋白浓度比值表示caspase-3活性,并以对照组进行标准化。

1.3.3 SIRT1含量测定 蛋白质免疫印迹试验(Western blotting)检测SIRT1含量。将细胞充分裂解后,4℃,12 000转/min,20 min,取上清,BCA法检测蛋白浓度。凝胶电泳后转膜,免疫化学发光法曝光并进行灰度值分析。以目的蛋白与内参GAPDH的灰度值比值表示目的蛋白的相对表达量,并以对照组进行标准化。

1.3.4 线粒体膜电位测定 处理后的HUVECs经PBS洗后重悬,与JC-1在37℃孵育15 min后酶标仪检测。荧光探针JC-1的工作浓度为5 μmol/L。共聚焦显微镜下观察荧光变化,计算绿色荧光与红色荧光的比值,并以对照组进行标准化。该比值用来衡量线粒体去极化的程度:比例越高表明线粒体去极化增加,膜电位下降;反之则去极化下降,膜电位上升。

1.3.5 细胞凋亡测定 采用TUNEL法检测细胞凋亡,处理后的HUVECs经PBS洗后重悬,根据说明书步骤进行处理,hoechest标记细胞核,荧光显微镜下观察。

1.4 体内指标检测

1.4.1 血管通透性检测 参照文献[6],大鼠经苯巴比妥30 mg/kg肌注麻醉后正中剖腹,将一小段空肠缓慢拉出并寻找合适观察的肠系膜静脉,Kreb液持续缓慢滴注维持血管内环境稳定,予FITC-白蛋白50 mg/kg注射,并持续性滴注FITC-白蛋白0.15 mg/(kg·min)维持荧光稳定,荧光显微镜下连续观察60 min内血管FITC-白蛋白渗出情况,并分别测定血管内的荧光密度值(Ii)和血管外渗出的荧光密度值(Io),血管通透性由血管内外荧光强度变化(ΔI)决定,ΔI=Io/Ii,其ΔI值越大表示通透性越高。另外,大鼠麻醉后,一侧股动脉置入PE50管,并连接于PowerLab八通道生理记录仪连续记录血压。

1.4.2 Western blotting检测组织SIRT1蛋白表达 肠系膜血管组织充分裂解,4℃,12 000转/min,20 min,取上清,BCA法检测蛋白浓度。凝胶电泳后转膜,免疫化学发光法曝光并进行灰度值分析。以目的蛋白与内参GAPDH的灰度值比值表示目的蛋白的相对表达量,并以对照组进行标准化。

1.5 统计学处理

应用SPSS 20.0软件进行统计分析。计量资料方差齐时以均数±标准差($\bar{x}\pm s$)表示,组间比较使用单因素方差分析(one-way ANOVA)和LSD两两比较;方差不齐时使用Tamhane's $T2$检验。组内血压比较采用单因素重复测量的方差分析。$P<0.05$为差异有统计学意义。

2 结 果

2.1 京尼平对内皮细胞通透性及caspase-3的影响

与对照组比较,模型组、治疗组及抑制剂组细胞Pa值及caspase-3显著增加。与模型组比较,治疗组Pa及caspase-3显著下降。与治疗组比较,抑制剂组Pa及caspase-3显著增加,差异均有统计学意义($P<0.05$;表1)。

2.2 京尼平对内皮细胞SIRT1表达的影响

与对照组(100.0±8.9)%比较,模型组[(61.0±8.5)%]、治疗组[(86.7±7.6)%]及抑制剂组[(64.3±4.8)%]的SIRT1表达水平显著降低;与模型组比较,治疗组SIRT1表达显著升高;与治疗组比较,抑制剂组SIRT1表达显著下降,差异均有统计学意义($P<0.05$;图1)。

表 1 4 组细胞内皮通透性及 caspase-3 活性比较

Table 1 Comparison of endothelial permeability and caspase-3 activity among four groups ($\bar{x}\pm s$)

Item	Control group	LPS group	Treatment group	Inhibitor group
Pa	1.00±0.04	1.68±0.09*	1.35±0.07*#	1.60±0.10*△
caspase-3 activity(%)	100.0±4.4	216.0±23.5*	165.0±15.0*#	202.0±16.5*△

LPS: lipopolysaccharide; caspase-3: cysteine-containing aspartate-specific proteases 3. Compared with control group, *P<0.05; compared with LPS group, #P<0.05; compared with treatment group, △P<0.05.

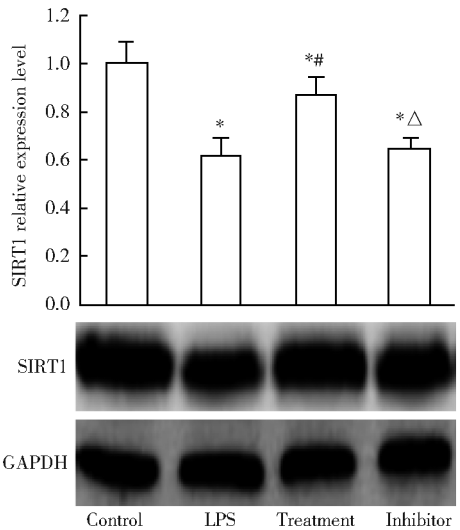

图 1 4 组细胞 SIRT1 表达比较

Figure 1 Comparison of SIRT1 expression among four groups

SIRT1: silent information regulator 1; LPS: lipopolysaccharide; GAPDH: glyceraldehyde-3-phosphatede hydrogenase. Compared with control group, *P<0.05; compared with LPS group, #P<0.05; compared with treatment group, △P<0.05.

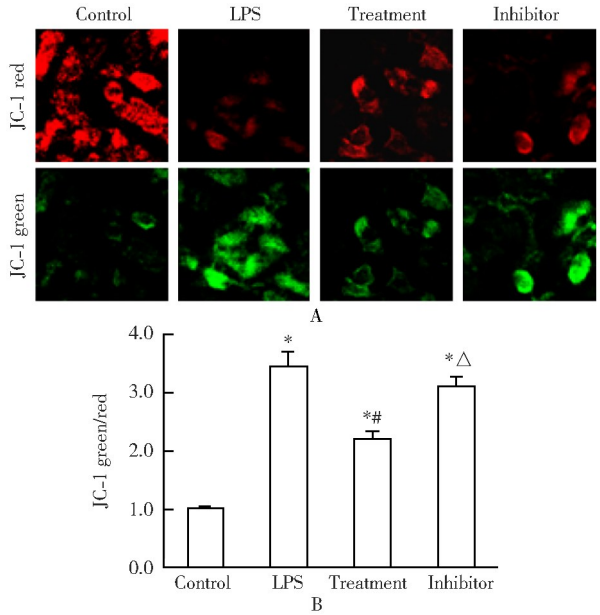

图 2 4 组细胞线粒体膜电位的比较

Figure 2 Comparison of mitochondrial membrane potential among four groups

A: fluorescent staining(×600); B: quantitative analysis results. LPS: lipopolysaccharide. Compared with control group, *P<0.05; compared with LPS group, #P<0.05; compared with treatment group, △P<0.05.

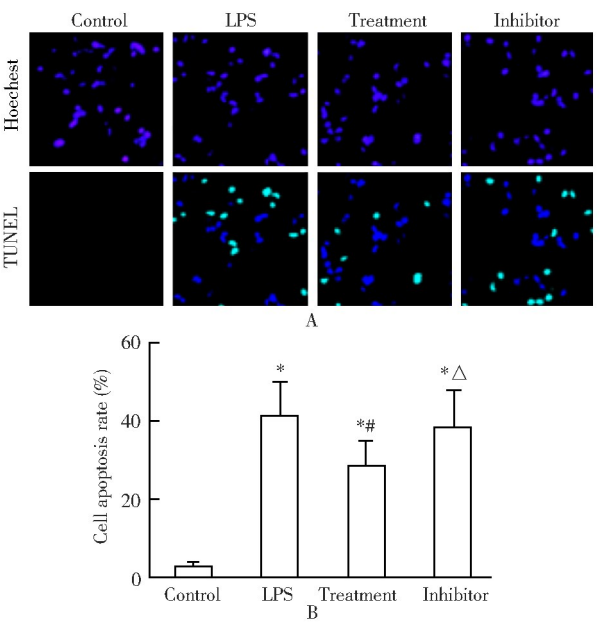

图 3 4 组细胞凋亡情况比较

Figure 3 Comparison of apoptosis among four groups

A: TUNEL staining (×100); B: quantitative analysis results. LPS: lipopolysaccharide. Compared with control group, *P<0.05; compared with LPS group, #P<0.05; compared with treatment group, △P<0.05.

2.3 京尼平对内皮细胞线粒体膜电位的影响

与对照组[(100.0±4.8)%]比较,模型组[(343.0±28.3)%]、治疗组[(220.0±13.3)%]及抑制剂组[(309.0±18.5)%]的 JC-1 绿色/红色荧光比例显著增加;与模型组比较,治疗组绿色/红色荧光比值显著下降;与治疗组比较,抑制剂组 JC-1 绿色/红色荧光比例显著增加,差异均有统计学意义(P<0.05;图 2)。

2.4 京尼平对内皮细胞凋亡的影响

与对照组[(2.3±1.4)%]比较,模型组[(40.8±8.9)%]、治疗组[(28.2±6.6)%]及抑制剂组[(38.0±9.5)%]细胞凋亡率显著增加;与模型组比较,治疗组细胞凋亡率显著下降;与治疗组比较,抑制剂组细胞凋亡显著上升,差异有统计学意义(P<0.05;图 3)。

2.5 大鼠血压变化

给药前各组大鼠血压差异无统计学意义(P>0.05);注射后 30 min,各组大鼠血压较给药前差异无统计学意义(P>0.05);模型组、治疗组及抑

制剂组大鼠在LPS注射30 min及60 min后,与处理前及对照组比较血压明显下降($P<0.05$),但3组大鼠间血压比较差异无统计学意义($P>0.05$;表2)。

表2 各组大鼠不同时间点血压比较

Table 2 Blood pressure at different time points in four groups of rats ($n=6$, $\bar{x}\pm s$, mmHg)

Group	Pre-injection	30 min after injection	60 min after injection	90 min after injection
Control	112±13	111±10	110±9	108±7
LPS	116±12	114±12	92±8*#	92±8*#
Treatment	113±14	118±12	91±5*#	92±7*#
Inhibitor	120±11	118±10	90±6*#	91±5*#

LPS: lipopolysaccharide. 1 mmHg=0.133 kPa. Compared with control group, *$P<0.05$; compared with pre-injection, #$P<0.05$.

2.6 京尼平对肠系膜血管组织中SIRT1表达的影响

与对照组[(100.0±4.0)%]比较,模型组[(49.3±8.3)%]、治疗组[(87.3±4.7)%]及抑制剂组[(55.3±4.9)%]SIRT1表达量显著降低;与模型组比较,治疗组SIRT1表达显著上升;与治疗组比较,抑制剂组SIRT1表达显著降低,差异均有统计学意义($P<0.05$;图4)。

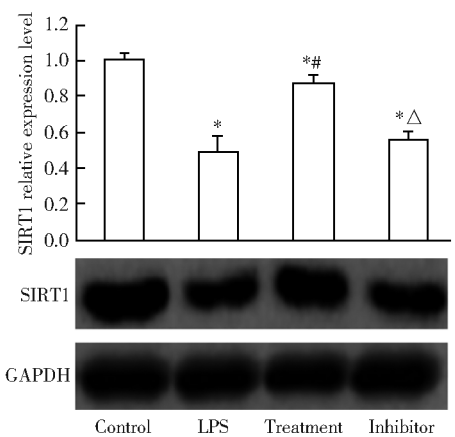

图4 4组大鼠肠系膜血管组织SIRT1表达

Figure 4 Comparison of SIRT1 expression in mesenteric vascular tissue among four groups of rats

SIRT1: silent information regulator 1; GAPDH: glyceraldehyde-3-phosphatede hydrogenase; LPS: lipopolysaccharide. Compared with control group, *$P<0.05$; compared with LPS group, #$P<0.05$; compared with treatment group, △$P<0.05$.

2.7 京尼平对血管通透性的影响

与对照组(0.12±0.03)比较,模型组(0.54±0.07)、治疗组(0.32±0.05)及抑制剂组(0.53±0.06)ΔI显著增加;与模型组比较,治疗组大鼠ΔI显著减弱;与治疗组比较,抑制剂组大鼠ΔI显著增强,差异均有统计学意义($P<0.05$;图5)。

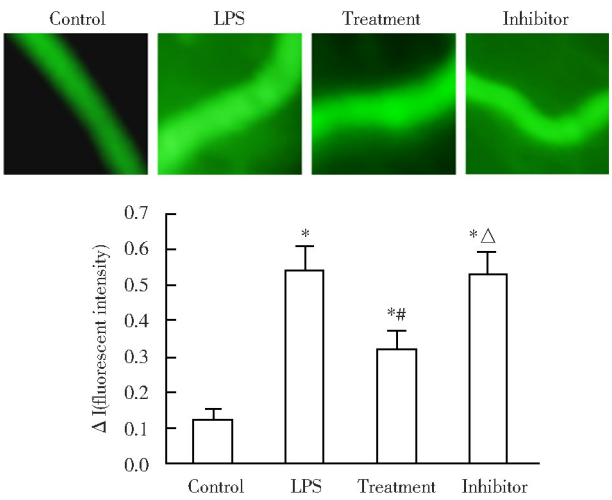

图5 4组大鼠血管通透性的比较

Figure 5 Comparison of vascular permeability among four groups of rats

LPS: lipopolysaccharide. A: fluorescent staining (×100); B: quantitative analysis results. Compared with control group, *$P<0.05$; compared with LPS group, #$P<0.05$; compared with treatment group, △$P<0.05$.

3 讨 论

脓毒症是感染导致的全身炎症反应综合征,是临床常见的危重症[8]。LPS是格兰阴性细菌细胞壁的成分之一,由于格兰阴性细菌在脓毒症致病菌中的重要地位,许多研究将LPS应用在脓毒症的模型中[9,10]。本研究中,我们在细胞水平发现经LPS刺激,HUVECs内皮通透性显著增加;在动物水平,通过在荧光显微镜下观察肠系膜血管白蛋白的渗出来检测血管通透性发现,注射LPS后,大鼠血管内的荧光白蛋白向外渗出明显,提示血管通透性增加。以往研究证实,京尼平可以显著改善血管内皮细胞功能障碍,在心血管疾病治疗方面有一定的疗效[4,5],但是其对于血管通透性是否有影响却少见报到。我们通过研究发现,京尼平可以显著减少LPS介导的内皮细胞及血管通透性增加。

SIRT1是沉默信息调节因子2相关酶家族中的一员,参与线粒体代谢及生成,而其在调节血管内皮细胞方面具有重要作用[11,12]。研究证实,SIRT1激活剂SRT1720可以显著抑制LPS介导的血管通透性增加[7,13],那么京尼平是否通过激活SIRT1发挥其对血管通透性的保护作用呢?我们进一步研究发现,LPS导致内皮细胞及血管组织中SIRT1的表达显著下降,但京尼平可以显著提高内皮细胞及血管组织中SIRT1的表达,而SIRT1抑制剂EX527则可

以显著抑制京尼平对 SIRT1 激活。另外我们还发现,EX527 可以抑制京尼平对内皮及血管通透性的保护效应,提示京尼平可能通过 SIRT1 发挥作用。

本课题以往研究证实,线粒体凋亡信号在血管通透性增加中起着重要作用,抑制线粒体凋亡信号可以显著改善血管通透性增加[14]。线粒体通透性转变孔开放后导致膜电位下降,细胞色素 C 等线粒体小分子蛋白释放进入细胞质,进而激活下游 caspase-3[15]。caspase-3 的众多底物中包括 β-catenin,而 β-catenin 是内皮之间紧密连接的重要组成部分,裂解后可导致内皮-内皮间链接破坏,间隙增大,最终引起血管通透性增加[16]。我们的研究证实,在内皮细胞中 LPS 可导致肠系膜血管线粒体膜电位下降、caspase-3 激活及细胞凋亡,提示线粒体凋亡信号激活。而京尼平可以显著抑制内皮细胞线粒体凋亡信号激活,从而发挥血管通透性的保护作用。加之 EX527 可以抑制京尼平对线粒体凋亡信号的抑制作用,进一步证实京尼平可能通过激活 SIRT1 发挥血管通透性保护作用。

综上,京尼平可能通过抑制线粒体凋亡信号激活,最终抑制 LPS 介导的血管通透性增加,而这些效应可能与激活 SIRT1 有关。下一步的研究,我们将探讨京尼平激活 SIRT1 的机制。

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