·基础研究·

艾塞那肽通过抑制黄嘌呤氧化酶-活性氧类降低内皮细胞缺氧复氧损伤

王红雷,韩延辉,贾静静,来利红,朱继红,董平栓*
(河南科技大学第一附属医院心血管内科,洛阳 471003)

【摘要】 **目的** 探究原代心肌微血管内皮细胞(CMEC)在缺氧复氧损伤(H/R)模型条件下的损伤信号通路,以及艾塞那肽(Exe)的保护机制。**方法** 双酶消化法体外分离培养SD大鼠CMEC,CD31和Ⅷ因子鉴定纯度。建立H/R模型并筛选Exe的最佳干预浓度。Fluo-3AM荧光探针检测H/R条件下胞内Ca^{2+}变化。Western blotting检测黄嘌呤氧化酶(XO)表达量。DCFH-DA标记的活性氧探针检测胞内活性氧类(ROS)水平。共聚焦显微镜观察JC-1染色和细胞色素-C(Cyt-C)释放。予以Exe干预和使用小干扰RNA(siRNA)干扰技术抑制XO的表达,同步观察各指标变化情况。计量资料以均数±标准差($\bar{x} \pm s$)表示,两组间比较采用t检验,多组间比较采用单因素方差分析。**结果** 10 nmol/L为Exe的最佳抗凋亡浓度。H/R导致胞浆内Ca^{2+}荧光强度和Ca^{2+}依赖的XO表达量升高,予以Exe处理可显著降低Ca^{2+}荧光($P<0.05$)。予以Exe处理或使用siRNA干扰技术可显著抑制XO的表达、降低XO-ROS生成、降低H/R损伤造成的线粒体膜电位、减少Cyt-C的释放,并伴随caspase-3和caspase-9活性的降低($P<0.05$)。**结论** H/R通过激活Ca^{2+}-XO-ROS损伤信号通路,导致CMEC的线粒体结构功能障碍,最终诱导细胞凋亡;而予以Exe处理可通过抑制上述信号通路保护CMEC,降低H/R模型引起的细胞凋亡。

【关键词】 黄嘌呤氧化酶;活性氧;细胞凋亡;缺氧复氧损伤;心肌微血管内皮细胞
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Exenatide attenuates hypoxia-reoxygenation injury in endothelial cells *via* inhibiting xanthine oxidase-reactive oxygen species

WANG Hong-Lei, HAN Yan-Hui, JIA Jing-Jing, LAI Li-Hong, ZHU Ji-Hong, DONG Ping-Shuan*
(Department of Cardiology, the First Affiliated Hospital of Henan University of Science and Technology, Luoyang 471003, China)

【**Abstract**】 **Objective** To explore the specific injured signal pathways under the condition of hypoxia/reoxygenation (H/R) injury in primary cardiac microvascular endothelial cells (CMEC), and investigate the protective mechanisms of exenatide. **Methods** Primary CMEC cells were islolated from SD rats with double-enzyme digestion and cultured *in vitro*. Immunocytochemical staining of CD31 antibody and Ⅷ factors were used to identify the purity of the obtained cells. The injury model of H/R was established. MTT assay was used to detect cell viability for the optimal concentrations of exenatide. The changes of intracellular calcium under H/R condition was detected with the aid of Fluo-3AM fluorescence probe. Western blotting was employed to measure the expression of xanthine oxidase (XO). The level of intracellular reactive oxygen species (ROS) was measured by DCFH-DA labeled ROS probe. Confocal microscopy was performed to observe the JC-1 staining and cytochrome C(Cyt-C) release. After XO siRNA and exenatide pre-treatment were applied to intervent the cells, the above indices were studied again. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Student's t test was used for mutual groups comparison, and one-way ANOVA for multiple groups comparison. **Results** The optimal anti-apoptotic concentration of exenatide was 10 nmol/L. H/R injury induced the increases in the intracellular calcium level and calcium-dependent XO expression level, while exenatide pre-treatment could decrease the calcium level ($P<0.05$). Both siRNA and exenatide pre-treatment significantly suppressed the expression of XO, reduced the level of XO-induced ROS, decreased the H/R injury induced nitochrondrial membrane potential, reduced Cyt-C release, and decreased caspase-3 and caspase-9 activities ($P<0.05$). **Conclusion** H/R activates Ca^{2+}-XO-ROS signaling pathway, leads to mitochondrial structural dysfunction, and finally induces cell apoptosis. exenatide pre-treatment can protect the CMEC cells by suppressing the signaling pathway and reduce apoptosis induced by H/R injury.

【**Key words**】 xanthine oxidase; reactive oxygen species; cell apoptosis; hypoxia-reoxygenation injury; cardiac microvascular endothelial cells

Corresponding author: *DONG Ping-Shuan*, *E-mail*: *Dongpingshuan@163.com*

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通信作者: 董平栓, E-mail: Dongpingshuan@163.com

经皮冠状动脉介入治疗(percutaneous coronary intervention,PCI)是目前治疗急性心肌梗死(acute myocardial infarction,MI)的最有效手段[1,2]。然而开通血管后,虽然消除了血管的机械性阻塞,但远端的分支及末端微血管并未得到有效灌注[3],各分支血管供应的心肌组织仍处于缺氧状态,甚至开通血管后,梗死面积进一步扩大,即PCI术后的缺血/再灌注损伤(ischemia/reperfusion injury,IRI)[4]。IRI的发生减少了血运重建带给患者的益处。再灌注阶段活性氧类(reactive oxygen species,ROS)的爆发以及随后发生的细胞凋亡是IRI发生重要因素。冠状动脉分支末梢的心肌微血管内皮细胞(cardiac microvascular endothelial cells,CMEC)是血管与心肌细胞之间的第1道屏障,在IRI发生时最早受到影响[5,6]。因此,在防治PCI术后的二次打击中,降低IRI诱导的CMEC氧化应激损伤起到至关重要的作用。胰高血糖素样肽-1(glucagon-like peptide-1,GLP-1)是由肠道内分泌细胞分泌的肠促胰岛素,可被二肽基肽酶-4(dipeptidyl peptidase-4,DPP-4)迅速降解[7,8]。艾塞那肽(exenatide,Exe)是GLP-1受体激动剂,能更好地耐受DPP-4降解,使低血糖事件发生率降低[9]。此外,Exe还具有减少心肌梗死面积、抑制心肌细胞凋亡等作用[10,11]。但Exe对IRI诱导的CMEC氧化应激损伤的保护机制尚未完全明确。因此,我们通过建立体外缺氧复氧模型(hypoxia/reoxygenation model,H/R),探究氧化应激损伤诱导CMEC凋亡的信号通路及Exe的保护机制。

1 材料与方法

1.1 试剂与仪器

小干扰RNA(small interfering RNA,siRNA)(Santa Cruz,上海),一抗黄嘌呤氧化酶(xanthine oxidase,XO)(1∶1000,Abcam,上海),β-actin(1∶2000,Santa Cruz,上海),Fura-3AM荧光探针试剂盒和DCFH-DA荧光探针试剂盒(Molecular probe,美国),JC-1染色试剂盒(碧云天,上海)。共聚焦显微镜(OLYMPUS DX51,日本),酶标仪(Epoch 2,美国)。

1.2 CMEC的提取及鉴定

在文献基础上进行改良[12]。5只5~6 d的SD大鼠处死后取出左心室,75%乙醇中浸泡15 s,清洗后剪碎,采用Ⅰ型胶原酶和胰蛋白酶37℃水浴消化10 min,离心后重悬于培养液中,培养4 h,去除悬浮未贴壁的细胞。细胞固定封闭后,分别滴加Ⅷ因子多克隆抗体和CD31单克隆抗体(均为1∶100),孵育过夜。倒置荧光显微镜观察,计数3个视野及均数。

1.3 H/R模型的建立以及Exe干预

CMEC缺氧处理4 h,换氧含量正常的复箱放置4 h,建立H/R模型。为了考察Exe对H/R模型的影响,分别5组:正常对照组、H/R模型组、1 nmol/L Exe组、10 nmol/L Exe组和100 nmol/L Exe组。采用完全培养基[20%胎牛血清(fetal bovine serum,FBS)+高糖DMEM)],37℃、5% CO_2条件下放置12 h。

1.4 Ca^{2+}和siRNA干扰技术对H/R模型的影响

1.4.1 Ca^{2+}对XO表达量的影响 采用Western blotting检测XO的表达量。分为5组:正常对照组、H/R模型组、H/R+Exe组、H/R+BAPTA组和H/R+Iono组。其中BAPTA为胞浆Ca^{2+}螯合剂,而Iono为胞浆Ca^{2+}激活剂。

1.4.2 siRNA干扰技术对ROS的影响 为证实XO是胞浆过量ROS的主要来源,采用2',7'-二氯荧光黄双乙酸盐(DCFH-DA)荧光探针检测各组胞浆ROS荧光强度,分为5组:正常对照组、H/R模型组、H/R+Exe组、H/R+siRNA组和H/R+siRNA对照组。其中H/R+siRNA组采用siRNA干扰技术敲低XO蛋白。将SD大鼠XO的siRNA序列修改为5'-CCACCUCCAAGAUUCAUAUTT-3'。CMEC细胞培养至80%融合,梯度更换低浓度血清培养基,培养24 h并同步化细胞。加入Lipo2000和对应的siRNA,确定最终转染时间。XO表达的抑制时间为48 h。

1.5 ROS对线粒体结构和功能的影响

分为6组:正常对照组、H/R模型组、H/R+Exe组、H/R+siRNA组、H/R+siRNA对照组和H/R+N-乙酰半胱氨酸(NAC)组。其中NAC为胞浆ROS清除剂。

1.5.1 JC-1染色 依据JC-1染料在正常细胞线粒体中为红色荧光、而在受损线粒体中为绿色荧光的特质,考察ROS对线粒体结构和功能的影响。各组中添加JC-1染料于37℃条件下孵育30 min,荧光显微镜下拍照。Jmage Pro软件荧光半定量检测膜电位。使用四甲基若丹明乙酯酸铵染料,于细胞处理结束后,添加染料与细胞共同孵育30 min,随后用酶标仪测定520 nm处吸光度值($A_{520\ nm}$),计算相对值为膜孔道开放程度。

1.5.2 细胞色素-C释放 细胞色素-C(cytochrome-C,Cyt-C)释放是线粒体膜电位受损、膜孔道

开放比例增加的结果。在培养板中将已爬好细胞的玻片用4%的多聚甲醛固定爬片15 min;0.5% Triton X-100室温通透20 min;在玻片上滴加正常山羊血清,室温封闭30 min;吸水纸吸掉封闭液,滴加足够量稀释好的Cyt-C一抗并放入湿盒,4℃孵育过夜;加荧光二抗;滴加DAPI避光孵育5 min,对标本进行染核,封片。共聚焦显微镜观察并检测caspase-3和caspase-9的活性。

1.6 TUNEL检测

处理组:50 μl脱氧核苷酸末端转移酶(terminal deoxynucleotidyl transferase,TdT)+450 μl荧光素标记的脱氧尿嘧啶核苷三磷酸(dUTP)。对照组:50 μl荧光素标记的dUTP。37℃孵育细胞30 min,50 μl TdT介导的dUTP缺口末端标记测定法(TdT-mediated dUTP nick end labeling,TUNEL)反应混合液加入标本中,暗湿盒中反应60 min。每组细胞随机选10个不同视野,荧光显微镜下计数凋亡细胞均值,重复3次。

1.7 免疫荧光检测

细胞爬片固定、通透、封闭后,分别加入CD31抗体(1∶500,Abcam)、Ⅷ因子(1∶1000,Abcam)和Cyt-C(1∶500,Abcam)孵育,4℃过夜,加入驴抗兔二抗(1∶2000,Sigma),孵育2 h。加细胞核染料4',6-二脒基-2-苯基吲哚(DAPI)后孵育3 min,荧光显微镜观察。

1.8 统计学处理

采用SPSS 17.0软件进行数据处理。计量资料以均数±标准差($\bar{x} \pm s$)表示,两组间比较采用t检验,多组间比较采用单因素方差分析。$P<0.05$为差异具有统计学意义。

2 结 果

2.1 CMEC的培养、鉴定及Exe对CMEC的毒性作用

CMEC于培养瓶中贴壁生长24 h后逐渐铺展;72~96 h铺满瓶底,呈典型铺路石样结构。CD31和Ⅷ免疫细胞荧光染色检测结果显示,双阳性率>95%,证实为CMEC(图1)。MTT检测示,各组细胞细胞间活性差异无统计学意义(图2)。

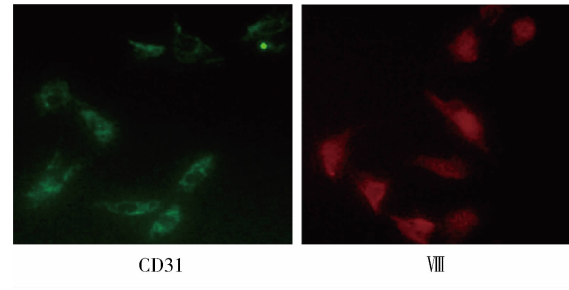


图1 CMEC的免疫组织化学染色鉴定

Figure 1 Identification of CMEC by immunohistochemistry (×400)

CMEC: cardiac microvascular endothelial cells

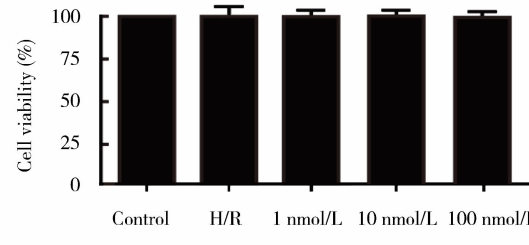


图2 Exe对CMEC活性的影响

Figure 2 Effect of Exe on cell viability ($n=3$)

Exe: exenatide; CMEC: cardiac microvascular endothelial cells

2.2 Exe对H/R诱导的细胞凋亡的影响

与正常组相比,H/R模型组表现为TUNEL绿色荧光显著增强。予以Exe预处理12 h,其以浓度依赖的方式降低TUNEL绿色荧光($P<0.05$)[13]。荧光半定量实验结果表明,10 nmol/L为Exe的最佳抗凋亡浓度,因此作为后续干预的最佳处理条件(图3)。

2.3 Ca^{2+}对H/R模型内XO表达量的影响

与H/R组和H/R+Iono组相比,H/R+Exe组和H/R+BAPTA组的XO表达量均显著降低,差异均具有统计学意义($P<0.05$;图4)。

2.4 siRNA干扰技术对H/R模型内ROS的影响

与H/R组和H/R+siRNA对照组相比,H/R+Exe组和H/R+siRNA组的ROS荧光强度均显著降低,差异均具有统计学意义($P<0.05$;图5)。

2.5 JC-1染色结果

与H/R组和H/R+siRNA对照组相比,H/R+Exe组、H/R+siRNA组和H/R+NAC组的线粒体膜电位均显著增加,而膜孔道开放程度均显著降低,差异均具有统计学意义($P<0.05$;图6)。

2.6 Cyt-C释放

通过共聚焦显微镜观察Cyt-C的亚细胞定位发现,H/R损伤模型导致Cyt-C荧光在胞质内呈弥散状分布,并伴随caspase-3和caspase-9活性的显著上升,提示H/R模型使得线粒体凋亡通路激活。而与H/R组和H/R+siRNA对照组相比,H/R+Exe组、H/R+siRNA组和H/R+NAC组均可在一定程度上减少Cyt-C的释放,并伴随caspase-3和caspase-9活性显著降低,差异均具有统计学意义($P<0.05$;图7)。

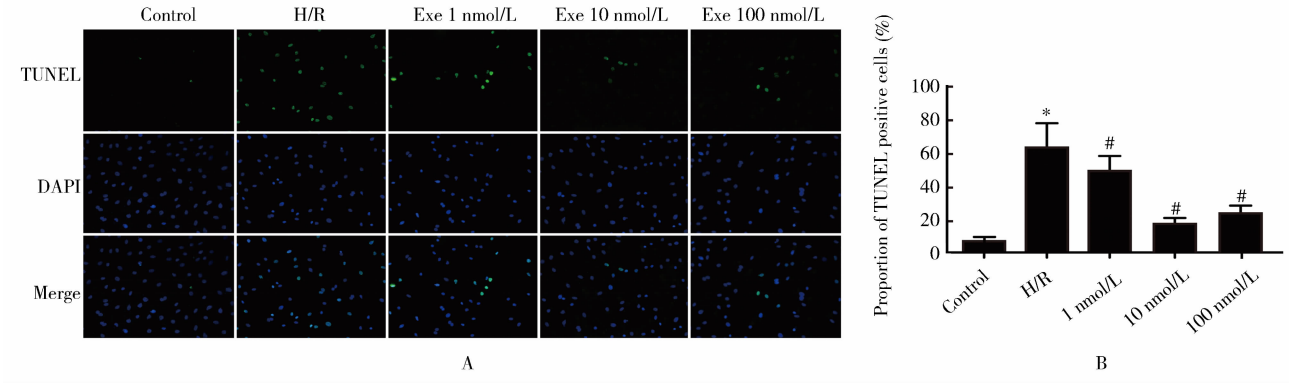


图 3 Exe 对 H/R 诱导的 CMEC 凋亡的影响

Figure 3 Effect of Exe on apoptosis of CMEC induced by H/R

A: results of staining (TUNEL ×200); B: quantitative analysis ($n=5$). Exe: exenatide; H/R: hypoxia/reoxygenation model; CMEC: cardiac microvascular endothelial cells. Compared with control group, $^{*}P<0.05$; compared with H/R group, $^{\#}P<0.05$

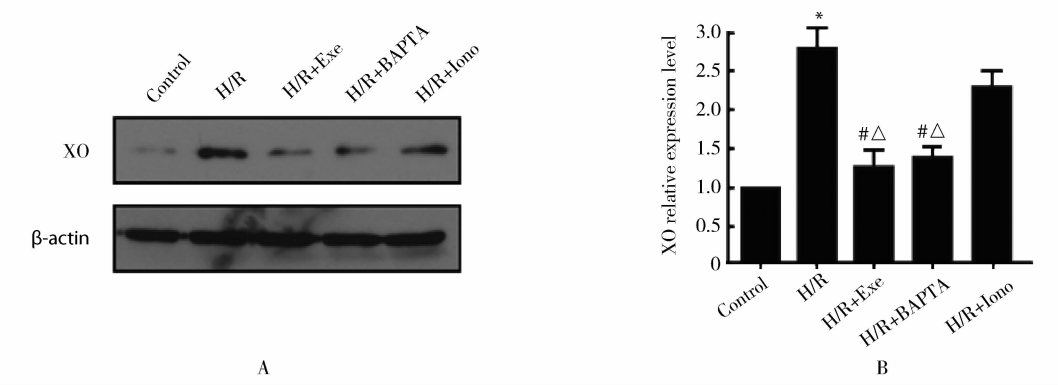


图 4 Ca^{2+} 对 XO 表达量的影响

Figure 4 Influence of Ca^{2+} on expression of XO ($n=3$)

A: western blotting; B: quantitative analysis. XO: xanthine oxidase; Exe: exenatide; H/R: hypoxia/reoxygenation model. Compared with control group, $^{*}P<0.05$; compared with H/R group, $^{\#}P<0.05$; compared with H/R + Iono group, $^{\triangle}P<0.05$

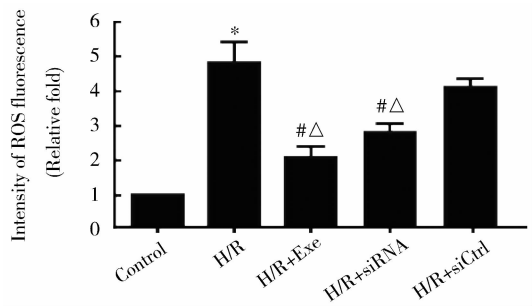


图 5 siRNA 干扰技术对 ROS 荧光强度的影响

Figure 5 Influence of siRNA on intensity of ROS ($n=5$)

ROS: reactive oxygen species; Exe: exenatide; H/R: hypoxia/reoxygenation model. Compared with control group, $^{*}P<0.05$; compared with H/R group, $^{\#}P<0.05$; compared with H/R + siCtrl group, $^{\triangle}P<0.05$

3 讨 论

尽管 PCI 术能迅速开通堵塞血管、恢复冠状动脉血流灌注,但也增加了组织发生 IRI 的可能[14,20]。ROS 爆发可导致组织细胞呈现氧化应激状态,是损伤细胞亚结构、诱导细胞进入程序性死亡的关键点[15]。

内皮细胞中 ROS 的来源较多,如线粒体呼吸链损伤、胞浆中的 XO,以及蛋白底物代谢过程中产生的负离子电荷[16,17]。内皮细胞中线粒体的含量相对缺乏,在 CMEC 中,线粒体主要作为信号传导器发挥作用。已有研究证实[18],在黄嘌呤脱氢酶大量转变为 XO、以及 XO 自身转录增加的作用下,H/R 环境中 XO 的表达量显著升高,其生成的 ROS 最终可诱导细胞凋亡的发生。

正常情况下,胞浆和线粒体内 ROS 的生成和清除处于动态平衡,但在病理情况下,CMEC 胞浆的 ROS 生成过量,渗漏进入线粒体,导致线粒体呼吸链Ⅰ~Ⅳ型复合物的氧化,发生电子传递功能障碍,线粒体膜电位下降导致膜孔道开放,线粒体内膜与 Cyt-C 结合力下降,渗漏至胞浆,从而激活 caspase-9/3 线粒体凋亡蛋白家族[16]。已有研究证实[17],H_2O_2

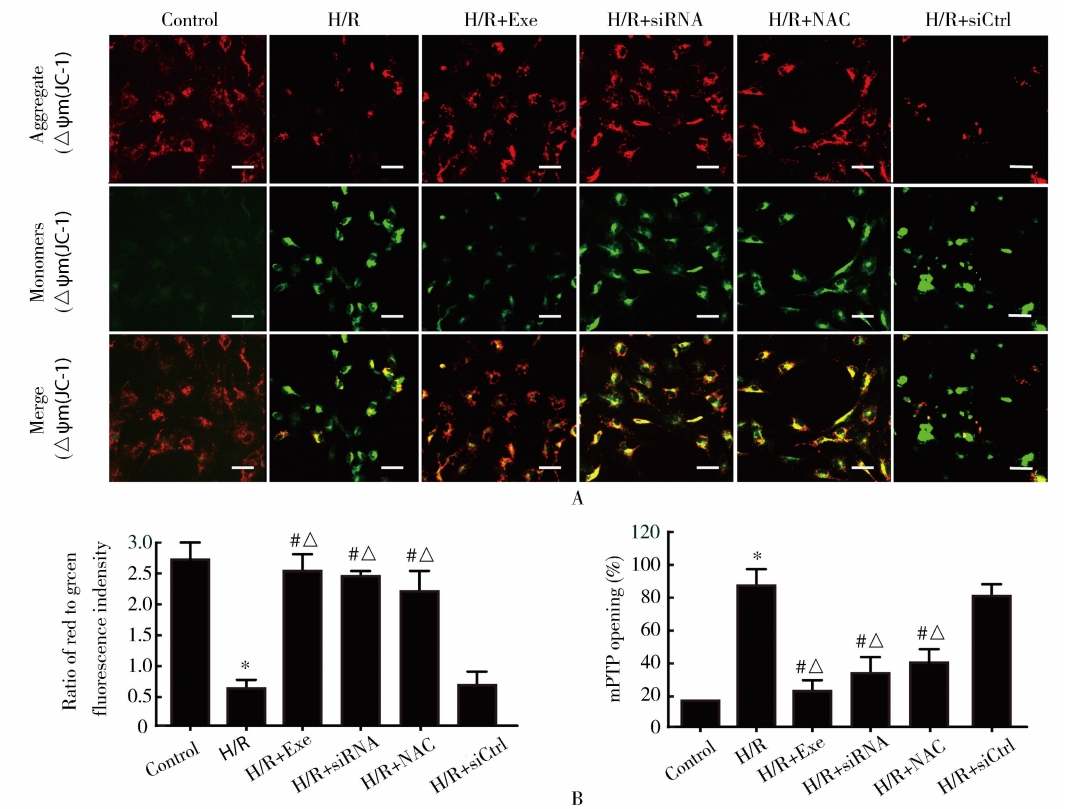


图6 JC-1染色结果及mPTP开放程度检测

Figure 6 Results of JC-1 staining and mPTP tests

A: results of staining (×200); B: quantitative analysis. Exe: exenatide; H/R: hypoxia/reoxygenation model; mPTP: mitochondrial permeability transition pore. Compared with control group, $^{*}P<0.05$; compared with H/R group, $^{\#}P<0.05$; compared with H/R + siCtrl group, $^{\triangle}P<0.05$

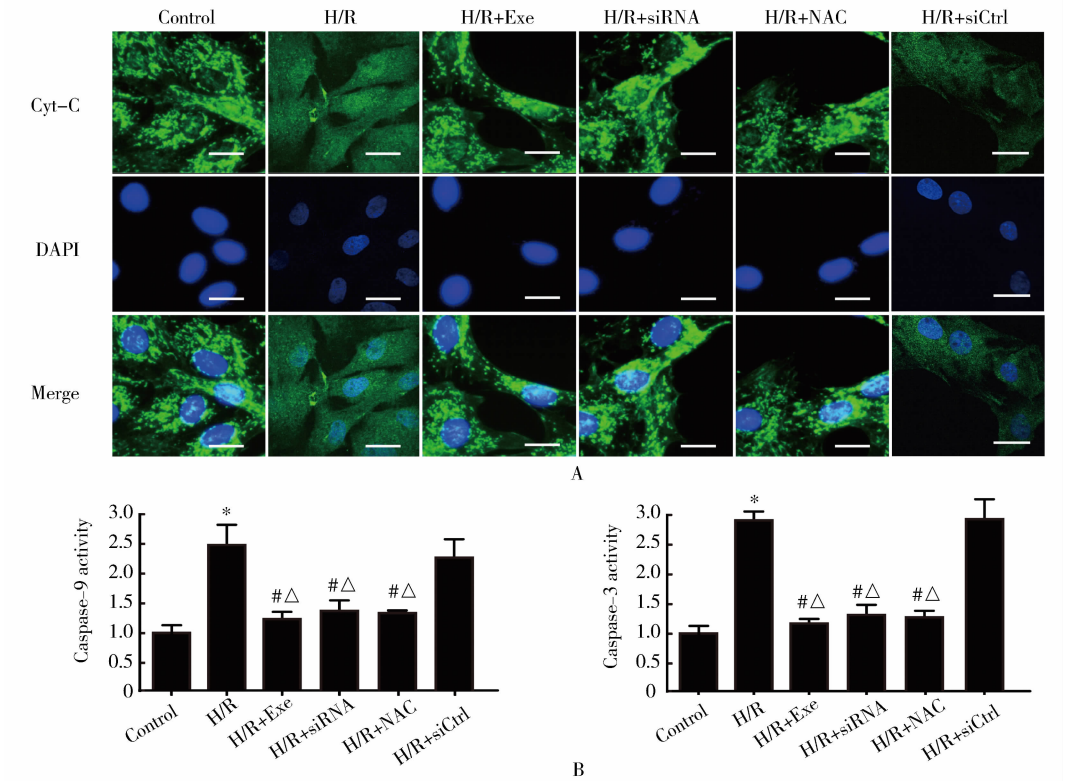


图7 Cyt-C释放检测结果

Figure7 Results of Cyt-C discharge

A: results of staining (×400); B: quantitative analysis ($n=5$). Exe: exenatide; H/R: hypoxia/reoxygenation model. Compared with control group, $^{*}P<0.05$; compared with H/R group, $^{\#}P<0.05$; compared with H/R + siCtrl group, $^{\triangle}P<0.05$

诱导的干细胞氧化应激损伤模型可以激活线粒体经典凋亡通路、诱导干细胞凋亡,这一过程也是通过上述途径完成的。而予以 Exe 则可逆转这一损伤过程,显著降低干细胞凋亡。本研究证实,在 CMEC 的 H/R 损伤过程中,施加 Exe 预处理能够有效逆转 XO-ROS 诱导的线粒体损伤。

Exe 为 GLP-1 受体激动剂,作用于胰岛细胞,降糖作用明显[18]。近年来的研究发现[19],在循环血液中,Exe 还可以与心脏上的 GLP-1 受体结合,进入细胞内,发挥抗凋亡作用,主要通过 Camp/PKA 信号转导途径,可显著改善线粒体功能。

综上所述,本研究证实了 H/R 损伤模型通过激活 XO-ROS 损伤信号,激活线粒体凋亡通路,引起 CMEC 结构和功能障碍。而予以 Exe 预处理可有效逆转此损伤信号通路,降低细胞的氧化应激损伤,提示 Exe 在 I/R 损伤过程中具有保护作用,不仅丰富了临床药物选择范围,也为应用 Exe 提供了可靠的理论基础。

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