

· 论 文 ·

羧甲司坦 L-精氨酸盐的合成及其对支气管上皮细胞的保护作用

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摘 要 羧甲司坦(CMC)是治疗慢性阻塞性肺病的常用药,长期服用对胃肠道产生严重刺激。L-精氨酸是一氧化氮(NO)合酶(NOS)的底物,在体内可转化为对心血管及胃肠道等有益的NO。L-精氨酸属碱性氨基酸,能与某些含羧基的化合物成盐以改善原药的水溶性,并可能由于促进NO的释放带来活性的提高或毒副作用的缓解。因此,本文设计、合成了CMC的L-精氨酸盐(CMCA),并测试其理化性质以及在香烟烟雾诱导的人支气管上皮细胞损伤模型中清除活性氧(ROS)、抗细胞凋亡和NO释放的能力。结果表明,CMCA能有效捕获ROS,释放NO,并抑制细胞凋亡,效果优于CMC或L-精氨酸,提示该化合物值得深入研究和开发。

关键词 慢性阻塞性肺疾病;羧甲司坦;一氧化氮;L-精氨酸;活性氧;凋亡

中图分类号 R914 **文献标志码** A **文章编号** 1000-5048(2022)02-0171-07

doi: 10. 11665/j. issn. 1000-5048. 20220206

引用本文 贾健,吴建兵,张奕华,等.羧甲司坦L-精氨酸盐的合成及其对支气管上皮细胞的保护作用[J].中国药科大学学报,2022,53(2):171-177.

Cite this article as: JIA Jian, WU Jianbing, ZHANG Yihua, et al. Synthesis and protective effect of carboxymethylcysteine L-arginate in bronchial epithelial cells[J]. J China Pharm Univ, 2022, 53(2): 171-177.

Synthesis and protective effect of carboxymethylcysteine L-arginate in bronchial epithelial cells

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Abstract Carboxymethylcysteine (CMC) is a common drug for the clinical treatment of chronic obstructive pulmonary disease, yet its long-term use can cause severe irritation to the gastrointestinal tract. As the substrate of nitric oxide (NO) synthase (NOS), L-arginine can be converted in the body into NO beneficial to the cardiovascular system, the gastrointestinal tract and so on. As a basic amino acid, L-arginine can be salified with some compounds containing acidic groups to improve the water solubility of the parent drug and may enhance the activity and alleviate side effects due to NO release. In this study, we designed and synthesized carboxymethylcysteine L-arginate (CMCA), and tested its physico-chemical properties, and the abilities to scavenge reactive oxygen species (ROS), inhibit apoptosis and release NO in cigarette smoke-induced injury model of human bronchial epithelial cells. The results revealed that CMCA is superior to CMC or L-arginine in that it could capture ROS, release NO and suppress apoptosis, suggesting that CMCA is worthy of further research and development.

Key words chronic obstructive pulmonary disease; carboxymethylcysteine; nitric oxide; L-arginine; reactive oxygen species; apoptosis

This study was supported by the National Natural Science Foundation of China (No. 81822041, No. 82173681, No. 21977116, No. 82104004, No. 81773573)

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基金项目 国家自然科学基金资助项目(No. 81822041, No. 82173681, No. 21977116, No. 82104004, No. 81773573)

慢性阻塞性肺疾病(chronic obstructive pulmonary diseases, COPD)是一种常见的慢性气道疾病,具有高发病率、致残率和致死率的特点^[1-4]。据报道我国20岁及以上成人COPD患病率为8.6%,40岁以上人群患病率高达13.7%,提示我国COPD发病率呈现高态势^[5-7]。吸烟是造成COPD最主要的原因,80%~90%的COPD患者都与吸烟密切相关^[8-10]。

羧甲司坦(carboxymethylcysteine, CMC)是临床常用的黏痰溶解药。临床研究发现,应用CMC可明显减少COPD患者病情急性加重并延长发作间歇^[11-12]。CMC治疗COPD的疗效不仅与其黏液溶解性有关,更多地与其抗氧化、抗炎作用相关^[13]。CMC还可通过抑制促炎因子白细胞介素1 β 、白细胞介素6及肿瘤坏死因子 α 等的生成,减少炎性介质的释放,减轻体内的炎症反应^[14-16]。遗憾的是,CMC化学结构中的两个羧基导致该药物对消化道有刺激作用,可引起胃部不适、恶心、呕吐、肠胃道出血等不良反应。

L-精氨酸(L-arginine, L-Arg)是一种半必需氨基酸,可被内源性NOS代谢为NO和瓜氨酸,在维持气道张力和功能方面起着重要作用。值得一提的是,只有L-精氨酸才能作为NOS的底物释放NO,而D-精氨酸不具有产生NO的能力^[17-18]。NO缺乏也被证明是COPD、哮喘和囊性肺纤维化患者肺功能恶化的原因之一^[19-21]。在胃肠道系统中,NO可通过抑制胃酸分泌、促进胃黏液分泌、调节黏膜血流量等发挥胃肠道保护作用^[22-27]。传统非甾体抗炎药(NSAIDs)具有严重胃肠道不良反应,而NO供体型NSAIDs(NO-NSAIDs)如阿司匹林-L-精氨酸盐、NO-阿司匹林、NO-布洛芬等不仅具有强效的抗炎、镇痛作用而且具有NO介导的胃肠道和心血管保护作用^[28-33]。

基于上述背景,本研究设计、合成了一种CMC的L-精氨酸盐化合物(CMCA),并测试了CMCA的理化性质及其在人支气管上皮细胞(16-HBE)中抗

氧化、抗凋亡和NO释放的作用。

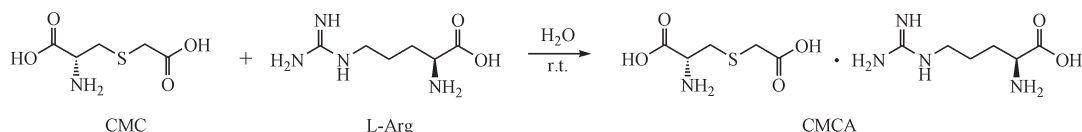
1 实验部分

1.1 试剂与仪器

羧甲司坦(成都西亚化工股份有限公司, Lot: 20200710); L-精氨酸[梯希爱(上海)化成工业发展有限公司, Lot: 22RPO]; 商用卷烟(万宝路红标); 16-HBE细胞系、DCFH-DA活性氧检测试剂盒、Annexin V-APC/7-AAD细胞凋亡检测试剂盒、DAF-FM DA一氧化氮检测试剂盒(江苏凯基生物科技股份有限公司); 6孔培养板(美国Corning Costar公司); AV-500核磁共振仪(德国Bruker公司); MAT95XP型高分辨质谱仪[赛默飞世尔科技(中国)有限公司]; X-4精密显微熔点测定仪(北京福凯仪器有限公司); pH计[梅特勒-托利多(中国)有限公司]; FACS Calibur流式细胞仪(美国Becton-Dickinson公司)。

1.2 CMCA的制备与表征

CMCA的合成途径如路线1所示,将L-精氨酸(1.74 g, 10 mmol)溶于蒸馏水5 mL中,逐量加入CMC(1.79 g, 10 mmol)始终保持反应液为澄清状,于室温25℃下搅拌2 h,向反应液中滴加无水乙醇50 mL,开始时滴速稍快,至有白色晶体析出时减慢滴速,滴毕,搅拌0.5 h,抽滤,用少量乙醇洗涤滤饼,40℃真空干燥,得白色CMCA结晶3.20 g,收率约90.7%。mp 203.8~205.2℃; UV(H₂O) λ_{\max} 211.5(lg ϵ 3.717) nm; IR(KBr, ν): 918.6, 1 223.9, 1 380.0, 1 690.7, 3 336.9, 3 438.0 cm⁻¹; MS(m/z): 175 [M₂+H]⁺, 178 [M₁-H]⁻; ¹H NMR(500 MHz, D₂O): δ 1.86~1.66(2H, m), 2.04~1.91(2H, m), 3.11(1H, dd, J_1 = 14.6, J_2 = 8.5 Hz), 3.23(1H, d, J = 14.8 Hz), 3.31(2H, t, J = 6.6 Hz), 3.39(2H, s), 3.84(1H, t, J = 5.7 Hz), 3.98(1H, d, J = 6.6 Hz); ¹³C NMR(125 MHz, D₂O): δ 175.38, 172.13, 170.66, 52.20, 51.61, 38.37, 34.83, 31.33, 25.39, 21.75。



Scheme 1 Synthetic route of carboxymethylcysteine L-arginate (CMCA)

1.3 香烟烟雾提取物(cigarette smoke extract, CSE)的制备及模型的建立

香烟烟雾溶液的制备方法如文献所述^[34]。在真空烧瓶中每 25 mL PBS 使用一支香烟燃烧 5 min 以生成 CSE-PBS 溶液。再使用 0.22 μm 孔径的过滤器过滤 CSE 溶液,以去除细菌和大颗粒。然后将烟雾溶液的 pH 调节至 7.4,其溶液设为 100% CSE,并在每次实验中稀释至所需浓度。

本研究采用 16-HBE 细胞系建立 CSE 模型,参照文献方法^[35]所述,将受试药物与 16-HBE 细胞预孵育 1 h 后,加入 10% CSE 继续孵育 18 h。每组实验重复进行 3 次。

1.4 DCFH-DA 荧光探针检测细胞内活性氧含量

DCFH-DA 是一种被广泛用于检测 ROS 的荧光探针。用 PBS 洗涤细胞 1 次(离心 200 r/min, 5 min)收集并调整细胞浓度为每毫升 1 × 10⁶ 个细胞,按照 1:1 000 用无血清培养液稀释 DCFH-DA,使终浓度为 10 μmol/L。细胞收集后悬浮于稀释好的 DCFH-DA 中,37 ℃ 细胞培养箱内孵育 20 min。每 3 ~ 5 分钟颠倒混匀一下,使探针和细胞充分接触,用无血清细胞培养液洗涤细胞 3 次,以充分去除未进入细胞内的 DCFH-DA。用流式细胞仪检测(λ_{Ex} = 488 nm; λ_{Em} = 530 nm)细胞内的活性氧。

1.5 Annexin-V APC/7-AAD 双染法检测细胞凋亡

将对数生长期的 16-HBE 细胞接种到 6 孔板中,次日,待细胞贴壁后,根据组别设置相应的含药培养基,同时设立阴性对照组。药物作用 18 h 后,用 0.25% 胰酶(不含 EDTA)消化收集细胞,用 PBS 洗涤细胞两次(离心 200 r/min, 5 min)调整细胞浓度为每毫升 5 × 10⁵ 个,加入结合缓冲液 500 μL 悬浮细胞,加入 Annexin V-APC 5 μL 混匀后,再加入 7-AAD 5 μL 混匀,室温、避光、反应 5 ~ 15 min。使用流式细胞仪检测细胞凋亡情况。

1.6 DAF-FM DA 荧光探针检测细胞内 NO 含量

DAF-FM DA 是一种可用于检测细胞内低浓度 NO 的荧光探针。将细胞消化、计数、配制成浓度为每毫升 5 × 10⁴ 个细胞的细胞悬液,接种到六孔板中,次日,待细胞贴壁后,根据组别设置加入相应的含药培养基,同时设立阴性对照组。加药 18 h 后,用 0.25% 胰酶消化收集细胞,用 PBS 洗涤细胞 1 次(离心 200 r/min, 5 min)收集并调整细胞

浓度为每毫升 1 × 10⁶ 个。按照 1:1 000 用无血清培养液稀释 DAF-FM DA,使终浓度为 5 μmol/L,细胞收集后悬浮于稀释好的 DAF-FM DA 中,37 ℃ 细胞培养箱内孵育 20 min,每 3 ~ 5 分钟颠倒混匀一下,使探针和细胞充分接触。用无血清细胞培养液洗涤细胞 3 次,以充分去除未进入细胞内的 DAF-FM DA;用流式细胞仪检测(λ_{Ex} = 495 nm; λ_{Em} = 515 nm)细胞内 NO 的情况。

1.7 统计学处理

计量数据以平均数 ± 标准差($\bar{x} \pm s$)表示, $P < 0.05$ 认为有显著性差异。两组间统计学差异采用 Tukey's 检验,多组间数据使用单因素方差分析(One-Way ANOVA)分析检验。所有分析和绘图均使用统计软件 GraphPad Prism 8。

2 结 果

2.1 CMCA 改善 CMC 的理化性质

将 CMC 与 CMCA 配制成为 1 mg/mL 的水溶液,使用 pH 计测试二者 pH,结果如表 1 所示,CMCA 改善了 CMC 自身的强酸性。以脂水分配系数 lg *P* 作为评价指标,采用分配系数(正辛醇-水)摇瓶法试验,结果如表 1 所示,CMCA 的亲水性较 CMC 好。

Table 1 pH and lg *P* of CMC and CMCA ($\bar{x} \pm s$, $n = 3$)

Compd.	pH	RSD	lg <i>P</i>	RSD
CMC	2.18 ± 0.67	30.47	-1.47 ± 0.09	-6.05
CMCA	4.22 ± 0.25	5.92	-2.03 ± 0.31	-15.05

CMC: Carboxymethyleysteine; CMCA: Carboxymethylcysteine L-arginate

2.2 CMCA 抑制 CSE 引起的 16-HBE 细胞内的 ROS 积聚

首先,本研究测试了 CMCA 清除 CSE 诱导 16-HBE 细胞内 ROS 的能力,以证明其抗氧化作用。如图 1 所示,10% CSE 刺激 18 h 后,16-HBE 细胞内产生大量 ROS($P < 0.001$)。通过给予 1 × 10⁻⁴ mol/L CMC 或 L-精氨酸治疗后,与模型组相比 ROS 的生成明显减少。采用不同浓度的 CMCA 治疗后 ROS 的生成也显著降低,并呈现剂量依赖性关系,其中 1 × 10⁻⁴ mol/L 的 CMCA 组 ROS 蓄积程度最低,且低于等摩尔浓度的 CMC 组($P < 0.001$),与 1 × 10⁻⁴ mol/L L-精氨酸组相比无显著性差异。

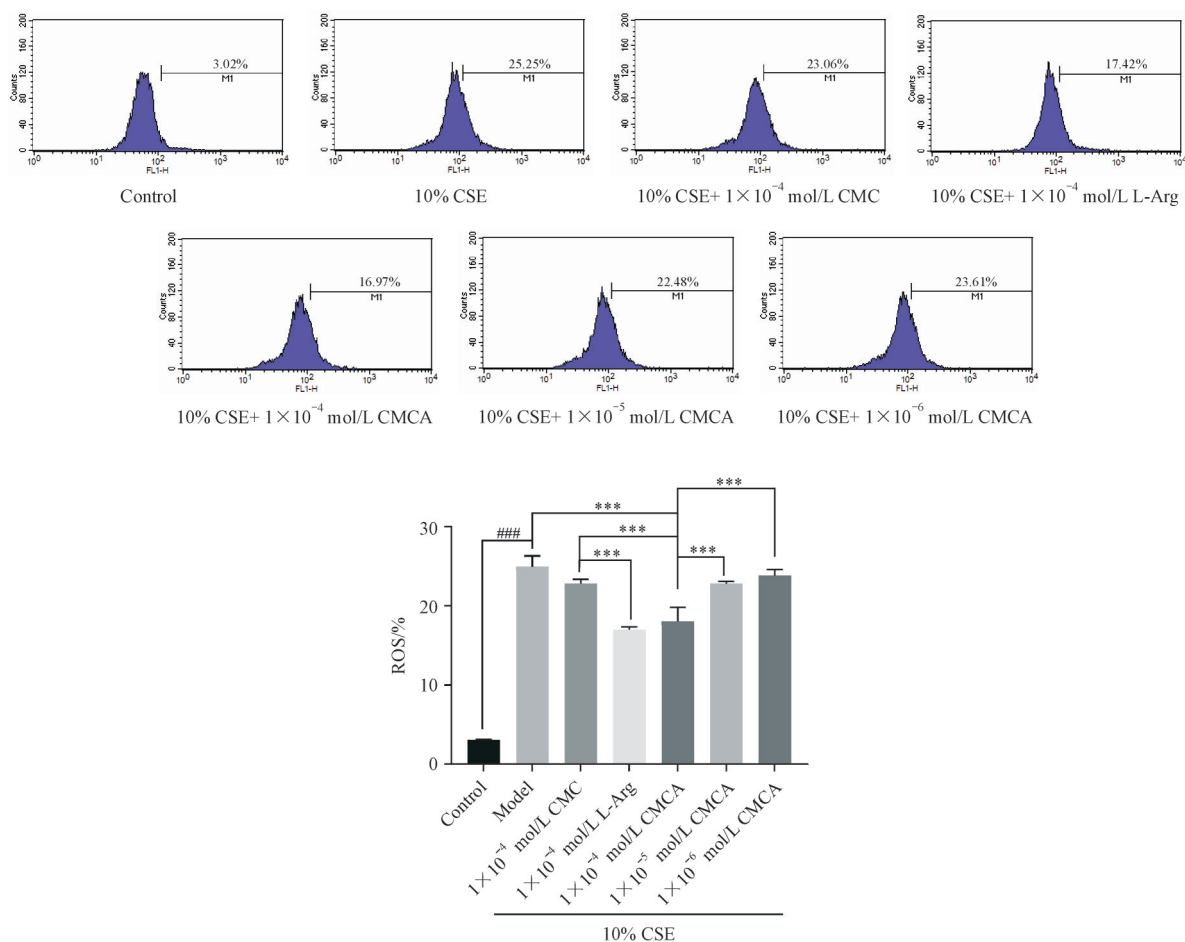


Figure 1 Effect of CMCA on cigarette smoke extract (CSE)-induced reactive oxygen species (ROS) generation in 16-HBE cells ($\bar{x} \pm s$, $n = 3$)

$P < 0.001$ vs control group; *** $P < 0.001$ vs 1×10^{-4} mol/L CMCA group or 1×10^{-4} mol/L L-Arg group

2.3 CMCA抑制CSE引起的16-HBE细胞凋亡

CMCA抑制CSE引起的16-HBE细胞凋亡情况见图2, 10% CSE刺激18 h后, 导致16-HBE细胞大量凋亡($P < 0.001$)。通过给予 1×10^{-4} mol/L CMC或 1×10^{-4} mol/L L-精氨酸治疗后, 与模型组相比细胞凋亡情况明显好转。采用不同浓度的CMCA治疗后能剂量依赖性的抑制细胞凋亡, 其中 1×10^{-4} mol/L CMCA治疗效果最佳, 优于 1×10^{-4} mol/L CMC组($P < 0.001$)和 1×10^{-4} mol/L L-精氨酸组($P < 0.001$)。

2.4 CMCA促进16-HBE细胞内NO的生成

为了验证NO在16-HBE细胞中发挥的作用, 进一步研究了16-HBE细胞内NO的含量情况(图3), 10% CSE刺激后, 16-HBE细胞内NO含量明显降低($P < 0.001$)。通过给予 1×10^{-4} mol/L CMC治疗后, 细胞内NO含量显著性增加($P < 0.001$)。给予 1×10^{-4} mol/L CMCA治疗后, 细胞内NO含量增

加, 显著多于模型组和 1×10^{-4} mol/L CMC组($P < 0.001$)。

3 结果与讨论

香烟烟雾中含有醛类、尼古丁类、氰化物、氧自由基、焦油等大量有害成分, 可直接对气道和肺组织造成损伤, 产生并释放TNF- α 、IL-8及IL-6等炎症介质, 促进气道炎症, 是导致COPD最主要的原因。CSE从香烟烟雾中提取并溶于细胞培养基中, 被广泛用于体外研究中^[36-38]。支气管上皮细胞在协调COPD病理进程中起着关键作用, 刺激物首先与之接触引起气道和肺部的炎症反应, 释放各种炎症介质和细胞因子, 进而导致疾病加重和各种相关症状的产生^[39]。本研究采用10% CSE诱导支气管上皮细胞系16-HBE病变, 以模拟体外COPD损伤。结果表明, 模型组ROS大量积聚, 细胞发生明显凋亡, 提示造模成功。

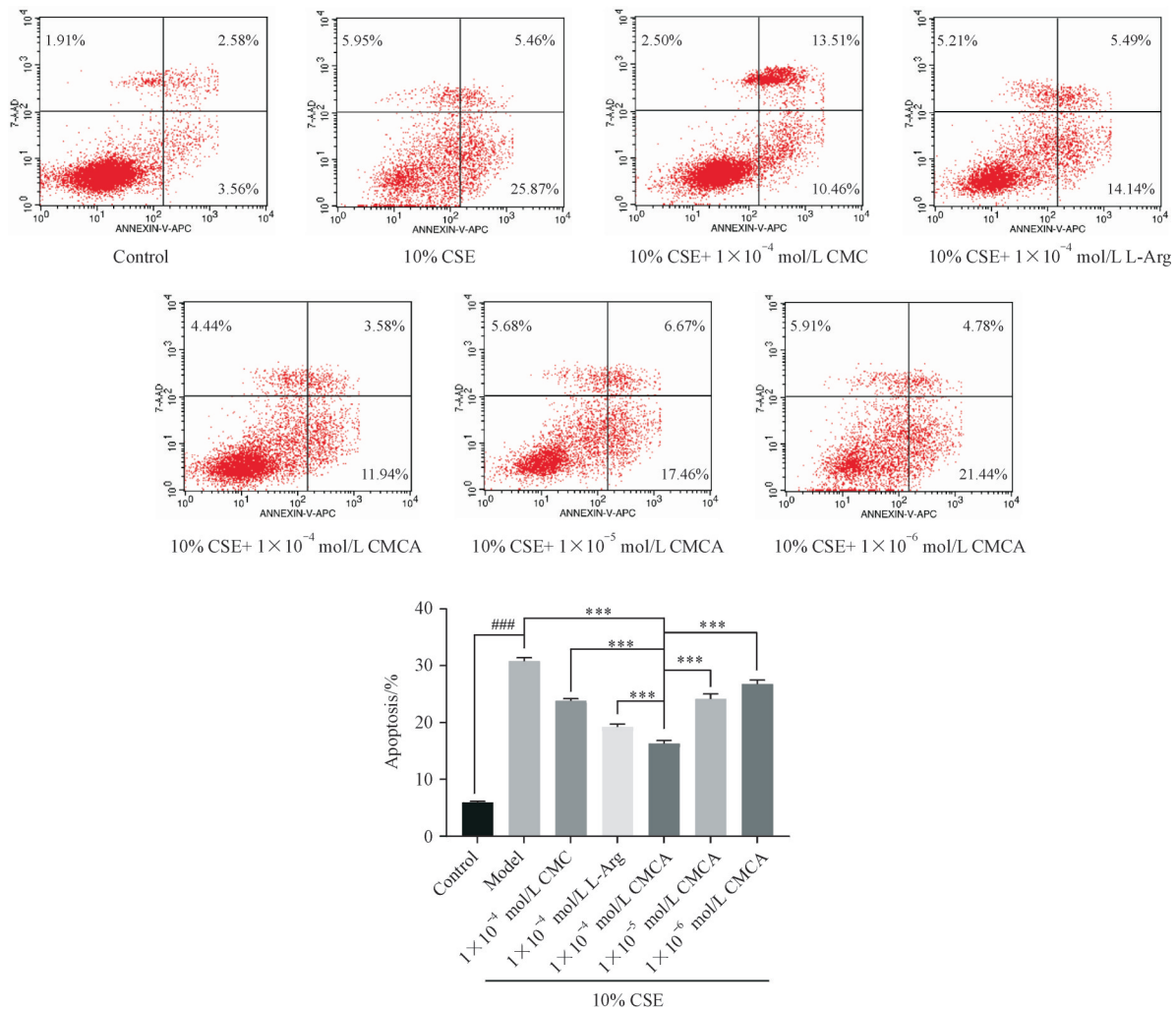


Figure 2 Effect of CMCA on CSE-induced 16-HBE cells apoptosis ($\bar{x} \pm s, n = 3$)

$P < 0.001$ vs control group; *** $P < 0.001$ vs 1×10^{-4} mol/L CMCA group

实验结果表明,CMCA显著改善了CMC本身的强酸性,从而可能减轻对消化道的刺激作用。此外,在等物质的量浓度下CMCA较CMC或L-精氨酸有着更好的清除ROS和抗细胞凋亡的能力,并且呈现浓度依赖性关系,提示在体外CSE诱导的细胞损伤模型中CMCA较CMC有着更好的治疗效果。

随后使用DAF-FM DA荧光探针检测细胞内NO的含量,考察CMCA细胞保护作用是否依赖于NO。研究发现,模型组较对照组NO含量显著下降,可能是由于10% CSE的加入,使细胞内自身的NO与CSE刺激产生的ROS进一步反应生成NO₂、

NO₂O₃和ONOO⁻等其他活性氮形式,无法被荧光探针所检测。CMC的加入,减轻细胞内炎症和抗氧化应激损伤,恢复细胞内部分NO含量($P < 0.001$ vs 模型组),而CMCA给药后细胞内NO含量进一步增加($P < 0.001$ vs CMC组)。以上提示NO对CSE诱导的细胞损伤可能具有保护作用。

总之,本研究合成的CMCA较CMC具有更优的理化性质,在体外CSE诱导的细胞损伤模型中较CMC有着更好的清除ROS和抗细胞凋亡的作用。此外,CMCA还可能降低应用CMC带来的胃肠道不良反应,改善患者的依从性,较CMC具有更广泛的应用前景,值得深入研究。

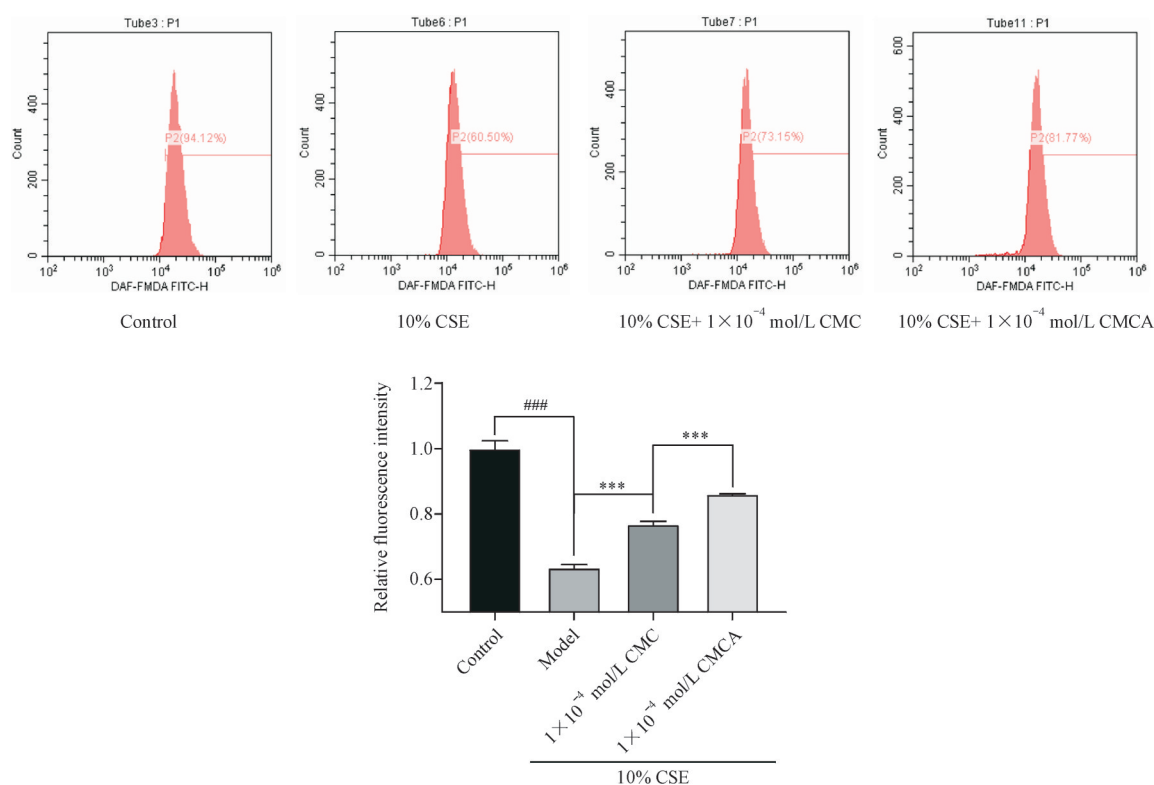


Figure 3 Effect of CMCA on CSE-induced NO concentration in 16-HBE cells ($\bar{x} \pm s$, $n = 3$)

$P < 0.001$ vs control group; *** $P < 0.001$ vs 1×10^{-4} mol/L CMC group

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