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MPC30-DEA70 载 AMO-miR-222 对大鼠颈总动脉球囊损伤后血管狭窄的影响 *

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摘要 目的：阳离子磷酸胆碱聚合物(MPC30-DEA70)为非病毒类转基因载体，可与 AMO-miR-222 络合，通过导管球囊系统将 MPC30-DEA70/AMO-miR-222 基因复合物导入大鼠颈内动脉球囊损伤处，观察对血管平滑肌细胞增生及血管狭窄程度的影响。**方法**：90 只雄性 SD 大鼠随机分为未损伤组、多聚赖氨酸（PLL 组）、MPC30-DEA70/AMO-miR-222 组、PLL/AMO-miR-222 组、PLL/MPC30-DEA70 组、AMO-miR-222 组、单纯损伤组、PLL 载 P/A=3:1 组和 P/A=5:1 组，每组各 10 只。构建大鼠颈总动脉球囊损伤模型，予血管损伤段行 PLL、MPC30-DEA70/AMO-miR-222、PLL/AMO-miR-222、PLL/MPC30-DEA70、AMO-miR-222、PLL 载 P/A=3:1 和 P/A=5:1 复合物的转运。4 周后通过光学显微镜观察 HE 染色血管段组织形态学改变。Western blot 法检测各组血管段 p57Kip2、p27Kip1 蛋白的表达情况。RT-PCR 法检测各组血管段 miR222 扩增情况。**结果**：光学显微镜下，多聚赖氨酸（PLL 组）、裸 MPC30-DEA70/AMO-miR-222 组、PLL/AMO-miR-222 组、PLL/MPC30-DEA70 组、AMO-miR-222 组、MPC30-DEA70 组可见内膜显著增生，新生内膜 / 中膜比值无组间差异，较 PLL 载 P/A=3:1 组和 P/A=5:1 组有组间差异，后两组比较无组间差异，未损伤组未见新生内膜增殖。Western blot 法检测显示 PLL 载 P/A=3:1 组和 P/A=5:1 组 P57kip2、P27kip1 蛋白表达含量较未损伤组降低（P<0.05），较余六组增高（P<0.05），组间比较无显著差异（P>0.05）。RT-PCR 法检测显示，miR-222 表达在未损伤组很低，PLL 载 P/A=3:1 组和 P/A=5:1 组增高，余六组过表达（组间比较无显著差异）。**结论**：MPC30-DEA70 可与 AMO-miR-222 络合，有效抑制球囊损伤后血管 miR222 表达，从而抑制新生内膜增生及血管狭窄。

关键词：磷酸胆碱聚合物；反义 miR-222；内膜增生；血管狭窄

中图分类号：R-33；R318.08；R543 **文献标识码**：A **文章编号**：1673-6273（2017）01-36-06

Effects of AMO-miR-222 Carried by MPC30-DEA70 on Rats' Hemadostenosis after Common Arteria Carotis Balloon Injury*

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ABSTRACT Objective: As a non-viral transgenic carrier, the cationic phosphorylcholine polymer (MPC30-DEA70) can form a complexation with AMO-miR-222. The MPC30-DEA70/AMO-miR-222 gene compound is imported through catheter-balloon system into places where rats' internal arteria carotid balloon injury occurs to observe their effects on vascular smooth muscle cell proliferation and hemadostenosis. **Methods:** 90 male SD rats are randomly divided into 9 groups with 10 rats per group, as the groups respectively are: the uninjured group, polylysine group (PLL group), nude MPC30-DEA70/AMO-miR-222 group, PLL/AMO-miR-222 group, PLL/MPC30-DEA70 group, AMO-miR-222 group, only injured group, PLL-loaded P/A =3:1 group and P/A=5:1 group. Rats' common arteria carotis balloon injury model is built to transfer PLL, MPC30-DEA70/AMO-miR-222, PLL/AMO-miR-222, PLL/MPC30-DEA70, AMO-miR-222, MPC30-DEA70, PLL-loaded P/A=3:1 and P/A=5:1 compounds for injured vascular. Four weeks later, histologic changes of HE stained blood vessel will be observed by optical microscope. The proteins expressions of p57Kip2 and p27Kip1 in blood vessels of those groups are detected by Western blot method. Amplification of miR222 in blood vessels of those groups is detected by RT-PCR method. **Results**: Under optical microscope, remarkable intimal hyperplasia can be observed in the polylysine (PLL) group, nude MPC30-DEA70/AMO-miR-222 group, PLL/AMO-miR-222 group, PLL/MPC30-DEA70 group, AMO-miR-222 and MPC30-DEA70, while the ratio of neointima/medial membrance among those groups shows no inter-group difference; however, compared with that of PLL-loaded P/A=3:1 and P/A=5:1 two groups, which are identical between the two groups, the ratio shows no difference; no neointimal hyperplasia is observed in the uninjured group. According to the Western blot method, compared with the uninjured group (P<0.05), the expressed contents of protein P57kip2 and P27kip1 in PLL loaded P/A=3:1 group and P/A=5:1 group are lower but higher than that of the rest six

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groups (P<0.05), while there is no remarkable difference between these two groups. As shown by the detection results of RT-PCR method, the expressed contents of miR-222 in the injured group is low; it is higher in PLL-loaded P/A=3:1 group and P/A=5:1 group, while too much of it is expressed in the rest six groups (there is no remarkable intergroup difference). **Conclusions:** MPC30-DEA70 and AMO-miR-222 can form a complexation together and effectively inhibit the expression of miR222 in blood vessel after balloon injury, so that inhibit neointimal hyperplasia and hemadostenosis can be inhibited.

Key words: Phosphorylcholine Polymer; Antisense Mir-222; Intimal Hyperplasia; Hemadostenosis

Chinese Library Classification(CLC): R-33; R318.08; R543 Document code: A

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前言

相关研究已经证明,p27Kip1 和 p57Kip2 对大鼠颈动脉血管平滑肌的增殖起到负调控作用,而 p27Kip1 和 p57Kip2 在大鼠颈动脉血管平滑肌中是 miR-221 和 miR-222 的靶基因。而反义 miR222(AMO-miR-222)可同时抑制 miR221 与 miR222 的表达[1,2]。我们的研究经验及其他学者们早已发现,磷酸胆碱聚合物(phosphorylcholine polymer,简称 PC polymer)是一种良好生物相容性和血液相容性的药物控释涂层材料。PC 涂层模仿人体自然细胞膜的特性,可降低摩擦系数,具有内在的抵御生物侵袭的能力,减少纤维蛋白原和血小板激活,减少血栓形成,无明显炎症反应[3]。几个较大型研究组的试验结果已经表明 PC 涂层支架是可行、安全和有效的[4-6]。PC 材料不仅在支架表面形成了一层生物屏障,其水凝胶样的特性更可作为药物的 " 储存库 " 来贮存和释放药物,既往已有研究通过在 PC 聚合物骨架引入一定量的正电基团而形成阳离子 PC 薄膜[7,8],使得对带有负电的小分子量在负载量及释放量均得到大大改善。应用正电基团修饰的阳离子 PC 聚合物与带负电的 miRNA 分子通过静电平衡作用相结合。然而,PC 聚合物涂层在负载 RNA 这样带有负电性的生物分子还是十分有限的,需借助多聚赖氨酸的作用。多聚赖氨酸带正电荷,与带负电荷的 RNA 分子结合,可产生较强的粘合力。多聚赖氨酸这一特殊性质被广泛应用于免疫组织化学、冰冻切片、细胞涂片、原位杂交等防脱片处理,并且用于细胞培养,增加细胞帖壁能力[9-11]。国内已有学者成功运用多聚赖氨酸包被的球囊转运腺病毒载体[12],本研究以阳离子磷酸胆碱聚合物 MPC30-DEA70(PC 聚合物)为转基因载体,利用多聚赖氨酸(PLL)涂层球囊将 MPC30-DEA70/AMO-miR-222 导入大鼠颈总动脉球囊损伤处,观察 miR-222 的表达及对血管狭窄程度的影响。

1 材料和方法

1.1 材料

健康雄性 SD 大鼠 90 只,体重为 250-350g,由徐州医学院动物实验中心提供,分笼饲养。AMO-miR-222 序列为 5'-AC-CCAGUAGCCAGAUGUAGCU-3' 由苏州吉马公司合成,5' 末端加 FAM 标记,0.1 % PLL 采购予上海生工生物工程公司,P57(kip2)单克隆抗体购予 AB 公司,P27(kip1)抗体购予北京博奥森公司,GAPDH 抗体购予巴戊德公司,碱性磷酸酶标记的山羊抗兔二抗购予北京中杉金桥科技有限公司,MPC30-DEA70 由中国矿业大学曹希传教授惠赠,1.2 mm× 6 mm 球囊导管等介入器材由美敦力公司提供。

1.2 基因球囊的制备

取 0.1 % PLL 溶液 30 μL,置于 1.5 mL EP 管中,以双蒸水稀释至 100 μL,将球囊导管避光浸泡于 EP 管中 30 min,后拿出吹干备用。取 90 μL、150 μL MPC30-DEA70 分别加入到 30 μL AMO-miR-222 溶液中,形成 P/A 比值为 3:1 和 5:1(MPC30-DEA70/AMO-miR-222)的混合溶液,室温下静置 30 min,以去离子水补充至 200 μL,均匀涂抹于球囊导管表面,吹干备用,同时注意无菌操作。

1.3 局部基因转运

参照文献建立大鼠颈总动脉球囊损伤模型[13],将 90 只大鼠随机分为 9 组,每组 10 只,对照组不予处理,PLL 组仅将多聚赖氨酸涂抹的球囊送至血管损伤段,裸 MPC30-DEA70/AMO-miR-222 组将 MPC30-DEA70/AMO-miR-222 络合的复合物未经多聚赖氨酸黏附,直接送至大鼠颈总动脉损伤表面,AMO-miR-222 组仅将球囊黏附的 AMO-miR-222 送至大鼠颈总动脉损伤处,MPC30-DEA70 组同理,P/A=3:1 组、P/A=5:1 组分别将已络合好的 2 种不同比例 MPC30-DEA70/AMO-miR-222 经多聚赖氨酸黏附在球囊,送至血管损伤段。上述各组球囊用 4atm 保持 10 min,给球囊负压,结扎颈内动脉,逐层缝合,予以普通饲养。

1.4 Western blot 法检测 p57Kip2、p27Kip1 蛋白的表达

48 h 后,每组取 4 只大鼠颈总动脉损伤段血管,严格按照凯基全蛋白提取试剂盒提取总蛋白,BCA 试剂盒测量每组蛋白浓度,采取 Western blot 法检测 P57、P27 蛋白的表达,利用 Bandscan 5.0 图像分析系统对 Western blot 结果进行灰度值分析。

1.5 qRT-PCR 法检测基因血管 miR-222 的表达

48 小时后,每组取 3 只大鼠颈总动脉损伤段血管,通过 TRIzol 进行总 RNA 提取,严格按照 TIANScript cDNA 第一链合成试剂盒进行 cDNA 合成,TIANGEN 公司 SuperReal 荧光定量预混试剂增强版试剂盒进行目的基因的扩增,利用 ABI 7500 软件进行结果分析。

1.6 组织病理形态检测

术后三周处死剩余大鼠,取转基因血管段制作病理标本,行苏木精伊红染色,光镜下观察各组血管内膜增生及官腔狭窄程度,并进行图像分析,测定各组血管新生内膜面积(NIA)中膜面积(MA),并计算内膜 / 中膜(I/M)面积比值。

1.7 统计学处理

采用 Graphpad Prism 5 软件进行统计学分析,实验数据用($\bar{x}\pm s$)表示,△ Ct 值表示 miR-222 的表达,荧光定量 PCR 法应用单因素方差分析,两两比较。P≤ 0.05 为数据有统计学意义。

2 结果

2.1 Western blot 法检测球囊损伤后目的血管 p57Kip2、p27Kip1 蛋白的表达

p57Kip2 蛋白予 55kPa 处出现目的条带，p27Kip1 蛋白予 27kpa 处出现目的条带，GAPDH 内参蛋白予 36kpa 处出现目的条带，如图 1 所示。

2.2 qRT-PCR 法检测球囊损伤后 miR-222 的表达(见图 4)

2.3 21 天后，各组血管新生内膜增生情况、内膜面积、中膜面积及内中膜面积比值(见表 1 及图 5)

3 讨论

自从 1977 年 Cruntizing 首次成功实施了经皮冠状动脉成形术，经皮冠状动脉介入治疗已经逐步成为冠心病治疗不可替

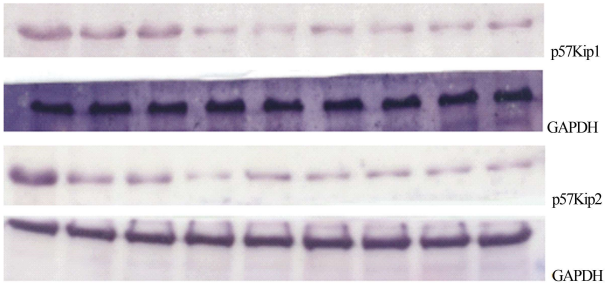

图 1 p57Kip2、p27Kip1 蛋白的表达

Fig.1 The proteins expressions of p57Kip2 and p27Kip1

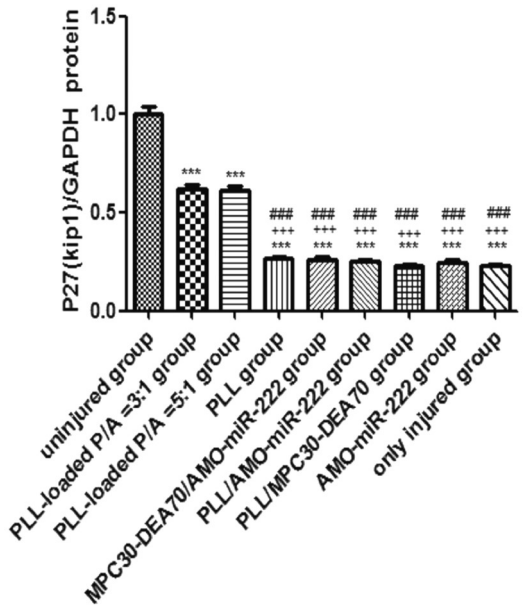

图 2 p27Kip1 与 GAPDH 比值

Fig.2 Ratio of p27Kip1 and GAPDH

注：*** 表示与未损伤组相比较 ***P<0.05 差异有统计学意义，+++ 表示与 P/A=3:1 组相比较 +++P<0.05 差异有统计学意义，### 表示与 P/A=5:1 组相比较，###P<0.05 差异有统计学意义。

Note: *** show that compared with uninjured group, ***P<0.05, these was a significant difference, +++ show that compared with P/A=3:1 group, +++P<0.05, these was a significant difference, ### show that compared with P/A=5:1 group, ###P<0.05, these was a significant difference.

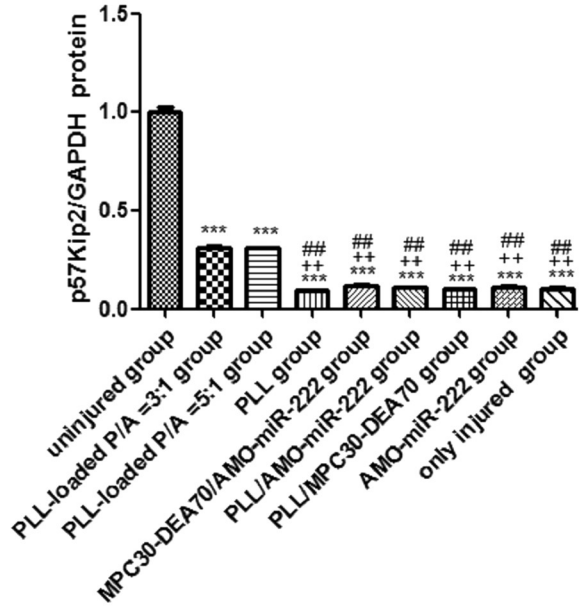

图 3 p57Kip2 与 GAPDH 比值

Fig.3 Ratio of p57Kip2 and GAPDH

注：*** 表示与未损伤组相比较 ***P<0.05 差异有统计学意义，++ 表示与 P/A=3:1 组相比较 ++P<0.05 差异有统计学意义，## 表示与 P/A=5:1 组相比较，## P<0.05 差异有统计学意义。

Note: *** show that compared with uninjured group, ***P<0.05, these was a significant difference, ++ show that compared with P/A=3:1 group, ++P<0.05, these was a significant difference, ## show that compared with P/A=5:1 group, ##P<0.05, these was a significant difference.

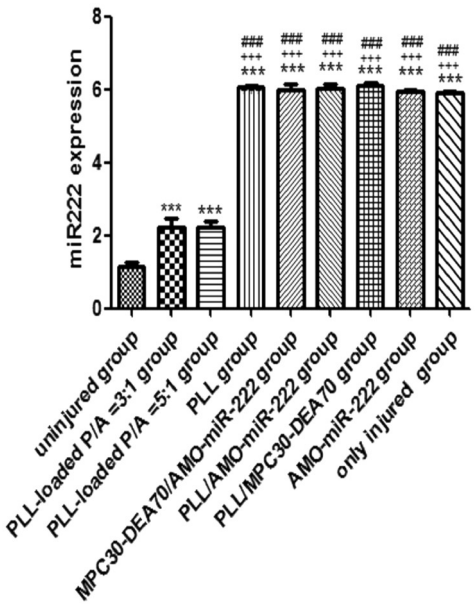

图 4 miR222 的表达

Fig.4 The expressions of miR222

注：*** 表示与未损伤组相比较 ***P<0.05 差异有统计学意义，+++ 表示与 P/A=3:1 组相比较 +++P<0.05 差异有统计学意义，### 表示与 P/A=5:1 组相比较，###P<0.05 差异有统计学意义。

Note: *** show that compared with uninjured group, ***P<0.05, these was a significant difference, +++ show that compared with P/A=3:1 group, +++P<0.05, these was a significant difference, ### show that compared with P/A=5:1 group, ###P<0.05, these was a significant difference.

表 1 各组新生血管内膜面积、中膜面积及内中膜面积的比较(n=3，$\bar{x}\pm s$)

Table 1 Comparison of the neointimal area and media area and ratio of neointima/media membrance among different groups (n=3, $\bar{x}\pm s$)

Groups	NIA (mm^2)	MA (mm^2)	I/M
Only injured group	0.148± 0.003#*	0.053± 0.003	2.798± 0.200#*
AMO-miR-222 group	0.153± 0.003#*	0.058± 0.002	2.637± 0.116#*
PLL/MPC30-DEA70 group	0.153± 0.004#*	0.056± 0.006	2.789± 0.442#*
PLL/AMO-miR-222 group	0.152± 0.005#*	0.057± 0.005	2.675± 0.176#*
MPC30-DEA70/AMO-miR-222group	0.143± 0.005#*	0.059± 0.004	2.461± 0.267#*
PLL group	0.144± 0.004#*	0.054± 0.002	2.658± 0.145#*
P/A=3:1 group	0.079± 0.006	0.056± 0.005	1.422± 0.055
P/A=5:1 group	0.085± 0.004	0.056± 0.003	1.532± 0.109

注：与 P/A=3:1 组相比，#P <0.05；与 P/A=5:1 组相比，*P<0.05。

Note: compared with P/A=3:1group, #P <0.05; compared with P/A=3:1group, *P<0.05.

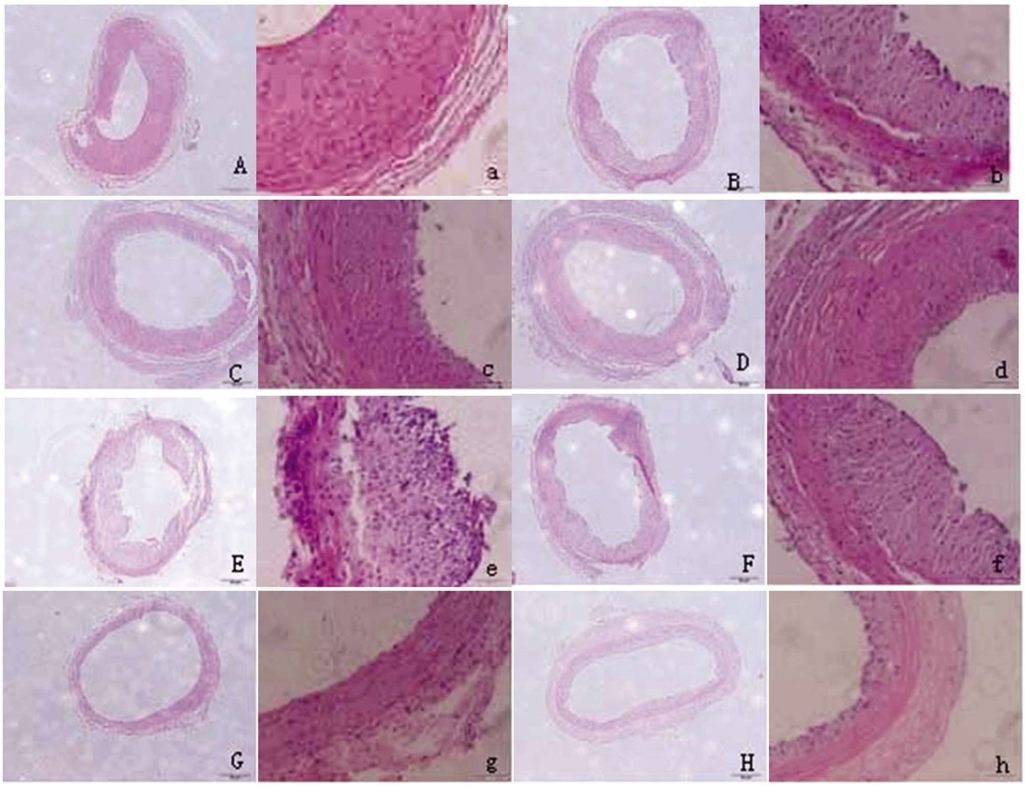

图 5 各组光镜下 HE 染色

Fig.5 Each group HE staining under light microscope

注：A. 单纯损伤组；B. 多聚赖氨酸组；C. MPC30-DEA70/AMO-miR-222 组；D. PLL/AMO-miR-222 组；E .PLL/MPC30-DEA70 组；F.AMO-miR-222 组；G.PLL 载 P/A=3:1 组；H. PLL 载 P/A=5:1 组，均为 40× ，图 a、b、c、d、e、f、g、h 为对应组高倍镜视野，均为 400× 。

Note: A. Only injured group; B. PLL group; C. MPC30-DEA70/AMO-miR-222 group; D.PLL/AMO-miR-222 group; E. PLL/MPC30-DEA70 group; F. AMO-miR-222group; G. PLL-loaded P/A =3:1 group; H. PLL-loaded P/A=5:1 group, these are 40× , Fig.a, b, c, d, e, f, g, h are high magnification, 400× .

代的组成部分[14]。然而，尽管新的技术层出不穷，各种并发症也接踵而至[15-17]。现在冠状动脉介入治疗面临的最重要的问题就是药物洗脱支架(DES)植入后的晚期支架内血栓形成[18-20]。药物洗脱支架的药物涂层主要为大环内酯类抗生素如西罗莫司及紫杉醇[21]，前者可抑制处于 G 期的细胞向 S 期转换，后者可阻断细胞的分裂，使细胞的发育停留在 G_0/G 期，从而达到抑制细胞分裂增殖的共同目的。但是目前的 DES 的金属小梁能在置入血管后的很长时间裸露在血液循环中，因此，也抑制了支架表面内皮化覆盖过程[22]。临床研究已经证明患者基因突变可导致对上述药物产生抵抗，其次，患者敏感体质可能会对支架或者药物产生过敏反应，而药物洗脱球囊(DEB)由于所携带的高浓度药物作用迅速，分布范围均匀，无异物存留体内，一定程度上避免了异物刺激所导致的内皮细胞愈合不良及炎症反应[23-25]。此外，DEB 可能缩短冠心病患者抗血小板治疗的疗程，降低出血风险和治疗成本，DEB 还具有治疗慢性复杂分叉病变等 DES 所不具备的优势[26-29]。因此，DEB 作为一种新兴治疗手

段,显示出了巨大前景。

近期的研究发现,许多 miRNA 在脉管系统中高度表达,并在血管发生病变时表达改变,如血管生成,动脉粥样硬化,血管重构,血管损伤、新生内膜增生及狭窄[30-32]。体外实验已经证实 MPC30-DEA70 可以安全有效负载反义寡核苷酸片段进入兔髂动脉损伤血管段[33],腺病毒载体可以载入 miRNA222 进入体内血管,而国外学者提取大鼠颈总动脉球囊损伤后血管段,对其做 miRNA 芯片,与未损伤血管段做比较,可发现 miRNA222 的表达含量明显增加,表明 miRNA222 与血管内皮细胞增加存在某种联系,而 miRNA222 与 p57Kip2、p27Kip1 存在负调控关系。

本实验将 PLL 涂于球囊上,将 MPC30-DEA70/AMO-miR-222 复合物黏附至球囊上,将球囊送至大鼠颈总动脉血管损伤段,观察对大鼠颈总动脉血管损伤段的影响。实验结果表明,球囊损伤后各组血管较未损伤组血管相比较内膜均有不同程度的增生及 p57Kip2、p27Kip1 蛋白表达含量的减少;转染 P/A=3:1 与 P/A=5:1 基因复合物治疗组血管官腔狭窄程度及新生内膜面积较未损伤组高,但较其他组明显减低,p57Kip2、p27Kip1 蛋白表达含量较损伤组余组高,荧光染料 PCR 法结果显示转染 P/A=3:1 与 P/A=5:1 基因复合物治疗组血管 miR222 的表达较其他损伤组相比明显减低,这证明 MPC30-DEA70 可以有效负载 AMO-miR222 进入大鼠颈总动脉血管内膜,通过反义寡核苷酸技术与 miRNA222 结合,阻止 p57Kip2 蛋白、p27Kip1 蛋白的表达、抑制新生内膜增生及血管平滑肌细胞增生。

目前心血管疾病的基因治疗已经成为一种重要的发展趋势,而药物球囊的临床应用也弥补了药物洗脱支架的不足。本实验将药物球囊与基因沉默技术相结合,通过 MPC30-DEA70 载体的应用,有效的抑制了与血管内皮增殖相关 miRNA 的表达,从而起到抑制相应引起血管增殖蛋白的表达,从而为药物球囊治疗相应狭窄血管病变提供一种新的思路。

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