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巨噬细胞来源外泌体介导心肌梗塞后心脏重塑的作用研究 *

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摘要 目的:研究活化的巨噬细胞来源外泌体在心肌梗塞后心脏重塑中的作用。**方法:**采用超高速离心分离提取溶血磷脂酸作用下巨噬细胞来源的外泌体,将其与心脏成纤维细胞共同孵育 48 小时,利用 Edu 细胞增殖实验、Transwell 实验及免疫荧光等方法检测溶血磷脂酸刺激(LPS)下巨噬细胞来源外泌体对心脏成纤维细胞的增殖、迁移以及分化的影响。选取正常 C57 雄性小鼠 32 只,根据其是否结扎左侧冠状动脉前降支及是否进行心脏原位外泌体注射,将实验小鼠随机分为:正常组,假手术组,心梗+空白外泌体组及心梗组+LPS 刺激外泌体组。手术完成 4 周后行心脏超声、Masson 染色以检测各组实验小鼠心功能状态及心脏纤维化程度。**结果:**在细胞实验中,LPS 刺激的巨噬细胞来源外泌体可以显著增加心脏成纤维细胞的增殖、迁移以及分化能力;在动物实验中,相对于正常组、假手术组及心梗+空白-外泌体组,心梗+LPS-外泌体组小鼠的左心室射血分数及短轴收缩率显著下降,左心室舒张末及收缩末内径显著增加。Masson 染色检测提示心肌梗塞+LPS-外泌体组小鼠心脏纤维化程度显著高于其余三组。**结论:**活化的巨噬细胞来源的外泌体可以显著加速心梗后心脏重塑的进程。

关键词:心肌梗塞;巨噬细胞;心脏纤维化;外泌体

中图分类号:R-33;R542.22 **文献标识码:**A **文章编号:**1673-6273(2020)05-808-05

Effects of Macrophage-derived Exosomes on the Cardiac Remodeling after Myocardial Infarction*

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ABSTRACT Objective: To explore the role of macrophage-derived exosomes on the cardiac remodeling following myocardial infarction (MI) and the possible molecular mechanism. **Methods:** Exosomes isolated from supernatant of macrophage stimulated by LPS were incubated with cardiac fibroblasts for 48 h, and then the ability of cardiac fibroblasts proliferation, migration and differentiation were detected by Edu assay, Transwell assay and immunofluorescence assay. Additionally, thirty-two male mice were divided into 4 groups as Control group, Sham group, MI+NC-Exos group and MI+LPS-Exos group according to whether the left anterior descending coronary artery was ligated and exosomes were injected in the tissue of heart. After four weeks, cardiac function and the extent of cardiac fibrosis were assessed by echocardiography and Masson's trichrome staining. **Results:** Exosomes derived from macrophage stimulated by LPS significantly elevated the ability of cardiac fibroblasts migration, proliferation and differentiation; Additionally, Echocardiographic analysis showed that exosomes derived from macrophage stimulated by LPS induced deterioration of cardiac dysfunction post-MI, as indicated by the smaller fractional shortening (FS%) and ejection fraction (EF%), and the larger left ventricular end diastolic diameter (LVEDD) and left ventricular end systolic diameter (LVESD). Similarly, a more remarkable intestinal fibrosis in MI+LPS-Exos group were detected than in other three groups as assessed by Masson's trichrome staining. **Conclusions:** Activated Macrophage-derived exosomes promotes cardiac remodeling following MI.

Key words: Myocardial infarction; Macrophages; Cardiac remodeling; Exosomes

Chinese Library Classification(CLC): R-33; R542.22 **Document code:** A

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前言

冠状动脉粥样硬化性心脏病是由于冠状动脉粥样硬化造成冠状动脉功能性异常或者器质性狭窄或者阻塞,进而导致心肌缺血缺氧而引起的心脏疾病,是目前世界范围内导致人类死

亡的首要病因^[1,2]。由于药物及冠状动脉支架植入术等治疗方式的普及,该类患者的预后明显改善,但心梗后心脏重塑的持续进展仍然是导致该类患者心功能渐进性恶化及预后不佳的重要原因^[3,4]。因此,进一步阐明心肌梗塞后心脏重塑的机制对于改善该类患者的远期预后具有重要的临床意义。

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外泌体是一种直径介于 30-150 nm 的微小囊泡,内含多种生物活性分子,如环状 RNA (circRNA)、mRNA、微小 RNA (miRNA)、DNA、功能蛋白、转录因子等,是细胞与细胞之间及组织与组织间相互联系的重要的信号分子^[5-8]。近年来,研究显示巨噬细胞来源的外泌体在多种心血管疾病、代谢功能障碍以及肿瘤细胞侵袭中均发挥着重要的作用^[9-12]。鉴于巨噬细胞在心肌梗后心肌坏死触发的无菌性炎症中大量聚集^[13-15],我们推测巨噬细胞来源的外泌体可能在心肌梗塞后心脏重塑的病理生理过程中发挥着重要的作用。因此,本研究拟采用分子生物学技术及梗动物模型,系统性研究巨噬细胞来源的外泌体对梗后心脏重塑中的作用,旨在为心肌梗塞后心脏重塑的治疗寻找新的靶点及预防措施。

1 材料与方法

1.1 动物与分组

健康成年 C57 小鼠 32 只,雄性,体重 20~25 g,由上海市第一人民医院实验动物中心提供。根据是否结扎小鼠左侧冠状动脉前降支近端及原位注射外泌体(2 mg/只),将实验小鼠随机分为四组:正常组(n=8),假手术组(n=8),梗+空白-外泌体组(n=8)及梗+LPS-外泌体组(n=8)。

1.2 主要实验仪器与试剂

莱卡倒置荧光显微镜(Leica,美国);小动物心脏超声显像仪(Siemens 公司,美国);DTX880 多功能酶标仪全波长酶标(美国 Beckman-Coulter 公司);小动物人工呼吸机(DW-2000 型,美国);蛋白电泳仪、转膜设备(Bio-Red 公司,美国);胎牛血清(Gibco,美国);溶血磷脂酸(Sigma,美国);II 型胶原酶(Sigma,美国);胰酶(Gibco,美国);兔源抗平滑肌抗(abcam,美国);Edu 细胞增殖试剂(锐博,中国),鬼笔环肽(Invitrogen,美国);PKH67(Invitrogen,美国);DAPI(碧云天,中国);Transwell 小室(康宁,中国)。

1.3 实验方法

1.3.1 梗模型建立 构建小鼠梗模型,术前 10 小时禁食,禁水,异氟烷(400 mL/min)气麻,并予以气管插管,潮气量为 200 μ L,呼吸频率设为 110 次/分。开胸,暴露小鼠心脏,并用 7.0 无创缝线结扎小鼠左侧冠状动脉前降支近端,缝合胸腔,采集心电图评估手术效果。

1.3.2 外泌体鉴定 ① 电镜检查,将 8 μ L 外泌体滴至 300 nm 的铜网上沉淀 3-5 分钟,8 μ L 2%醋酸双氧铀滴至铜网静置 3 分钟后,观察外泌体形态及粒径大小。② NTA:另取 8 μ L 外泌体悬液稀释 10000 倍,利用传感上纳米颗粒跟踪分析仪 Zate View 检测外泌体的粒径分布和浓度。外泌体表面分子标记检查:利用 Western blot 检测外泌体特有的分子标记物。

1.3.3 细胞迁移实验 取对数生长期的心脏成纤维细胞,利用细胞计数板调整细胞浓度为 1×10^5 /mL,取 200 μ L 细胞悬液接种到以孔径为 8 μ m 的聚碳酸酯微膜孔分隔 Transwell 小室,取 600 μ L 含 10%血清的 DMEM 加入下室,37 $^{\circ}$ C、5% CO_2 培养箱中培养 24 h 后取出聚碳酸酯微膜,PBS 洗涤 2 次。4%多聚甲醛固定 15 min 后利用结晶紫染色 20 min,PBS 洗净多余的染料,室温凉干,每个小室用显微镜观察记录 5 个视野细胞数量,取其平均值。

1.3.4 EdU 法检测细胞增殖实验 将含有 Edu 染料(终浓度为 10 μ M)的培养基提前处理心脏成纤维细胞 4 小时,弃去细胞培养基,PBS 洗涤 2-3 遍,而后依次用 4%多聚甲醛固定,0.3%的 Triton $\times$ 100 通透细胞,并用 Edu 检测发光盒检测处于增殖活跃期的细胞比例,拍照记录。

1.3.5 免疫荧光 将心脏成纤维细胞接种在共聚焦培养皿中,细胞密度适度,4%多聚甲醛固定,0.3%的 Triton $\times$ 100 通透细胞 15 分钟,5%的 BSA 室温封闭 1 小时,兔源抗 α -SMA 抗体 4 $^{\circ}$ C 孵育过夜,次日用抗兔荧光二抗室温 2 小时,DIPA 标记细胞核,荧光显微镜下拍照观察。

1.3.6 Masson 染色 观察小鼠梗周边区纤维化程度改变:梗 4 周后,对各实验组小鼠进行安乐死处理,4%多聚甲醛固定,石蜡包埋,依次经过 Bouin's 溶液 56 $^{\circ}$ C 水浴、Weigert 苏木素溶液处理、丽春红溶液处理,1%的磷钨酸溶液和苯胺蓝溶液处理、1%的冰醋酸溶液处理后,经脱水透明后进行图像采集及分析。

1.3.7 心脏超声 检测各组实验小鼠的心功能状态,脱毛,麻醉处理后,取胸骨旁左室长轴、心尖四腔为切面,利用彩色多普勒超声连续记录 3 个心动周期中小鼠左室舒张末内径(LVEDd),收缩末内径(LVESd)的平均值、并计算左室射血分数(EF)及短轴收缩率(FS)。

1.4 统计学方法

所有数据均采用 SPSS 19.0 软件进行统计分析,计量资料以均数 $\pm$ 标准差($\bar{x} \pm s$)表示,四组实验数据采用单因素方差分析,两组间比较采用 t 检验,以 $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 溶血磷脂酸对巨噬细胞细胞的影响

研究资料显示巨噬细胞在梗后心肌组织中大量聚集^[13],同时梗后心脏内细胞坏死可以产生大量溶血磷脂酸(LPS)^[16],为了模拟梗后心脏组织内 LPS 对巨噬细胞的影响,我们对体外培养的巨噬细胞予以外源性 LPS(1 μ g/ml)刺激,从图 1 可见,LPS 刺激下,巨噬细胞内的炎症相关的基因如 iNOS、IL-1 β 、IL-6 以及 TNF- α 表达量显著升高,同时巨噬细胞体积显著增大。

2.2 溶血磷脂酸刺激下巨噬细胞来源外泌体的鉴定

外泌体是一种微小的囊泡状结构,是机体内器官与器官,细胞与细胞之间沟通的重要工具^[5,6]。为明确巨噬细胞培养上清提取物为外泌体,我们首先利用 Western blot 检测其是否带有外泌体表面的蛋白标记物^[17,18],结果如图 2(A)所示,利用超高速离心提取的巨噬细胞培养基内物质表明携带有外泌体形成所必需的分子标志物 CD63、CD81 以及 TSG101,提示外泌体提取成功。另外,如图 2B 所示,透射电镜下可见巨噬细胞来源的外泌体外形呈茶托状圆形囊泡,与既往的文献报道一致^[19,21]。采用 NTA 检测外泌体的粒径大小,可见其范围波动在 30-15 nm,且直径主要集中在 115 nm 左右,见图 2(C)。

2.3 溶血磷脂酸刺激下巨噬细胞来源外泌体具有显著的促纤维化效应

为明确 LPS 刺激下巨噬细胞来源的外泌体对心脏成纤维细胞的影响,我们将 LPS 刺激下巨噬细胞来源的外泌体与心脏成纤维细胞共培养 48 小时,如图 3(A)所示,巨噬细胞来源外泌

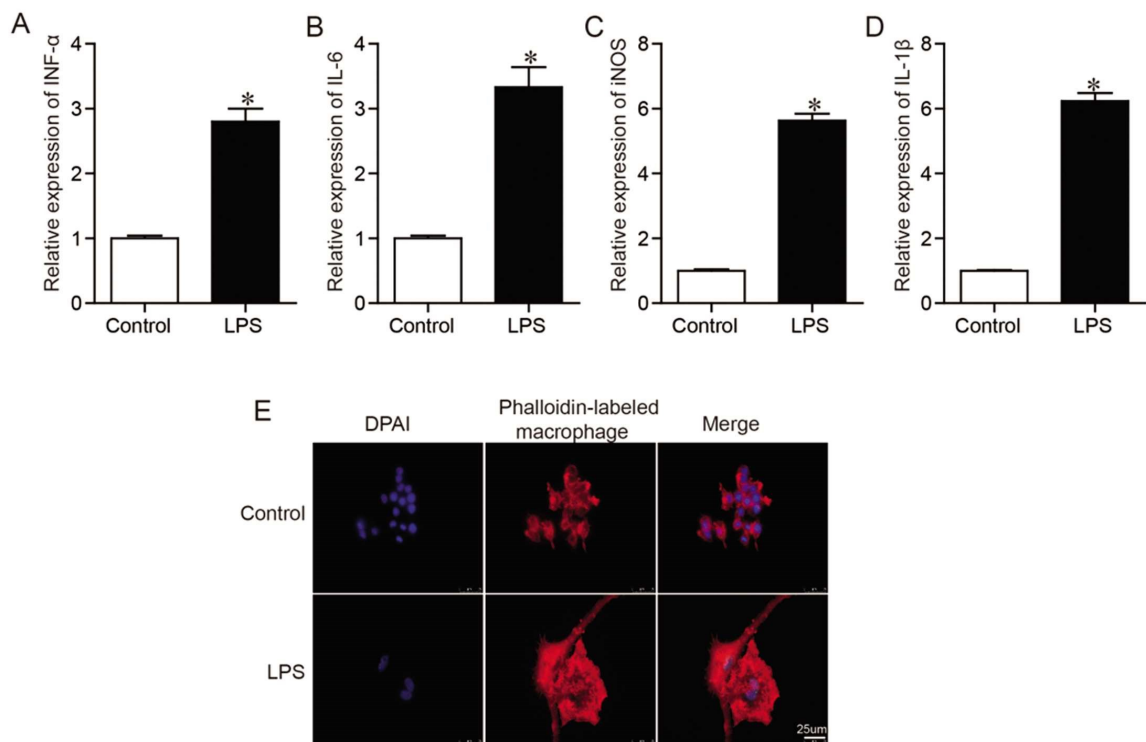


图 1 LPS 刺激下巨噬细胞炎症因子表达及形态学改变

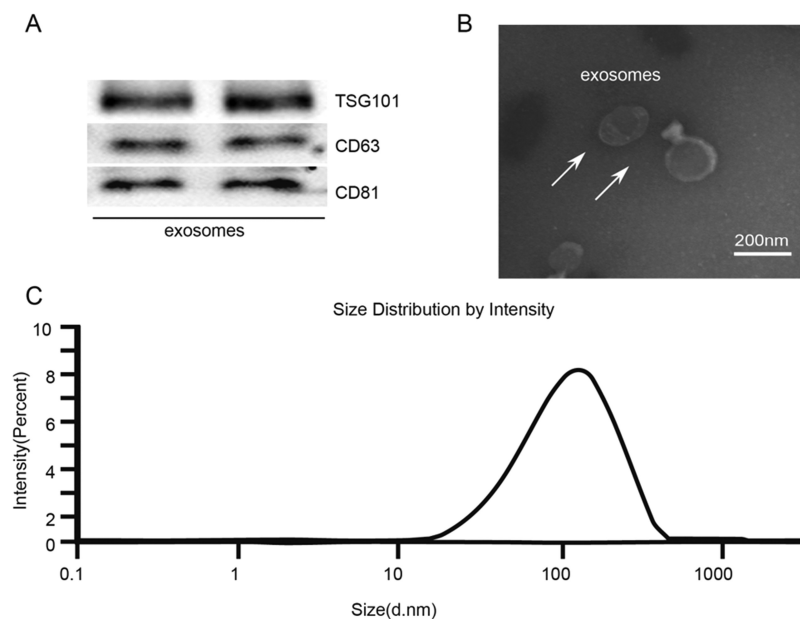
Fig.1 LPS stimulation upregulated the expression of iNOS, IL-1 β , IL-6 and TNF- α in macrophages and induced the size increase

图 2 巨噬细胞来源的外泌体特征

Fig.2 Characterization of Macrophage-derived exosomes

(A) Western blot analysis of several exosome biomarkers in Macrophage-derived exosomes. (B) Transmission electron microscopic images of exosomes.

Scale bar =200 nm; (C): Distribution of Macrophage exosome sizes.

体可以被成纤维细胞吞噬 (孵育 2 小时), 通过 Edu 染色标记法、Transwell 法及细胞免疫荧光法分别检测外泌体对成纤维细胞增殖、迁移及分化的影响, 从图中 3(B-D) 可见, LPS 刺激下巨噬细胞来源的外泌体具有显著促进心脏成纤维细胞增殖、迁移及分化的能力。

2.4 溶血磷脂酸刺激下巨噬细胞来源外泌体对小鼠心功能的影响

为进一步明确 LPS 刺激下巨噬细胞来源的外泌体对心脏

成纤维细胞的影响, 我们通过超声引导下心脏组织原位注射, 将外泌体注入小鼠心脏组织, 如图中 4(A-E) 所见, 相较于心梗 + 对照外泌体组, 心梗 + LPS- 外泌体组中小鼠的心功能显著恶化, 表现为 EF 及 FS 的下降, LVEDD 及 LVESD 显著增加。另外, 我们通过对心梗组织周边区域行 Masson 染色检测, 如图 4F 所示, 心梗 + LPS- 外泌体组小鼠的心脏纤维化程度显著高于其余三组。

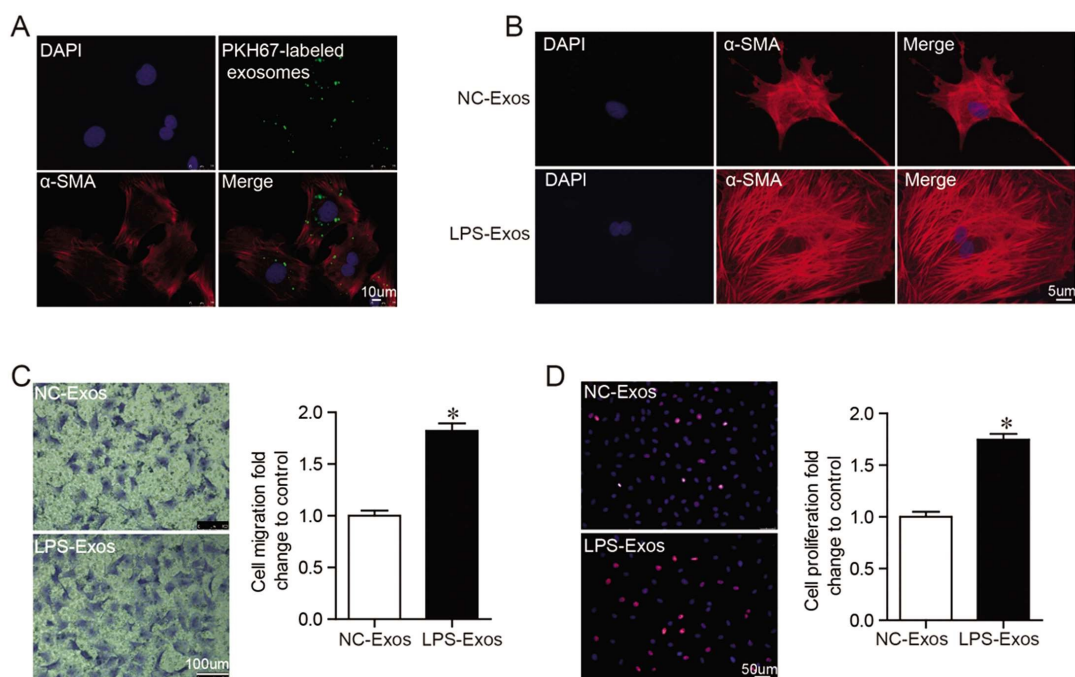


图3 LPS刺激下巨噬细胞来源的外泌体促进心脏成纤维细胞分化、迁移以及增殖

Fig.3 Exosomes derived from LPS-stimulated macrophages promoted CFs differentiation, migration and proliferation.

(A) Immunofluorescence imaging analysis PKH67-labeled exosomes were taken up by cardiac fibroblasts. Scale bar = 10 μm.

(B) Immunofluorescent analysis of myofibroblast activation. Scale bar = 5 μm. (C) Transwell assay of cardiac fibroblast migration. Scale bar = 100 μm.

(D) EdU incorporation detection of cardiac fibroblast proliferation. Scale bar = 50 μm. * $P < 0.05$.

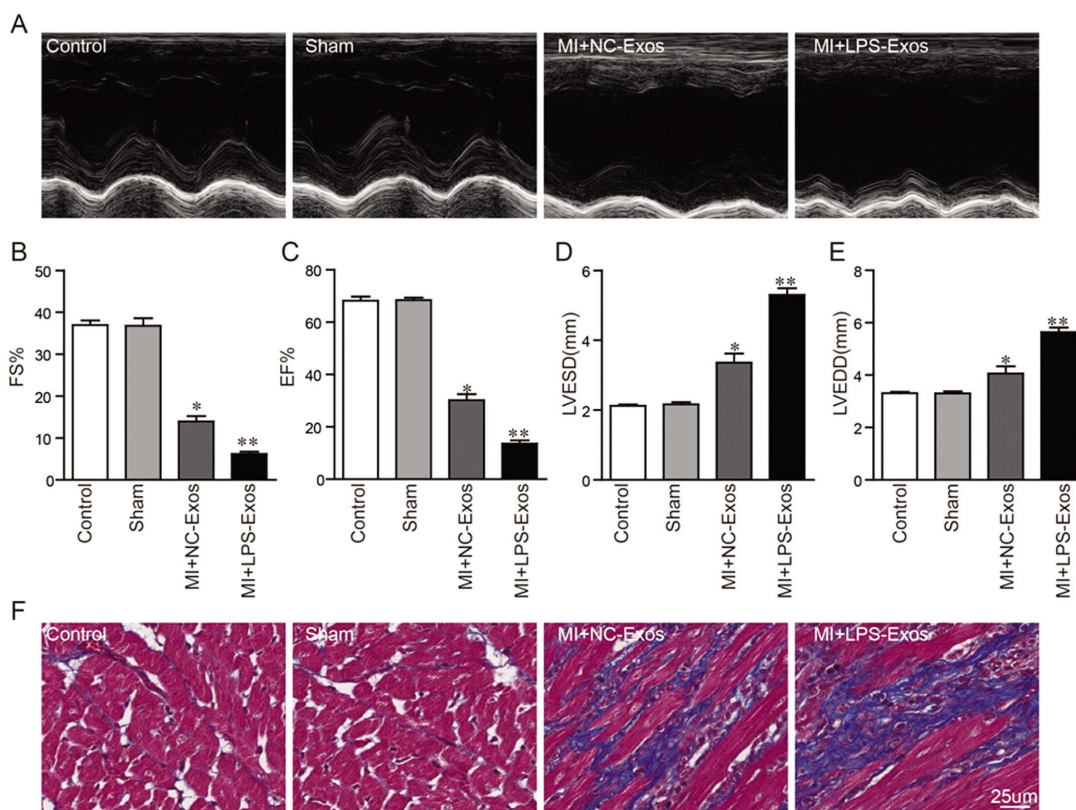


图4 LPS刺激下巨噬细胞来源的外泌体促进心梗小鼠心脏功能恶化及胶原的沉积

Fig.4 Exosomes derived from LPS-stimulated macrophages aggravated cardiac remodeling post myocardial infarction

(A) Representative echocardiography at the fourth week post-MI. (B-E) Statistic summary from (A). EF: ejection fraction;

FS: fractional shortening; LVESD: left ventricular end-systolic dimension; LVEDD: left ventricular end-diastolic dimension (n = 5).

* $P < 0.05$ vs. Sham; ** $P < 0.05$ vs. MI+NC-Exos. (F) Masson's trichrome staining. Scale bar = 25 μm.

3 讨论

心肌梗塞后心脏重塑的持续进展仍然是导致该类患者远期预后不佳的重要因素^[3,4]。急性心肌梗死一旦发生即可促发一系列无菌性炎症反应^[13],进而招募一系列免疫细胞在心肌组织中的大量聚集,尽管目前的研究表明心肌梗塞早期巨噬细胞以及 CD4⁺ T 细胞的聚集有利于心肌梗塞后心脏组织的修复^[22,23]。然而,心梗后炎症细胞的持续存在是导致心梗患者远期预后不佳的重要指标,这或许与炎症因子 TNF- α , IL-6 等诱导心肌细胞病理性肥大、凋亡,促进心脏成纤维细胞激活,促进胶原合成有关^[24-26]。

巨噬细胞在心肌梗塞后心肌组织中的浸润数量远远大于较其他炎症细胞的总和,故本研究将目光聚焦于巨噬细胞在心肌梗塞中的作用。目前的多项研究资料表明巨噬细胞激活以后释放的外泌体内所含的生物活性分子,如 miRNA 等,后者被受体细胞接收后,可以显著改变受体细胞在器官、组织中的功能活性,进而介导多项疾病的发生^[27-30]。鉴于心肌梗塞后巨噬细胞在心脏组织中的大量激活,故我们推测巨噬细胞来源的外泌体或许在心肌梗塞后心脏重塑的发生、发展中发挥着重要的作用。我们采用细胞坏死后细胞膜的消化产物 LPS 模拟心肌梗塞后巨噬细胞的病理环境,因其含量在心肌梗塞后心脏组织含量急剧增加,结果显示 LPS 刺激后的巨噬细胞细胞形态发生显著改变,且其分泌的外泌体具有显著促进心脏成纤维细胞增殖、迁移、分化及合成胶原的能力。另外,本研究通过心脏原位注射技术,结合小动物心脏超声及 Masson 染色技术,进一步明确 LPS 刺激下巨噬细胞来源的外泌体具有促进心肌梗塞后心脏重塑,加速心功能恶化的作用。在本研究中,我们利用 LPS 刺激静止状态的巨噬细胞向促炎型(M1)巨噬细胞转变,进而研究其分泌的外泌体的功能特点。然而,需要指出的是,巨噬细胞在心梗后期,在局部微环境及其他免疫细胞的调节下,其细胞分型将逐渐从促炎型的 M1 型向抑炎型的 M2 型转变,鉴于外泌体内生物活性物质的种类与含量与细胞的属性具有的密切,因此进一步明确 M2 型巨噬细胞来源外泌体的功能特点是本研究下一步研究的重点。此外,目前的研究资料提示外泌体内含有多重生物活性成分,如受体蛋白、Circular RNA、miRNA 等,进一步鉴定并识别介导巨噬细胞来源外泌体生物功能的活性分子,可以为心梗后心脏重塑的治疗提供新的线索及干预靶点。

综上所述,本研究表明巨噬细胞在心肌梗塞后心脏重塑中发挥着重要的作用,这可能与心肌梗塞后心脏组织局部心肌细胞缺血、缺氧,细胞崩解坏死,导致组织内 LPS 及 TGF- β 等因子释放增加,巨噬细胞释放外泌体等促纤维化因子有关。本研究进一步丰富了心肌梗塞后心脏重塑的机制,为心肌梗塞后心脏重塑的治疗提供了新的理论依据及干预靶点。

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(上接第 812 页)

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