论著

脂联素对流体切应力下骨髓间充质干细胞损伤的保护作用观察

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【摘要】目的 探讨脂联素(APN)是否可减轻流体切应力(SS)对骨髓间充质干细胞(BMSCs)的损伤及其可能机制。方法 采用全血贴壁法分离、培养BMSCs并进行鉴定。将BMSCs分为对照组、SS组、SS+APN组，采用平板液体流动
学模型给予BMSCs流体切应力作用24h，显微镜下观察细胞形态，TUNEL染色检测BMSCs的凋亡情况，Western blotting
检测Akt、p-Akt及Bax蛋白的表达。结果 与对照组比较，SS组BMSCs凋亡明显增加，Bax表达明显上调。与SS组比较，SS+APN组细胞形态明显改善，细胞增殖显著增加，Bax表达显著下调，p-Akt表达显著上调。三组Akt的表达无明
显差异。结论 脂联素可通过激活Akt信号通路抑制流体切应力对BMSCs的损伤。

【关键词】脂联素；切应力；间质干细胞；组织工程

Protective effect of adiponectin on bone marrow mesenchymal stem cell injury caused under fluid shear stress
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This work was supported by the National Natural Science Foundation of China (81470477, 81470480), the National High
Technology Research and Development Program of China (863 Program) for Young Scholars (2014AA020514, 2015AA020919), and the
Postdoctoral Science Foundation of China (2013M532113)

【Abstract】Objective To explore whether adiponectin (APN) can attenuate the injury of bone marrow mesenchymal stem
cells (BMSCs) caused under fluid shear stress (SS) and the mechanism involved. Methods BMSCs were isolated and cultured by
whole blood adherent method, and then identified and divided into control group, SS (10dyne/cm2) group and SS+APN group.
BMSCs were subjected to fluid SS for 24 hours by using liquid plate flow chamber model, the cell morphology was observed by
microscopy, the apoptosis of BMSCs was detected by TUNEL staining, and Western blotting was performed to detect the protein
expressions of Akt, p-Akt and Bax. Results Compared with the control group, the apoptosis of BMSCs significantly increased, and
the expression of Bax was up regulated in SS group. Compared with SS group, the cell morphology was significantly improved, the
number of cells obviously increased, the Bax expression was down-regulated and the p-Akt expression was up-regulated markedly
in SS+APN group. There was no significant difference in Akt expression among the three groups, but the p-Akt expression significantly
increased in SS+APN pretreatment group. Conclusion Adiponectin may activate the Akt signaling pathway and inhibit the
apoptosis of BMSCs under fluid SS.

【Key words】adiponectin; shear stress; mesenchymal stem cells; tissue engineering

[基金项目] 国家自然科学基金(81470477, 81470480); 国家高技术研究发展计划(863计划)青年科学家专题课题(2014AA020514,
2015AA020919); 国家博士后科学基金(2013M532113)
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Clinical surgery often requires the use of blood vessels. However, as a result of vascular tissue limited, transplant the blood vessels is not affected by immunosuppression. Tissue-engineered blood vessels, TEBVs are currently being studied in the directions of bone marrow stromal cells (BMSCs), BMSCs and umbilical vein. BMSCs play a regulatory role in the regeneration of blood vessels.

1. Materials and Methods

1.1 BMSCs isolation and culture. BMSCs were isolated from the bone marrow of healthy rats. The bone marrow was flushed with phosphate buffered saline (PBS) and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed, and the bone marrow was digested with 0.25% trypsin-EDTA solution for 5 minutes at 37°C. The digested cells were then washed with PBS and resuspended in DMEM containing 10% fetal bovine serum (FBS) at a concentration of 1 x 10^6 cells/mL.

1.2 BMSCs were cultured in DMEM containing 10% FBS at 37°C in a humidified atmosphere of 5% CO₂. The culture medium was replaced every 3 days. The cells were passaged when they reached 80-90% confluence. The cells were harvested at 10^5/cm² density using a 0.25% trypsin-EDTA solution.

1.3 Western blotting of BMSCs. BMSCs were collected and washed with PBS. The total protein was extracted using RIPA buffer containing 1% protease inhibitor. The protein concentration was determined using the BCA method. The proteins were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk and incubated with primary antibody (anti-BMSCs) at 4°C overnight. The membrane was then washed and incubated with secondary antibody. The signals were detected using an enhanced chemiluminescence (ECL) system.

1.4 BMSCs were implanted into the rat abdominal cavity. After 14 days, the BMSCs were harvested and analyzed using Western blotting. The results showed that the expression of CD29 and CD34 in the BMSCs was significantly upregulated compared to the control group. The expression of CD44 was also increased, indicating that the BMSCs had successfully differentiated into fibroblasts.

1.5 BMSCs were co-cultured with human umbilical vein endothelial cells (HUVECs). The co-cultured cells were stained with Hoechst 33342 and imaged using confocal microscopy. The results showed that the BMSCs had integrated with the HUVECs and formed a continuous network of vessels.

These results indicate that BMSCs can be used as a source of cells for vascular transplantation. Further studies are needed to optimize the culture conditions and improve the differentiation efficiency.

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总蛋白。取10 μl蛋白样品行10%丙烯酰胺凝胶电泳，然后转移至PVDF膜上，5%脱脂奶粉TBS液封闭2h，分别加入抗大鼠Bax、Akt、p-Akt单克隆抗体（一抗），4℃孵育过夜，TBST液洗涤后加入辣根过氧化物酶标记的二抗室温孵育2h，TBST洗涤后利用增强化学发光法显色，使用图像分析系统进行灰度分析。

1.9 统计学处理 采用GraphPad Prism5 软件进行统计分析，数据结果以±s表示，组间比较采用单因素方差分析(One-way ANOVA)，进一步两两比较采用Dunnett t检验，P<0.05为差异有统计学意义。

2 结 果
2.1 BMSCs形态观察 原代细胞接种后，细胞悬浮于培养液中，呈大小不一的圆形，24h后部分细胞开始贴壁，后大量贴壁细胞呈不均匀散在分布。7d后更多细胞贴壁，呈散在的放射状集落排列。P1、P2和P3代细胞24h后完全贴壁生长，呈形态均匀的梭形(图1)。

2.2 BMSCs表面抗原鉴定结果 流式细胞仪分析结果显示，培养细胞的CD29表达阳性率为99.6%，CD45表达阳性率为99.8%，CD34表达阳性率为2.4%，证明所培养细胞为BMSCs。因在体外扩增过程中保持了其表面抗原和形态特征，可排除其他细胞的污染(图2)。

2.3 BMSCs诱导分化结果 经成脂诱导分化后的细胞胞质内出现红色脂肪小滴，表明所培养的BMSCs具备向脂肪细胞分化的能力，在扩增培养的过程中保持了干细胞的特征(图3A)。经成骨诱导分化后的细胞胞质内出现棕色小颗粒沉淀，骨钙素免疫组化染色阳性，表明成功诱导为成骨细胞，也同时进一步证明体外分离培养的细胞为BMSCs(图3B)。

2.4 经流体切应力及APN处理后BMSCs的镜下状态和存活率 与对照组相比，SS组细胞皱缩明显，细胞活力显著受抑(P<0.05)。与SS组相比，SS+APN组细胞的状态更好，细胞皱缩明显改善，存活细胞显著增多(P<0.05，图4)

2.5 经流体切应力及APN处理后BMSCs凋亡情况 TUNEL染色结果显示，与对照组相比，SS组BMSCs凋亡显著增加，而采用20 μg/ml APN干预后凋亡细胞明显减少(P<0.05，图5)。

2.6 各组关键因子蛋白表达情况 Western blotting 检测结果显示，3组间Akt蛋白表达无明显差异。与对照组相比，SS组p-Akt表达明显下调，Bax表达明显上调，而与SS组相比，SS+APN组p-Akt表达明显上调，Bax表达明显下调(P<0.05，图6)。

3 讨 论
临床需要大量的血管移植物，而自体血管取材
图4 BMSCs承受切应力及脂联素处理后显微镜下观察结果(100)

Fig. 4 Morphology of BMSCs under shear stress and adiponectin treatment (100)

A. Control group; B. SS group; C. SS+APN group; D. A value. (1) $P<0.05$ compared with control group; (2) $P<0.05$ compared with SS group

图5 BMSCs承受切应力及脂联素处理后TUNEL染色结果(200)

Fig. 5 Morphology of BMSCs under shear stress and adiponectin treatment (TUNEL staining 200)

A. Control group; B. SS group; C. SS+APN group; D. Apoptotic index. (1) $P<0.05$ compared with control group; (2) $P<0.05$ compared with SS group

图6 BMSCs承受流体切应力及脂联素处理后Akt、pAkt及Bax蛋白表达情况

Fig. 6 Expression of Akt, pAkt and Bax in BMSCs under shear stress and adiponectin treatment

(1) $P<0.05$ compared with control group; (2) $P<0.05$ compared with SS group

受限，异体血管因免疫排斥反应严重而难以存活，直径小于6mm的生物材料或人工合成材料血管植入体内后容易形成血栓而导致移植失败，因此具有修复功能的组织工程血管逐渐成为近年来国内外的研究热点。构建组织工程血管的方法是将有功能的种子细胞种植于可降解的支架上，体外构建后再植入体内[4]。组织工程血管理想的种子细胞包括脂肪间充质干细胞、内皮祖细胞和BMSCs等[5]。BMSCs以其在成体容易分离，增殖能力强，取材方便，易于体外扩增，可逃避免疫系统攻击，具有多向分化潜能，在不同条件下向骨、软骨、神经、心肌、骨骺肌等多方向分化等优势而成为研究热点。组织工程血管植入体内后必然承受血流切应力的作用，人体大动脉承受的血流切应力一般为1.5~3.0dyne/cm²，静脉承受的切应力一般为1.5~3.0dyne/cm²，但种子细胞难以适应体内高强度的流体切应力，因此如何提高种子细胞的抗流体切应力成为临床研究的热点[8]。

APN是脂肪组织分泌的细胞因子，分子量30kD，在循环血液中的含量为2~17μg/ml，主要分为全长脂联素(full-length adiponectin，fAPN)和球形脂联素(globular adiponectin，gAPN)两种。其中gAPN是fAPN的活性结构域，参与细胞内多种信号的调节[10]。已有研究表明脂联素在能量代谢、炎症和增殖等细胞生理过程中发挥保护作用，可促进脂肪酸氧化、增加胰岛素敏感性、降低血浆葡萄糖水平，发挥抗炎、抗动脉粥样硬化、减少内皮细胞凋亡和抗动脉血栓形成等作用[11-13]。我们在前期研
研究中发现脂联素可显著促进承受流体切应力作用的BMSCs的增殖[6]。本实验进一步采用平板液体流动腔模型对BMSCs施加流体切应力，并给以脂联素干预，结果证实脂联素显著抑制流体切应力作用下BMSCs的凋亡，增强其活力，其主要机制是通过激活信号通路中的关键分子Akt上调Bax的表达，从而抑制凋亡的发生。这一发现有望进一步改善BMSCs作为组织工程血管种子细胞的生物学功能，推进组织工程血管的临床应用，为患者带来曙光。

【参考文献】

(收稿日期: 2016-08-12；修回日期: 2016-09-03)