Ghrelin预处理骨髓间充质干细胞来源上清液对内皮细胞增殖、迁移及凋亡的影响

葛珊慧，张莉珊，林 山，曾 勉
（中山大学1.附属第一医院内科重症监护室；2.呼吸病研究所，广东 广州 510080）

摘 要：【目的】研究生长激素释放肽(ghrelin)预处理骨髓间充质干细胞(BMSCs)来源上清液对内皮细胞增殖、迁移及凋亡的影响，并初步探索其作用机制。【方法】全骨髓贴壁分离法提取大鼠BMSCs，利用流式细胞术鉴定BMSCs表面分子标记。收集BMSCs上清液及高、中、低浓度(100 nmol/L、10 nmol/L、1 nmol/L)ghrelin预处理BMSCs 24 h后上清液，以无血清培养基为对照，将上清液分别加入人脐静脉细胞融合细胞(EA.hy926)作用24 h，利用CCK8法检测各组细胞活力，划痕实验评估各组细胞迁移能力。上述各组细胞加入脂多糖处理24 h后，用Annexin V法检测各组细胞凋亡率，Western blot法检测各组细胞凋亡蛋白bax、caspase3及β-catenin表达情况。【结果】与未处理BMSCs相比，高浓度ghrelin预处理BMSCs来源上清液能增强内皮细胞活力及迁移能力，差异有统计学意义(P<0.05)，流式细胞结果显示，100 nmol/L ghrelin预处理BMSCs组内皮细胞凋亡率(34.51±3.51)%较未处理BMSCs组(41.11±1.83)%显著降低，差异有统计学意义(P<0.05)。Western blot结果显示，100 nmol/L ghrelin预处理BMSCs组较未处理BMSCs组凋亡蛋白bax及caspase 3与β-catenin表达下降。【结论】Ghrelin预处理BMSCs可以改善内皮细胞功能，其中抗内皮细胞凋亡作用可能与其抑制Wnt/β-catenin通路激活有关。

关键词：急性呼吸窘迫综合征；生长激素释放肽；骨髓间充质干细胞；内皮细胞
中图分类号：R56 **文献标志码：**A **文章编号：**1672-3554(2020)05-0681-09

Effect of Conditioned Medium of Bone Marrow Mesenchymal Stem Cells Pretreated by Ghrelin on Proliferation, Migration and Apoptosis of Endothelial Cells

GE Shan-hui, ZHANG Li-shan, LIN Shan, ZENG Mian
(1. Medical Intensive Care Unit, First Affiliated Hospital; 2. Institute of Respiratory Diseases, Sun Yat-sen University, Guangzhou 510080, China)
Correspondence to: ZENG Mian, E-mail: zengmian2004@163.com

Abstract:【Objective】To investigate the impact of conditioned medium derived from bone marrow mesenchymal stem cells (BMSCs) pretreated by ghrelin on proliferation, migration and apoptosis of endothelial cells (EA.hy926) and its underlying mechanism.【Methods】Rat BMSCs were isolated and cultured in vitro by the whole bone marrow adherence method, and surface markers of BMSCs were identified with flow cytometry. The conditioned medium of BMSCs and BMSCs pretreated by high, moderate and low concentration (100 nmol/L, 10 nmol/L and 1 nmol/L) of ghrelin for 24 hours was collected and co-cultured with EA.hy926 for 24 hours, and the serum-free medium was considered as the control group. Cell viability was detected by cell counting kit-8 while migration ability was analyzed by scratch assays. The cell apoptosis rates were measured by Annexin V method after treatment of lipopolysaccharide for 24 hours, and the protein expression of bax, caspase3, and β-catenin were detected by western blot.【Results】Compared with untreated BMSCs

收稿日期：2020-06-16
基金项目：国家自然科学基金(81670066)；广东省基础与应用基础研究基金(2019A1515011198)；广东省科技计划项目(2016A020216009)；2017年贝朗蛇牌学院重症科学研究基金
作者简介：葛珊慧，在读研究生，研究方向：呼吸与危重症医学，E-mail：geshh12@163.com；曾勉，通信作者，博士研究生导师，教授，主任医师，研究方向：呼吸与危重症医学，E-mail：zengmian2004@163.com

group, high concentration of ghrelin pretreated BMSCs group indicated higher cell viability and migration ability ($P<0.05$). In light of the result of flow cytometry, endothelial cells apoptosis rate of 100 nmol/L ghrelin pretreated BMSCs group (34.51±3.51%) were remarkably lower than that of untreated BMSCsgroup ($P<0.05$). As revealed by western blot, expression level of bax, caspase3, and β-catenin were reduced in 100 nmol/L ghrelin pretreated BMSCs group compared with untreated BMSCs group ($P<0.05$).【Conclusion】Endothelial cell function was improved when co-cultured in conditioned medium of BMSCs pretreated by ghrelin, and the attenuation of LPS-induced apoptosis was related with inhibition of Wnt/β-catenin signal path in endothelial cells.

Key words: acute respiratory distress syndrome; ghrelin; bone marrow mesenchymal stem cells; endothelial cell

[J SUN Yat-sen Univ(Med Sci), 2020, 41(5): 681-689]

急性呼吸窘迫综合征(acute respiratory distress syndrome, ARDS)是临床上常见的一种高死亡率疾病,其临床特点为短期进展的呼吸困难、低氧血症($PaO_2/FIO_2 \leq 300$ mmHg)及渗透性肺水肿[1-2],其发病机制复杂,而肺毛细血管内皮细胞受损是ARDS发生的重要事件[3]。间充质干细胞(mesenchymal stem cells, MSCs)是一种具有多向分化潜能及自我更新能力的成体干细胞,是组织工程及再生医学的种子细胞。研究发现,骨髓间充质干细胞(bone marrow mesenchymal stem cells, BMSCs)可通过其旁分泌途径减轻脂多糖(LPS)诱导的急性肺损伤[4-5]。生长激素释放肽(ghrelin)是生长激素释放激素受体的内源性配体,研究显示其参与调节食物摄入、能量代谢、肌肉萎缩、骨代谢以及细胞增殖与凋亡等病理生理过程[6-7],近年来研究指出,ghrelin对MSCs存在某些调控作用,如Abdanipour等[8]发现ghrelin可显著促进MSCs的增殖活力,Han等[9]指出ghrelin可通过激活PI3K/AKT通路减轻MSCs的凋亡,增强其在缺氧状态下的存活能力,提高其对缺血性心脏病的治疗效果。这种ghrelin对MSCs调控作用是否同样也能够增强其对急性肺损伤(acutelunginjury, ALI)的治疗效果,具体的作用机制是什么?这些问题目前尚未清楚,因此,本研究通过观察在ghrelin预处理BMSCs来源的上清液中培养的内皮细胞的增殖、迁移及凋亡情况探讨ghrelin预处理后BMSCs对内皮细胞的作用,为治疗ALI/ARDS提供一种新的思路。

1 材料与方法

1.1 材 料

1.1.1 实验动物 3周龄,SPF级雄性Sprague Dawley大鼠(平均体质量50 g),由中山大学实验动物中心提供,实验动物生产许可证号:SCXK(粤)2016-0029,本实验经中山大学附属第一医院实验动物伦理委员会批准,实验过程对动物处理符合动物伦理学要求。

1.1.2 实验试剂 低糖DMEM培养基(c11885500bt)、高糖DMEM培养基(c11995500bt)、胎牛血清(Gibco公司,美国)、青霉素-链霉素双抗(Thermo Fisher)、2.5 g/L EDTA胰酶(Thermo Fisher)、CCK8细胞增殖毒性检测试剂盒(东仁(DOJINDO), CK04)、Ghrelin-human(Tocris)、脂多糖(来源于大肠杆菌0127:B8,货号L3129,Sigma-Aldrich公司)、流式表面分子抗体:Anti-Mouse/Rat CD29 (eBioscience)、Anti-Mouse/Rat CD90.1(eBioscience)、FITC Mouse Anti-Rat CD45(BD Pharmingen);Annexin V-FITC/PI双染细胞凋亡试剂盒(BD Pharmingen)、Anti-甘油醛-3-磷酸脱氢酶(glyceraldehyde-3-phosphate dehydrogenase, GAPDH)抗体(cell Signaling Technology)、Anti-Bax抗体(cell Signaling Technology)、Anti-caspase3(cell Signaling Technology)、Anti-β-catenin抗体(cell Signaling Technology)、SDS-PAGE凝胶配制试剂盒(Beyotime)、RIPA裂解液(强)、PMSF试剂(Beyotime)、NuPAGE LDS Sample Buffer (4×)上样缓冲液(Thermo Fisher)、电泳液(自配)、转膜液(自配)、磷酸盐缓冲液(Phosphate Buffered Saline(PBS),自配)、TBST缓冲液(自配)、含2%牛血清白蛋白PBS溶液(PBA,自配)、PVDF膜(0.45 μm,Merck millipore公司)、特超敏ECL化学发光试剂盒(Beyotime)。

1.1.3 实验仪器 超净工作台,CO_2培养箱(Thermo Fisher)、低温高速离心机(Eppendorf公司)、化学发光成像分析系统(ImageQuant Las4000mini)、流式细胞仪(CytoFLEX, BECHMAN COULTER)、全自动倒置荧光显微镜(Leica DMi8)、酶标仪

（Sunrise）、电泳仪（Bio-Rad）、转膜装置。

1.2　间充质干细胞提取、培养与鉴定

1.2.1　大鼠BMSCs的提取与培养　本实验沿用之前课题组采用的全骨髓贴壁分离法[10]提取大鼠BMSCs。取3周龄SD大鼠，腹腔注射戊巴比妥钠处死后将大鼠放进75%乙醇浸泡5 min，在超净工作台内用高压灭菌过的手术器械分离胫骨、股骨，用无菌PBS冲洗骨髓至发白，将骨髓悬液收集至15 mL离心管，300 ×*g*离心5 min，弃去上清，用含10%胎牛血清低糖DMEM完全培养基重悬细胞沉淀，吹打混匀后转移至25 cm^2细胞培养瓶中，置于37℃，5%体积分数CO_2饱和湿度培养箱中，24 h后显微镜下观察可见大量漂浮圆形细胞，为大鼠骨髓中的血红细胞，换液，去除漂浮的血细胞，此后2~3 d换1次液，直至细胞融合80%~90%，可进行传代。

1.2.2　大鼠BMSCs的流式鉴定　胰酶消化BMSCs（P3），300 ×*g*离心5 min，弃上清液，预冷PBA 1 mL洗涤细胞一次，加入100 μL PBA重悬细胞，用枪头轻轻吹打混匀，4℃避光孵育30 min，然后用预冷PBA离心洗涤两次，以除去未结合的多余抗体成分，最后用100 μL PBA重悬细胞，吹打混匀，置于流式管中，上机检测（测量终浓度1×10^6/100 μL）。

1.3　内皮细胞培养

本实验采用人EA.hy926细胞（人脐静脉内皮细胞与人肺腺癌细胞A549融合细胞）作为替代模拟肺泡内皮细胞，EA.hy926细胞从American Type Culture Collection获取，具有内皮细胞的生物学特性，用含100 mL/L胎牛血清高糖DMEM完全培养基培养，置于37 ℃，5%体积分数CO_2饱和湿度培养箱中培养。

1.4　BMSCs上清液的提取

取处于对数生长期的第3代BMSCs，待细胞贴壁后分别加入高、中、低浓度ghrelin（100、10、1、0 nmol/L），24 h后换为无血清低糖DMEM培养基，培养24 h后取上清液，离心后取上清备用。

1.5　CCK8测定BMSCs上清对内皮细胞增殖能力的影响

内皮细胞（EA.hy926）以2 000/孔接种于96孔板中，待细胞贴壁后弃去原培养基，加入各组BMSCs上清液100 μL培养24 h，每孔加入10 μL CCK8溶液，37 ℃孵育2 h后于酶标仪450 nm处测量吸光度（OD值）。

1.6　划痕试验评估BMSCs上清对内皮细胞迁移能力的影响

在六孔板背面用marker笔每隔0.5~1 cm画一横线，取各组对数生长期EA.hy926细胞接种于六孔板，待六孔板细胞长至约90%融合后，用200 μL无菌枪头，垂直于横线画线，每孔至少穿过5条线，划痕完成后，用无菌PBS洗细胞3次，洗去不贴壁细胞，使划线后留下的痕迹清晰可见，最后加入1 mL各组BMSCs上清液，细胞放入37 ℃，体积分数5%CO_2培养箱培养，然后在0 h，24 h显微镜下观察并测量划痕之间的面积，并拍照，结果使用Image J软件分析。

1.7　BMSCs上清对脂多糖诱导EA.hy926凋亡的影响

EA.hy926接种于六孔板，待细胞长至70~80%融合后，加入1 mL各组BMSCs上清液，同时加入脂多糖（lipopolysaccharide，LPS）诱导凋亡，培养24 h后进行后续处理分析。

1.7.1　流式细胞计数分析凋亡细胞数量　上述各组细胞加不含EDTA胰酶消化后，300 ×*g*离心5 min，用预冷PBS缓冲液洗涤细胞一次，然后加入300 μL的1X binding buffer悬浮细胞，再加入5 μL的Annexin V-FITC避光，室温孵育15 min，上机前5 min再加入5 μL的PI（碘化丙啶，Propidium Iodide）染色，然后用CytoFLEX流式细胞仪分析早期及晚期凋亡情况。

1.7.2　western blot分析各组蛋白的表达情况　上述各组细胞用强RIPA裂解液裂解后，4 ℃ 14 000 ×*g*离心10 min，取上清，用BCA法测定各组蛋白浓度，加入上样缓冲液后于95 ℃变性15 min；每孔蛋白上样量取30 μg，10%SDS-PAGE凝胶电泳后进行转膜，转膜完成后用2%牛血清白蛋白（bovine serum albumin，BSA）溶液封闭PVDF膜1 h，TBST缓冲液洗涤3次，每次5 min，分别加GAPDH（1∶1 000）、Bax（1∶1 000）、caspase3（1∶1 000）、β-catenin（1∶1 000）一抗4度孵育过夜，TBST缓冲液洗涤3次，每次10 min，加二抗（1∶5 000）室温孵育1 h，TBST缓冲液洗涤3次，加ECL发光液显影，蛋白条带结果使用image J软件分析。

1.8　统计学分析

实验数据采用SPSS 25.0作统计学分析，呈正态分布的数据以均数±标准差（$\bar{x} \pm s$）表示，非正态分布的数据以四分位数M（P_{25}~P_{75}）表示。多组比

较,当数据呈正态分布且方差齐时,采用单因素方差分析(One-way ANOVA),方差分析有统计学意义时,两两比较采用LSD-t检验。当数据呈非正态分布或方差不齐时,则采用Kruskal Wallis H检验进行统计分析,两两比较采用Bonferroni法。以P<0.05为差异有统计学意义。

2 结 果

2.1 BMSCs形态及鉴定

利用全骨髓贴壁法提取BMSCs,多次传代提纯以后悬浮细胞大大减少,镜下可见P3 BMSCs贴壁生长,形态均匀一致,呈纺锤形(图1),流式抗体鉴定BMSCs表面分子可见,细胞高表达CD29(99.78%±0.19%)、CD90(98.92%±0.94%)表面抗原,而低表达造血干细胞相关的表面抗原CD45(4.92%±0.20%),符合间充质干细胞的生物学特性,可用于后续实验(图2)。

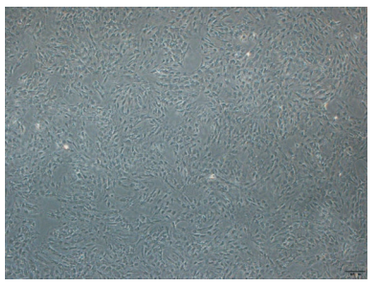
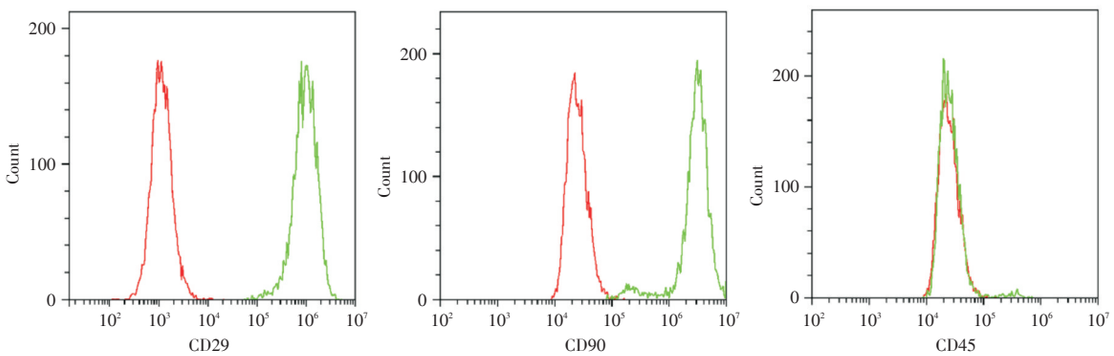


图1 光镜下大鼠BMSCs(P3)形态(40×)

Fig.1 The third generation of rat BMSCs in vitro(40×)



Surface antigens of the third generation BMSCs were detected by flow cytometry. Representative histograms are shown. Figures from the left to right revealed the expression of surface antigens CD29, CD90, and CD45, respectively.

图2 BMSCs表面标志物表达情况

Fig.2 Expression of Surface markers in BMSCs

2.2 梯度浓度ghrelin预处理BMSCs上清液对内皮细胞增殖能力影响

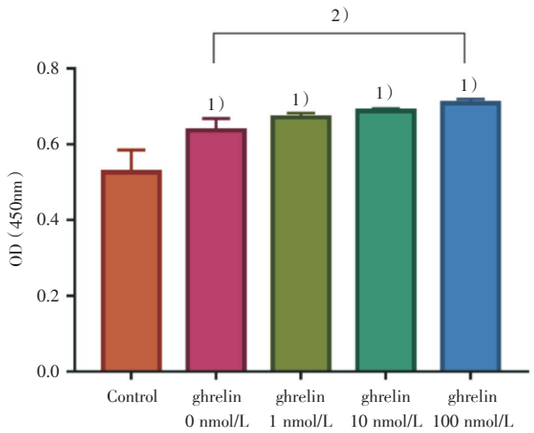
本实验采用CCK8法检测内皮细胞的活力,如图3所示,与无血清处理组相比,BMSCs上清可以增强内皮细胞活力,而相较于未给予ghrelin预处理的BMSCs,100 nmol/L ghrelin预处理BMSCs能促进内皮细胞的增殖,差异有统计学意义(P<0.05)。

2.3 梯度浓度ghrelin预处理BMSCs上清液对内皮细胞迁移能力的影响

划痕实验结果(图4)显示,对照组及各实验组之间差异有统计学意义(P<0.05)。具体而言,与无血清处理组(33.87±1.94)%相比,BMSCs(54.37±3.52)%、1 nmol/L ghrelin+ BMSCs(60.37±5.95)%、10 nmol/L ghrelin+ BMSCs(69.83±3.67)%、100 nmol/L ghrelin+BMSCs(73.70±2.10)%组24 h面积修复率明显升高,差异有统计学意义(P<0.05)。而与BMSCs处理组相比,10、100 nmol/L ghrelin预处理BMSCs组内皮细胞迁移能力增强,差异有统计学意义(P<0.05),10 nmol/L ghrelin预处理BMSCs组与100 nmol/L ghrelin预处理BMSCs组24 h面积修复率未见统计学差异(P>0.05)。

2.4 梯度浓度ghrelin预处理BMSCs上清液对LPS诱导内皮细胞凋亡的影响

流式细胞术是一种常见的检测细胞凋亡情况的手段,利用AnnexinV及PI双染法标记凋亡细胞,可以直观地了解细胞凋亡比例。结果如图5所示,空白对照组总凋亡率为(6.60±1.07)%,加入



Cell viability of endothelial cells cultured in serum-free medium and the conditioned media of BMSCs which were pretreated by ghrelin for 24 hours. One-way ANOVA was conducted for differences of cell viability among five groups (F=13.522, P<0.001) and LSD-t tests were performed for multiple comparison. 1) P<0.05 compared with control group. 2) P<0.05 compared between BMSCs group and 100 nmol/L ghrelin pretreated BMSCs group. Data wereexpressed as *mean* ± *SD*, n=3.

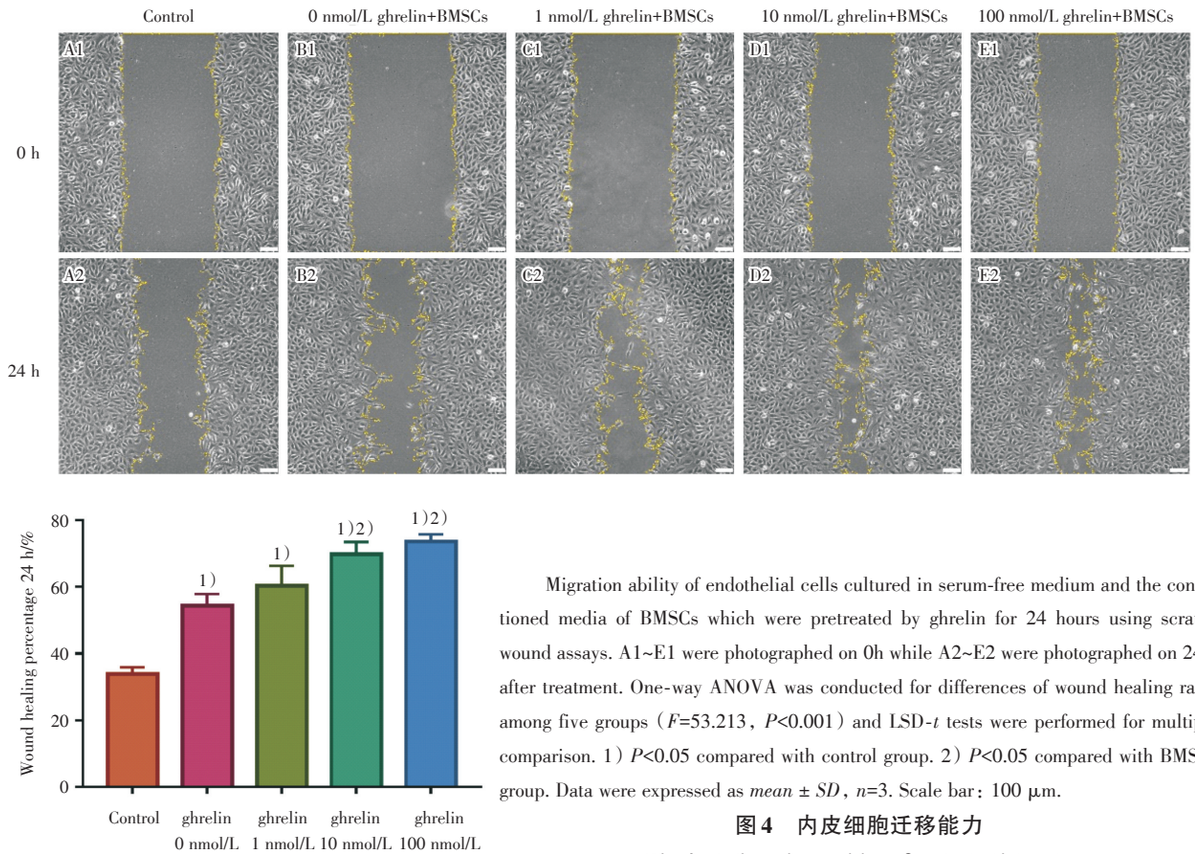
图3　CCK8检测内皮细胞活力

Fig.3　Analysis of cell viability of endothelial cells by cell counting kit-8

脂多糖诱导损伤后总凋亡率为(49.91±1.66)%，两组之间差异有统计学意义(P<0.05)，提示脂多糖(LPS)可以诱导内皮细胞凋亡。与损伤组相比，BMSCs+LPS 组(41.11±1.83)%、1 nmol/L ghrelin+BMSCs+LPS 组(40.64±1.52)%、10 nmol/L ghrelin+BMSCs+LPS 组(38.92±2.26)%及 100 nmol/L ghrelin+BMSCs+LPS 组(34.51±3.51)%总凋亡率均降低，差异有统计学意义(P<0.05)，说明 BMSCs 可以减轻 LPS 诱导的内皮细胞凋亡程度。而与 BMSCs+LPS 组相比，只有 100 nmol/L ghrelin+ BMSCs+ LPS 组细胞凋亡率明显降低(P<0.05)，提示高浓度 ghrelin 预处理后的 BMSCs 能更明显地降低 LPS 诱导的内皮细胞凋亡水平。

2.5　梯度浓度 ghrelin 预处理 BMSCs 上清液对内皮细胞相关蛋白表达的影响

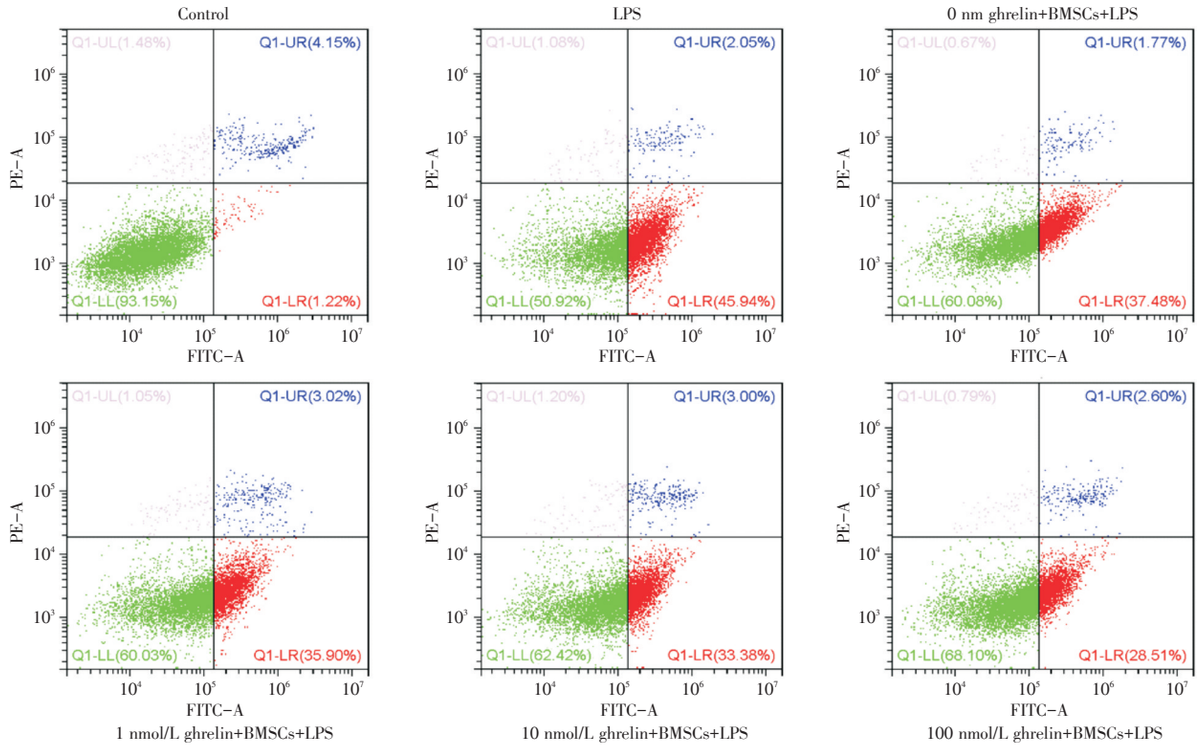
Western blot 实验结果(图6)表明，LPS 处理后内皮细胞凋亡蛋白 Bax 及 caspase 3 和β-catenin 的表达水平明显升高(P<0.05)，而 100 nmol/L ghrelin 预处理 BMSCs 能够显著降低上述蛋白的表达水平(P<0.05)。

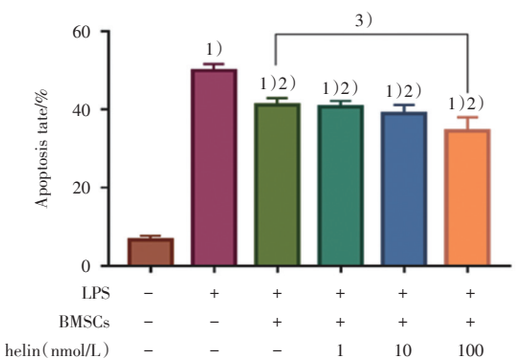


Migration ability of endothelial cells cultured in serum-free medium and the conditioned media of BMSCs which were pretreated by ghrelin for 24 hours using scratch wound assays. A1~E1 were photographed on 0h while A2~E2 were photographed on 24 h after treatment. One-way ANOVA was conducted for differences of wound healing rates among five groups (F=53.213, P<0.001) and LSD-t tests were performed for multiple comparison. 1) P<0.05 compared with control group. 2) P<0.05 compared with BMSCs group. Data were expressed as *mean* ± *SD*, n=3. Scale bar: 100 μm.

图4　内皮细胞迁移能力

Fig.4　Migration ability of endothelial cells





Apoptosis of endothelial cells cultured in serum-free medium and the conditioned media of BMSCs which were pretreated by ghrelin for 24 hours weredetected by staining of annexinV-FITC/PI with flow-cytometry.All groups were treated with LPS except the control group. One-way ANOVA was conducted for differences of apoptosis rates among six groups (F=148.576, P<0.001) and LSD-t tests were performed for multiple comparison. 1) P<0.05 compared with control group. 2) P<0.05 compared with LPS group. 3) P<0.05 compared between BMSCs+LPS group and 100 nmol/L ghrelin+ BMSCs+LPS group. Data was expressed as mean ± SD, n=3.

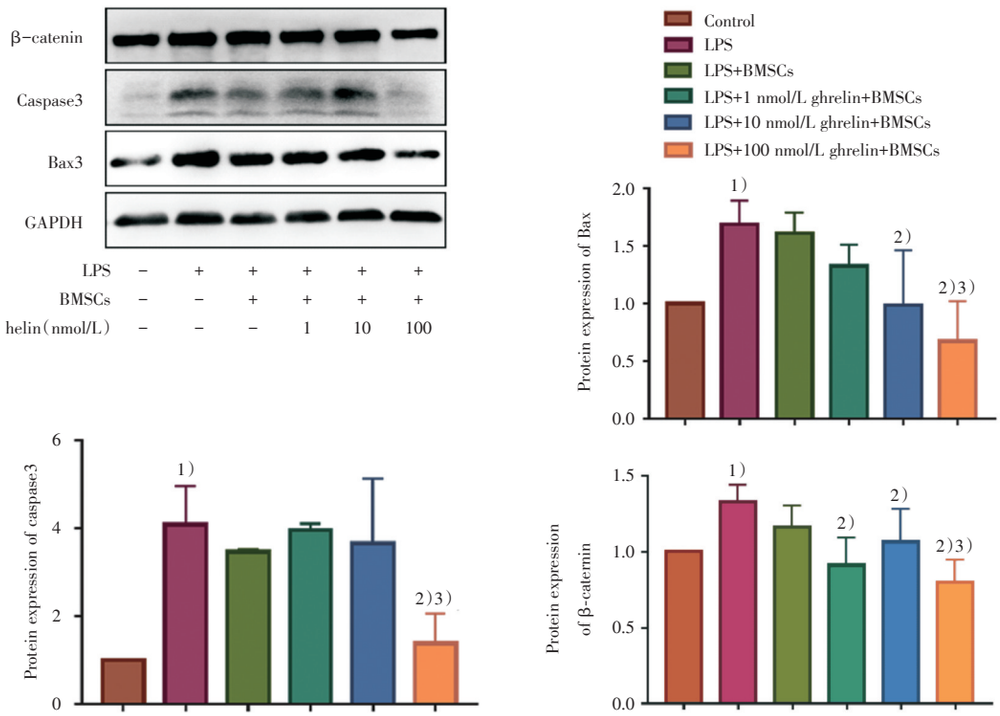
图5 流式细胞术分析内皮细胞凋亡率

Fig.5 Analysis of endothelial cells′ apoptosis rates with flow-cytometry

3 讨 论

目前ARDS病死率仍居高不下,大约为35%~46%,而其治疗手段主要依靠抗感染及对症治疗,缺少针对发病机制的治疗方法[11]。各种致病因子对肺泡上皮细胞及肺泡微血管内皮细胞的损伤导致的“血-气屏障”被破坏是ARDS发生的关键,由LPS或TNF-α、IL-1等促炎因子介导的免疫细胞应答紊乱,进而促炎/抗炎反应失衡、瀑布式炎症反应发生,炎症反应过程所产生的活性氧、蛋白酶及细胞因子等可以破坏肺微血管内皮细胞的完整性与通透性,导致凝血状态异常及微血栓形成[12-13],因此抑制肺泡内皮细胞凋亡及炎症反应是治疗ARDS的一个研究方向。BMSCs作为一种来源广泛、易于获得的多能干细胞,其分泌的一些细胞因子、生长因子及生物活性因子能够起到减轻炎症反应、修复损伤组织及免疫调节作用[14-15],目前已经有许多研究报道了间充质干细胞来源的外泌体或者细胞培养上清对ARDS肺泡血管内皮细胞的治疗价值[16-18],我们的实验结果也证实了这一点,将来源于BMSCs的上清液与内皮细胞共培养可以增强内皮细胞活力及迁移能力,并减轻脂多糖诱导的细胞凋亡。

ghrelin—生长激素释放激素受体的内源性配体,与肺损伤修复及炎症反应密切相关。我们课题组前期研究中发现ghrelin通过降低肺泡巨噬细胞[19]及肺泡上皮细胞[20]凋亡减轻脓毒症肺损伤。



Expression of bax, caspase 3, and β-catenin proteinof endothelial cells cultured in serum-free medium and the conditioned media of BMSCs which were pretreated by ghrelin for 24 hours were detected by western blot. All groups were treated with LPS except the control group. Kruskal Wallis *H* test (for Bax, H=19.730, P=0.001; for caspsae3, H=13.289, P=0.021) or One-way ANOVA (for β-catenin, F=4.698, P=0.013) was conducted for differences of protein expression level among six groups and multiple comparison was performed. 1) P<0.05 compared with control group. 2) P<0.05 compared with LPS group. 3) P<0.05 compared between BMSCs+LPS group and 100 nmol/L ghrelin+ BMSCs+ LPS group. Data was expressed as mean ± SD, n=3.

图6　免疫印迹法检测内皮细胞蛋白表达情况

Fig.6　Analysis of protein expression of endothelial cells with western blot

Ye等[21-22]发现，生长激素释放激素受体(GHSR)在BMSCs上高表达，ghrelin通过与GHSR结合激活ERK1/2通路促进BMSCs增殖、成骨分化及成软骨分化，也有研究指出，ghrelin预处理MSCs能够增强其对缺血性心脏病的治疗效果[9]，因此，ghrelin可能通过结合BMSCs上的GSHR改变了BMSCs的某些生物特性，从而影响了BMSCs的治疗效果。本实验利用梯度浓度ghrelin预处理BMSCs来源上清液与内皮细胞共培养，探索其对内皮细胞增殖、迁移及凋亡的影响，结果提示高浓度(100 nmol/L) ghrelin预处理能够增强BMSCs促进内皮细胞增殖、迁移以及抗脂多糖诱导细胞凋亡的能力。由此我们猜测，ghrelin预处理BMSCs可能促进其分泌某些生物因子，从而改善内皮细胞的功能，但具体是什么成分起到了作用，需要进一步的研究。

Wnt/β-catenin通路调控细胞增殖与凋亡、炎症及免疫反应[23-24]，是机体生长发育的重要信号通路之一。β-连环蛋白(β-catenin)是Wnt/β-catenin途径的关键转录共激活因子，研究发现其参与胚胎肺发育[25]，肺组织损伤修复[26]，肺纤维化[27]等肺部病理生理过程。为了进一步探索ghrelin预处理BMSCs减轻LPS诱导内皮细胞凋亡的具体机制，我们对内皮细胞β-catenin蛋白水平进行了检测。本实验结果显示，LPS处理后内皮细胞β-catenin蛋白表达上调，而100 nmol/L ghrelin预处理BMSCs能够下调内皮细胞中β-catenin蛋白的表达水平，同时细胞凋亡蛋白Bax、caspase 3表达降低，流式细胞术结果也证实ghrelin预处理BMSCs可使内皮细胞凋亡率下降。Villar等[28]研究指出Wnt/β-catenin通路在脓毒症诱导肺损伤早期即激活，参与肺损伤后的异常修复及肺纤维化过程。实际上，Wnt/β-catenin通路在调控细胞凋亡及炎症反应方面起到了重要作用，研究发现，β-catenin蛋白在细胞凋亡时表达上调，下调β-catenin蛋白

表达及抑制Wnt/β-catenin通路激活可以减轻细胞凋亡以及降低细胞中促炎因子IL-6，IL-8，TNF-α等表达水平，减轻LPS诱导的炎症反应[29-30]。我们的结果也显示ghrelin预处理BMSCs减轻了脂多糖诱导的内皮细胞损伤，同时下调了β-catenin蛋白的表达，与上述研究一致。因此，我们认为ghrelin预处理BMSCs可能通过抑制Wnt/β-catenin通路的激活来减轻LPS诱导的内皮细胞凋亡。

综上，本研究结果提示，ghrelin预处理BMSCs上清液能增强肺泡血管内皮细胞的活力及迁移能力，改善脂多糖诱导的内皮细胞凋亡，其中减轻脂多糖诱导的内皮细胞凋亡作用可能与下调β-catenin表达、抑制Wnt/β-catenin通路激活有关，但具体是BMSCs上清液的何种成分，具体如何调控Wnt/β-catenin通路的上游分子如p-GSK3β、GSK3β及下游靶基因Axin2，Cyclin D1和Cyclin E1等，后续实验将进一步探讨。

参考文献

[1] Bellani G, Laffey JG, Pham T, et al. Epidemiology, patterns of care, and mortality for patients with acute respiratory distress syndrome in intensive care units in 50 countries[J]. JAMA, 2016,315(8):788-800.

[2] Ranieri VM, Rubenfeld GD, Thompson BT, et al. Acute respiratory distress syndrome: the berlin definition[J]. JAMA, 2012,307(23):2526-2533.

[3] Nova Z, Skovierova H, Calkovska A. Alveolar-Capillary membrane-related pulmonary cells as a target in endotoxin-induced acute lung injury[J]. Int J Mol Sci, 2019,20(4):831.

[4] Rahbarghazi R, Keyhanmanesh R, Aslani MR, et al. Bone marrow mesenchymal stem cells and condition media diminish inflammatory adhesion molecules of pulmonary endothelial cells in an ovalbumin-induced asthmatic rat model[J]. Microvasc Res, 2019,121:63-70.

[5] Su VY, Lin C, Hung S, et al. Mesenchymal stem cell-conditioned medium induces neutrophil apoptosis associated with inhibition of the NF-κBpathway in endotoxin-induced acute lung injury[J]. Int J Mol Sci, 2019,20(9):2208.

[6] Pradhan G, Samson SL, Sun Y. Ghrelin: much more than a hunger hormone[J]. Curr Opin Clin Nutr Metab Care, 2013,16(6):619-624.

[7] Kurowska P, Mlyczynska E, Rak A. Effect of ghrelin on the apoptosis of various cells. a critical review[J]. J Physiol Pharmacol, 2019,70(1):3-13.

[8] Abdanipour A, Shahsavandi B, Alipour M, et al. Ghrelin upregulates hoxb4 gene expression in rat bone marrow stromal cells[J]. Cell J, 2018,20(2):183-187.

[9] Han D, Huang W, Ma S, et al. Ghrelin improves functional survival of engrafted adipose-derived mesenchymal stem cells in ischemic heart through PI3K/Akt signaling pathway[J]. Biomed Res Int, 2015,2015:1-12.

[10] 陈钦桂，郑海崇，何婉媚，等. 小鼠骨髓间充质干细胞的分离培养及其诱导分化为肺泡上皮细胞[J]. 医学研究生学报，2017,30(12):1283-1288.
Chen QG, Zheng HC, He WM, et al. Isolation and culture of mouse bone marrow mesenchymal stem cells and induced differentiation into alveolar epithelial cells[J]. Med Postgra, 2017, 30(12):1283-1288.

[11] Parhar K, Zjadewicz K, Soo A, et al. Epidemiology, mechanical power, and 3-year outcomes in acute respiratory distress syndrome patients using standardized screening an observational cohort study[J]. Ann Am Thorac Soc,2019,16(10):1263-1272.

[12] Millar FR, Summers C, Griffiths MJ, et al. The pulmonary endothelium in acute respiratory distress syndrome: insights and therapeutic opportunities[J]. Thorax, 2016,71(5):462-473.

[13] Frantzeskaki F, Armaganidis A, Orfanos SE. Immunothrombosis in acute respiratory distress syndrome: cross talks between inflammation and coagulation[J]. Respiration, 2017,93(3):212-225.

[14] Wu P, Zhang B, Shi H, et al. MSC-exosome: a

novel cell-free therapy for cutaneous regeneration [J]. Cytotherapy, 2018,20(3):291-301.

[15] Yang Y, Hu S, Xu X, et al. The vascular endothelial growth factors-expressing character of mesenchymal stem cells plays a positive role in treatment of acute lung injury in vivo [J]. Mediators Inflamm, 2016,2016:1-12.

[16] Meng SS, Guo FM, Zhang XW, et al. mTOR/STAT-3 pathway mediates mesenchymal stem cell-secreted hepatocyte growth factor protective effects against lipopolysaccharide-induced vascular endothelial barrier dysfunction and apoptosis [J]. J Cell Biochem, 2019,120(3):3637-3650.

[17] Potter DR, Miyazawa BY, Gibb SL, et al. Mesenchymal stem cell-derived extracellular vesicles attenuate pulmonary vascular permeability and lung injury induced by hemorrhagic shock and trauma [J]. J Trauma Acute Care Surg, 2018,84(2):245-256.

[18] Tang X, Shi L, Monsel A, et al. Mesenchymalstem cell microvesiclesattenuate acute lung injury in mice partly mediated byAng-1 mRNA [J]. Stem Cells., 2017,35(7):1849-1859.

[19] Li B, Zeng M, He W, et al. Ghrelin protects alveolar macrophages against lipopolysaccharide-induced apoptosis through growth hormone secretagogue receptor 1a-dependent c-Jun N-terminal kinase and Wnt/β-cateninsignaling and suppresses lung inflammation[J]. Endocrinology, 2015,156(1):203-217.

[20] Huang C, Zheng H, He W, et al. Ghrelin ameliorates the human alveolar epithelial A549? cell apoptosis induced by lipopolysaccharide [J]. Biochem Biophys Res Commun, 2016,474(1):83-90.

[21] Ye N, Jiang D. Ghrelin accelerates the growth and osteogenic differentiation of rabbit mesenchymal stem cells through the ERK1/2 pathway [J]. BMC Biotechnol, 2015,15:51.

[22] Ye N, Wang L, Dou Z, et al. Ghrelin accelerates the cartilagic differentiation of rabbit mesenchymal stem cells through the ERK1/2 pathway [J]. Cytotechnology, 2018,70(1):415-421.

[23] Wong D, Yiu WH, Chan KW, et al. Activated renal tubular Wnt/beta-catenin signaling triggers renal inflammation during overload proteinuria [J]. Kidney Int, 2018,93(6):1367-1383.

[24] Tan Z, Zheng H, Liu X, et al. MicroRNA-1229 overexpression promotes cell proliferation and tumorigenicity and activates Wnt/beta-catenin signaling in breast cancer[J]. Oncotarget, 2016,7(17):24076-24087.

[25] Hussain M, Xu C, Lu M, et al. Wnt/β-catenin signaling links embryonic lung development and asthmatic airway remodeling [J]. Biochim BiophysActa Mol Basis Dis, 2017,1863(12):3226-3242.

[26] Flozak AS, Lam AP, Russell S, et al. β-catenin/T-cell factor signaling is activated during lung injury and promotes the survival and migration of alveolar epithelial cells [J]. J Biol Chem, 2010,285(5):3157-3167.

[27] Zhu Y, Tan J, Xie H, et al. HIF-1αregulates EMT via the snail andβ-catenin pathways in paraquat poisoning-induced early pulmonary fibrosis [J]. J Cell Mol Med, 2016,20(4):688-697.

[28] Villar J, Cabrera-Benítez NE, Ramos-Nuez A, et al. Early activation of pro-fibrotic WNT5A in sepsis-induced acute lung injury [J]. Critical care, 2014,18(5):568.

[29] Li B, Zhang H, Zeng M, et al. Bone marrow mesenchymal stem cells protect alveolar macrophages from lipopolysaccharide-induced apoptosis partially by inhibiting the Wnt/beta-catenin pathway [J]. Cell Biol Int, 2015,39(2):192-200.

[30] Jang J, Jung Y, Chae S, et al. XAV939, a Wnt/beta-catenin pathway modulator, has inhibitory effects on LPS-induced inflammatory response [J]. Immunopharmacol Immunotoxicol, 2019,41(3):394-402.

（编辑　孙慧兰）