论著·基础医学

骨髓间充质干细胞通过抑制NLRP3/GSDMD介导的细胞焦亡减轻重症急性胰腺炎炎症与组织损伤

李亚希[1],金 晶[2],张雅婧[1],孟唤男[1],赵 航[1] [1]上海中医药大学附属市中医医院消化内科,上海;[2]海军军医大学第一附属医院消化内科,上海

［摘要］ **目的** 探索骨髓间充质干细胞(mesenchymal stem cells,MSCs)在抑制焦亡通路治疗重症急性胰腺炎(severe acute pancreatitis,SAP)大鼠模型的作用机制。**方法** 冲洗骨髓腔法分离提取4周龄SPF级雄性SD大鼠[体质量(100±20)g]骨髓MSCs,并进行培养鉴定。将24只6~8周龄SPF级雄性大鼠[体质量(200±20)g]采用随机数字表法随机分为4组(n=6):假手术组、SAP模型组、乌司他丁组、MSCs治疗组。SAP模型组、乌司他丁组、MSCs治疗组采用逆行胰胆管注射5%牛黄胆酸钠制备大鼠SAP模型,SAP模型组于造模后12 h注射100 μL PBS;乌司他丁组于模型制备完成后立即腹腔注射乌司他丁溶液(5×10^4 U/kg);MSCs治疗组于造模后12 h注射MSCs细胞悬液(5×10^6个/kg);假手术组切开腹腔并数次翻动胰腺后,关闭腹腔。HE染色比较各组间胰腺组织形态,并进行病理学评分;试剂盒检测血清淀粉酶、脂肪酶水平;ELISA法检测血清炎症因子IL-10、IL-1β、TNF-α水平;透射电镜(transmission electron microscopy,TEM)观察胰腺腺泡细胞超微形态相关变化;免疫荧光观察胰腺组织NLRP3、GSDMD、caspase-1蛋白表达情况;qRT-PCR法检测胰腺组织NLRP3、GSDMD、caspase-1的mRNA含量;Western blot法检测胰腺组织NLRP3、GSDMD、GSDMD-N、caspase-1、cleaved caspase-1、IL-1β的蛋白相对表达量。**结果** 骨髓MSCs具有成纤维细胞样纺锤体形状,并贴附在培养板的壁上;CD29(99.22%)呈高表达,CD45(0.42%)呈低表达;具有成脂、成骨分化能力,细胞状态良好。与假手术组相比,SAP模型组在胰腺组织病理学评分、血清淀粉酶、脂肪酶、炎症因子等方面均有显著差异(P<0.000 1)。与SAP模型组相比,乌司他丁组、MSCs治疗组胰腺组织病理学评分、血清淀粉酶、脂肪酶、IL-1β、TNF-α水平均降低(P<0.001),IL-10水平升高(P<0.05);且MSCs治疗组上述指标较乌司他丁组明显好转(P<0.05)。TEM显示MSCs治疗组腺泡细胞线粒体嵴恢复,线粒体形态改变,内质网肿胀减少;且与乌司他丁组相比,MSCs治疗组线粒体与内质网损伤程度减轻。免疫荧光结果显示,与SAP模型组相比,MSCs治疗组和乌司他丁组的胰腺组织NLRP3、GSDMD、caspase-1表达量下降(P<0.000 1)。Western blot结果示,与SAP模型组相比,MSCs治疗组和乌司他丁组胰腺组织NLRP3、GSDMD、GSDMD-N、caspase-1、cleaved caspase-1、IL-1β蛋白相对表达量均有不同程度下降(P<0.000 1)。qPR-PCR结果示,与SAP模型组相比,MSCs治疗组和乌司他丁组胰腺组织NLRP3、GSDMD、caspase-1 mRNA表达水平明显下降(P<0.01);且与乌司他丁组相比,MSCs治疗组上述指标均下降更为显著(P<0.001)。**结论** MSCs减轻炎症反应,减少胰腺组织损伤,可通过抑制NLRP3/GSDMD/IL-1β通路改善腺泡细胞焦亡并缓解SAP。

［关键词］ 重症急性胰腺炎;间充质干细胞;焦亡;腺泡细胞

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［通信作者］ 赵 航,E-mail:drzhaohang@163.com

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Bone marrow-derived mesenchymal stem cells alleviate inflammation and tissue injury in severe acute pancreatitis by suppressing NLRP3/GSDMD-mediated pyroptosis

LI Yaxi[1], JIN Jing[2], ZHANG Yajing[1], MENG Huannan[1], ZHAO Hang[1] [1]Department of Gastroenterology, Shanghai Municipal Hospital of Traditional Chinese Medicine Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai; [2]Department of Gastroenterology, the First Affiliated Hospital of Naval Medical University, Shanghai, China

[**Abstract**] Objective To investigate the mechanism of bone marrow-derived mesenchymal stem cells (MSCs) in alleviating severe acute pancreatitis (SAP) by inhibiting the pyroptosis pathway in a rat model. Methods Bone marrow-derived MSCs were isolated from 4-week-old male SPF SD rats [body weight, (100±20) g] using the bone marrow flush technique, followed by culture and characterization. Twenty-four male SPF SD rats [body weight (200±20) g] were randomly assigned to four groups (n=6 per group) using a random number table: a sham-operated group, an SAP model group, an ulinastatin-treated group, and an MSCs-treated group. SAP was induced in the latter three groups via retrograde injection of 5%sodium taurocholate into the biliopancreatic duct. The SAP model group received an injection of 100 μL PBS at 12 h post-modeling. The ulinastatin-treated group received an intraperitoneal injection of ulinastatin solution (5×10^4 U/kg) immediately after model induction. The MSCs-treated group received an injection of MSC suspension (5×10^6 cells/kg) at 12 h post-modeling. Sham-operated rats underwent laparotomy with gentle pancreatic manipulation before abdominal closure. Pancreatic histopathology was assessed by HE staining and pathological scoring. Serum amylase and lipase activities were measured using commercial assay kits. Serum levels of IL-10, IL-1β, and TNF-α were determined by ELISA. The ultrastructural changes of pancreatic acinar cells were observed under transmission electron microscopy (TEM). Protein expression of NLRP3, GSDMD, and caspase-1 in pancreatic tissue was evaluated by immunofluorescence. The mRNA levels of NLRP3, GSDMD, and caspase-1 were quantified by qRT-PCR. The relative protein expression levels of NLRP3, GSDMD, GSDMD-N, caspase-1, cleaved caspase-1, and IL-1β were analyzed by Western blotting. Results The isolated cells exhibited a typical adherent, spindle-shaped, fibroblast-like morphology. Flow cytometry confirmed they were highly positive for the mesenchymal marker CD29 (99.22%) and nearly negative for the hematopoietic marker CD45 (0.42%). These cells also demonstrated osteogenic and adipogenic differentiation potential and were maintained in good condition. Compared with the sham-operated group, the SAP model group showed significantly increased pancreatic histopathological scores, elevated serum amylase and lipase activities, higher levels of pro-inflammatory cytokines (IL-1β and TNF-α), and a lower level of IL-10 (all P<0.0001). Both ulinastatin and MSCs treatment significantly attenuated these changes, reducing histopathological scores, serum enzyme activities, IL-1β, and TNF-α (P<0.001), while increasing IL-10 (P<0.05). Importantly, the MSCs-treated group showed significantly greater improvement in all these metrics compared to the ulinastatin-treated group (P<0.05). TEM analysis revealed that MSCs treatment promoted the recovery of mitochondrial cristae structure, improved overall organelle morphology, and reduced endoplasmic reticulum swelling in acinar cells, with these restorative effects being more pronounced than in the ulinastatin group. The immunofluorescence results showed that compared with the SAP model group, the expression levels of NLRP3, GSDMD, and caspase-1 in pancreatic tissue were decreased in both the MSCs-treated group and the ulinastatin-treated group (P<0.0001). Western blot results indicated that, compared with the SAP model group, the relative protein expression levels of NLRP3, GSDMD, GSDMD-N, caspase-1, cleaved caspase-1, and IL-1β in pancreatic tissue were reduced to varying degrees in both the MSCs-treated group and the ulinastatin-treated group (P<0.0001). qPR-PCR results demonstrated that, compared with the SAP model group, the mRNA expression levels of NLRP3, GSDMD, and caspase-1 in pancreatic tissue were significantly decreased in both the MSCs-treated group and the ulinastatin-treated group (P<0.01). Moreover, compared with the ulinastatin-treated group, the MSCs-treated group exhibited a more significant decrease in all the aforementioned indicators (P<0.001). Conclusion Mesenchymal stem cells alleviate inflammation and tissue damage in severe acute pancreatitis. Their protective mechanism involves inhibiting the NLRP3/GSDMD/IL-1β signaling axis, thereby suppressing acinar cell pyroptosis.

[**Key words**] severe acute pancreatitis; mesenchymal stem cells; pyroptosis; acinar cells

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重症急性胰腺炎(severe acute pancreatitis, SAP)是一种常见的急性消化系统疾病,据统计,SAP的全球年发病率约为33.74/10万人,且呈逐年增长的趋势[1-2]。SAP病因复杂,其三大病因依次为:胆道疾病(58.3%)、高脂血症(13.6%)和饮酒(7.1%)[2]。临床SAP治疗仍是难点,部分患者预后不理想,病死率最高达50%[3]。作为一种病因复杂的危重急症,SAP仍是目前公共卫生系统的重要经济负担,消耗大量医疗资源[3-4]。因此,探索一种安全、高效的治疗手段是目前的研究重点。

腺泡细胞是胰腺组织的主要外分泌细胞,其死亡可引起胰酶释放和全身炎症,导致胰腺损伤[5]。腺泡细胞死亡可由腺泡细胞发生焦亡引起,GAO等[6]研究发现在急性胰腺炎期间,腺泡细胞内NOD样受体蛋白3(NOD like receptor protein 3, NLRP3)炎性小体被激活后,可进一步切割消皮素D(gasdermin D, GSDMD)产生其N端活性片段(GSDMD-N),后者在细胞膜上寡聚成孔,改变膜结构与渗透压,促进细胞焦亡加剧急性胰腺炎的病理进程。焦亡是一种新型形式的细胞程序性死亡,不同于凋亡和自噬,其特征为NLRP3炎性小体的形成以及半胱天冬酶(caspase)和gasdermin家族成员的激活,焦亡可以由caspase-1依赖性信号通路或caspase-1非依赖性信号通路诱导激活,从而诱导炎症因子大量表达,介导细胞过度焦亡,加重组织和器官损伤[7-8]。

间充质干细胞(mesenchymal stem cells, MSCs)是一种多潜能成体干细胞,其来源广泛,可从脐带、牙髓、脂肪、骨髓等组织中提取获得,因其低免疫原性、强大的自我更新及分化潜能,越来越多的研究将其作为一种新兴细胞治疗手段[9-10]。已有研究表明,MSCs不仅可通过分化能力修复受损细胞和组织,而且可通过抑制免疫,调控炎症微环境发挥独特的免疫调节功能[11-12]。骨髓是MSCs的重要来源,已有多项研究运用骨髓MSCs治疗SAP,均证实MSCs能抑制炎症反应、减少腺泡细胞损伤,改善SAP,然而其作用机制尚不明确[11-12]。基于此,本研究就骨髓MSCs对SAP大鼠的炎症抑制作用及其对NLRP3/GSDMD/IL-1β通路的影响进行研究,以探讨骨髓MSCs治疗SAP的潜在机制。

1 材料与方法

1.1 实验动物

24只6~8周龄SPF级雄性SD大鼠[体质量(200±20)g],5只4周龄SPF级雄性SD大鼠[体质量(100±20)g],均购自杰思捷实验动物有限公司[生产许可证号:SCXK(沪)2023-0004]。动物实验经上海中医药大学附属市中医医院实验动物伦理委员会批准(2025017)。实验用的所有SD大鼠在SPF级环境中饲养,大鼠自由饮水摄食,适应性喂养7 d。

1.2 药物、主要试剂和仪器

戊巴比妥钠购自天津市河东区红岩试剂厂,牛磺胆酸钠购自美国Sigma公司,乌司他丁购自广东天普生化药业公司,淀粉酶试剂盒、脂肪酶试剂盒购自南京建成公司,L-DMEM培养基、胎牛血清、青霉素、链霉素购自美国Gibco公司,PBS溶液购自白鲨公司,4%多聚甲醛购自江苏Vicmed公司,TRIzol试剂、反转录试剂盒购自诺唯赞公司,qPCR试剂购自日本TaKaRa公司,HE试剂盒购自碧云天公司,IL-10、IL-1β、TNF-α ELISA试剂盒购自武汉菲恩公司,NLRP3、IL-1β、GSDMD一抗购自美国Abcam公司,caspase-1、cleaved caspase-1购自美国CST公司,山羊抗兔二抗、一步法PAGE凝胶快速制备试剂盒、通用型抗体稀释液、超灵敏化学发光检测试剂盒购自雅酶公司,流式细胞仪购自美国安捷伦公司,酶标仪购自美国美谷分子公司,实时荧光定量分析仪购自德国罗氏Light Cycle 480公司。

1.3 骨髓间充质干细胞的提取、培养及鉴定

造模前1周,采用随机数字表法选取5只4周龄SD大鼠,经腹腔注射2%戊巴比妥钠(50 mg/kg)对动物实施麻醉,待麻醉后脱颈处死,自股骨、胫骨收集原代骨髓MSCs。骨髓腔以L-DMEM(含20%胎牛血清、100 U/mL青霉素、100 U/mL链霉素)基础培养基冲洗,在5%CO_2、37 ℃的培养箱中培养。当细胞生长密度达到90%时进行传代培养。使用流式细胞术鉴定MSCs的表面特异性抗原,包括CD29和CD45。使用成脂和成骨诱导的分化培养基(Cyagen,美国)来表征MSCs的分化能力。钙结节用0.1 mg/mL茜素红标记,脂滴用0.5%油红O染料标记。选择来自第3~5代的细胞进行后续实验。

1.4 动物模型制备

在整个手术过程中,研究人员严格遵循无菌技术的原则。为避免围术期出现胃肠道不良反应,所有大鼠在术前12 h禁食。术前2 h对动物手术室进行紫外线照射(紫外线辐射强度>120.0 μW/cm^2),使用前使用高压蒸汽灭菌器(0.14 MPa,126.0 ℃,30 min)对相关手术器械进行灭菌。在手术期间吸入异氟醚以诱导(2.5%)和维持(1.5%)所有大鼠的麻醉状态。24只6~8周龄SPF级雄性大鼠采用随机数字表法分为4组(n=6):假手术组、SAP模型组、乌司他丁组和MSCs治疗组。SAP模型组、乌司他丁组、MSCs治疗组进行SAP造模,采用2%戊巴比妥钠(50 mg/kg)腹腔注射麻

醉大鼠,随后将其置于仰卧位,固定在手术台上,随后用碘伏消毒大鼠腹部皮肤。将大鼠腹壁沿腹部前中线切开,使用微量注射泵逆行将5.0%牛黄胆酸钠溶液(1.0 mL/kg,0.1 mL/min)注射到胰管中,然后关闭腹腔。SAP模型组造模后12 h,通过尾静脉注射100 μL PBS溶液;乌司他丁组在模型制备后立即在大鼠腹腔注射乌司他丁(5×10^4 U/kg);MSCs治疗组在模型建立后12 h,通过大鼠尾静脉注射MSCs(100 μL PBS重悬,5.0×10^6个/kg)。假手术组如上所述打开腹腔后,用无菌棉签将胰腺翻转数次,然后闭合腹腔;在模型建立后12 h通过尾静脉将等体积PBS溶液注射到大鼠体内。

1.5 HE染色观察胰腺组织病理变化

在模型建立后72 h,以2%戊巴比妥钠(50 mg/kg)腹腔注射麻醉后处死所有大鼠,打开腹腔,以静脉采血针穿刺下腔静脉,抽取3 mL血液,以备检测血清淀粉酶、脂肪酶水平;打开腹腔,找到并分离胰腺组织,将胰腺组织分为5份。其中4份置于液氮保存,以备检测焦亡指标NLRP3、caspase-1、cleaved caspase-1、GSDMD、GSDMD-N、IL-1β蛋白;余1份放入4%多聚甲醛固定做HE染色。

HE染色观察确定SAP的严重程度。用体积分数10%的甲醛溶液固定胰腺组织以制备胰腺组织切片;将胰腺组织经梯度乙醇脱水,二甲苯透明后,浸蜡包埋。将包埋好的蜡块固定于切片机上,切成5~8 μm厚的薄片。将薄片烫平,再贴到载玻片上,45 ℃恒温烘干。脱蜡后行HE染色。染色后的切片经纯乙醇脱水,再经二甲苯使切片透明。将已透明的切片滴上树胶,盖上盖玻片封固。待树胶干燥后,荧光显微镜下观察各组小鼠胰腺组织病理形态变化。根据LYU等[13]的评分指南评估水肿、炎症浸润和坏死的程度。

1.6 检测血清淀粉酶、脂肪酶水平

造模后72 h取大鼠下腔静脉血,在4 ℃上以3 000 r/min离心10 min,取上层血清。血清淀粉酶和脂肪酶水平使用商业试剂盒进行分析。

1.7 ELISA法检测血清IL-1β、TNF-α、IL-10水平

用收集到的血清使用ELISA试剂盒检测血清IL-1β、TNF-α和IL-10的水平。

1.8 透射电镜(transmission electron microscopy,TEM)成像

将新鲜胰腺组织浸泡3.0%戊二醛溶液,用1.0%四氧化锇溶液固定,用丙酮浓度梯度脱水,包埋在Epon812中,制成半薄切片和厚度为70 nm的超薄切片。将超薄切片置于200目铜网格上,用醋酸铀酰和柠檬酸铅溶液染色,然后用透射电镜在80 kV下观察。

1.9 免疫荧光观察胰腺组织焦亡相关蛋白表达情况

将脱蜡水化后的切片在柠檬酸盐缓冲液(pH=6.0)中加热10 min以进行抗原修复。用含有Tween20(PBST)的磷酸盐缓冲盐水洗涤切片3次,然后在室温下用5.0%血清溶液封闭切片1 h,用0.2%Triton X-100溶液透化,用PBST洗涤3次。执行上述相关程序后,将切片与NLRP3(1∶50)、caspase-1(1∶50)和GSDMD(1∶100)一抗在4 ℃下共孵育过夜。第2天,用PBST洗涤切片3次。将切片按照上述过程与山羊抗兔IgG(Alexa Fluor 488)抗体(1∶500)和4,6-二氨基-2-苯基吲哚(DAPI)抗体在远离光线的情况下依次共孵育。孵育后,用PBST再洗涤切片3次,并在荧光显微镜下拍摄图像。最后,使用Image J软件对图像进行半定量分析。

1.10 Western blot检测胰腺组织焦亡相关蛋白表达

采用Western blot检测NLRP3、caspase-1、cleaved caspase-1、GSDMD、GSDMD-N、IL-1β蛋白表达。取液氮中保存的胰腺组织研磨匀浆,置于1.5 mL EP管内,加入1 mL含0.1%蛋白酶抑制剂的PIPA裂解液,冰上匀浆,超声粉碎机粉碎。在4 ℃下以12 000 r/min离心10 min,取上清液。然后用蛋白质测定试剂盒进行蛋白质定量,用5×上样缓冲液(雅酶,中国)进行蛋白质变性,在98 ℃下加热10 min。待冷却至室温后,将等量的样品在十二烷基硫酸钠-聚丙烯酰胺凝胶中电泳,然后将十二烷基硫酸钠-聚丙烯酰胺凝胶上分离的蛋白质转移到甲醇饱和聚偏二氟乙烯膜上,然后在室温下用5%脱脂奶粉室温封闭1 h。加入NLRP3、caspase-1、cleaved caspase-1、GSDMD、IL-1β一抗(1∶1 000),在4 ℃下孵育过夜;加入山羊抗兔二抗IgG(1∶5 000),室温下孵育1 h。孵育后,在室温下以TBST摇床洗膜10 min,共3次。用凝胶成像仪曝光显影,观察NLRP3、caspase-1、cleaved caspase-1、GSDMD、GSDMD-N、IL-1β蛋白表达。用Image J图像分析软件,对各目标条带的灰度值进行分析。以β-actin为内参,目的蛋白表达水平根据目的蛋白条带灰度值/β-actin条带灰度值计算。

1.11 qRT-PCR检测胰腺组织NLRP3、GSDMD、caspase-1 mRNA相对表达量

胰腺组织充分研磨,依次加入TRIzol、氯仿、异丙醇、75%乙醇提取总RNA,根据诺唯赞反转录试剂盒说明书分别反转录为cDNA,反转录条件为37 ℃、15 min,85 ℃、5 s,保持4 ℃。参照TB Green®Premix EX Taq™ Ⅱ试剂盒说明书进行qRT-PCR检测,引物由生工生物工程(上海)股份有限公司合成,扩增引物序列见表1。扩增条件为:95 ℃预变性30 s;95 ℃变性5 s,60 ℃退火,延伸320 s,40个循环。计算$2^{-\Delta\Delta Ct}$得出各组相对表达量。

表1　qRT-PCR引物序列

Table 1　Primer sequences for qRT-PCR

Genes	Sequences (5'→3')
NLRP3	Upsteam:TGCATGCCGTATCTGGTTGT
	Downsteam:ATGTCCTGAGCCATGGAAGC
GSDMD	Upsteam:GGACAGTACCAGGCAGTTCG
	Downsteam:GTCACCAAGTAGGGCTGTGT
caspase-1	Upsteam:GACCGAGTGGTTCCCTCAAG
	Downsteam:GACGTGTACGAGTGGGTGTT
GAPDH	Upsteam:CCCCCAATGTATCCGTTGTG
	Downsteam:TAGCCCAGGATGCCCTTTAGT

1.12　统计学分析

采用SPSS 19.0统计软件分析数据,服从正态分布的计量资料以$\bar{x}\pm s$表示,重复计量资料比较采用单因素方差分析,两组比较采用LSD-t检验,组内比较采用独立样本t检验。P<0.05为差异具有统计学意义。

2　结果

2.1　三系分化检测鉴定原代骨髓MSCs

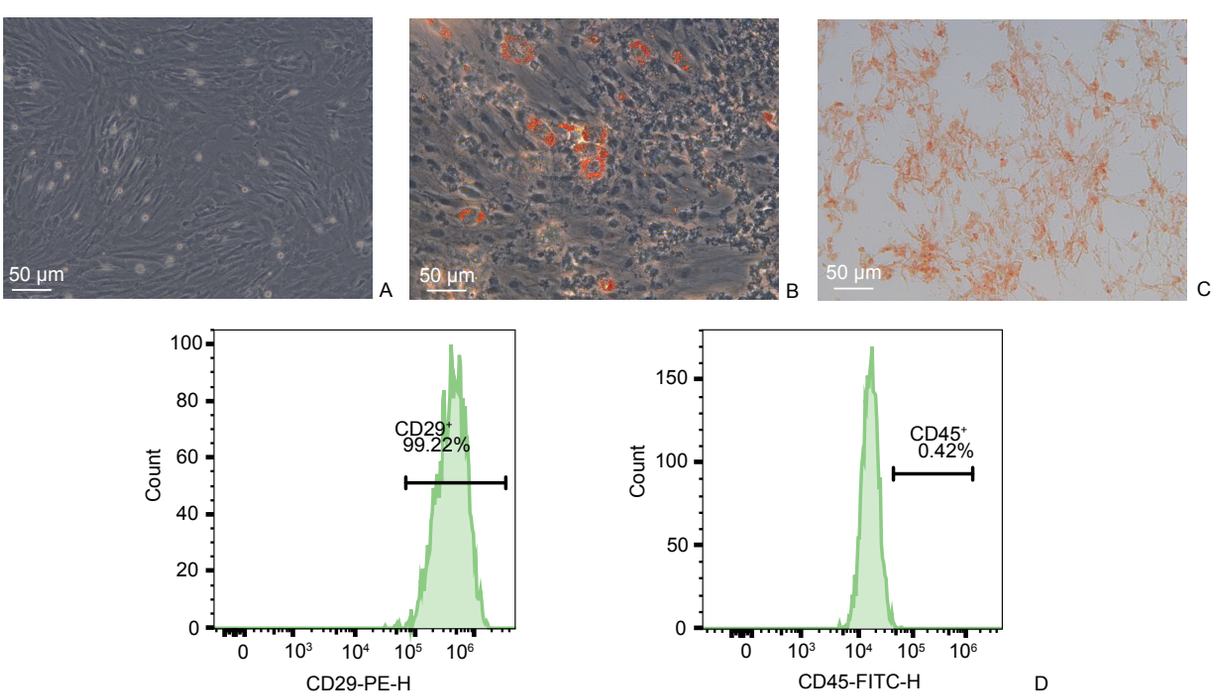
骨髓MSCs的三系分化鉴定如图1所示。骨髓MSCs具有成纤维细胞样纺锤体形状,并贴附在培养板的壁上(图1A)。脂肪分化诱导14 d后,油红O染色显示细胞质中出现橙红色脂滴(图1B),成骨分化诱导21 d后茜素红染色显示红色钙结节(图1C),表明MSCs可以分别分化为脂肪细胞和成骨细胞,具有分化能力。流式细胞术检测结果示CD29(99.22%)呈高表达,CD45(0.42%)呈低表达(图1D)。

2.2　骨髓MSCs改善胰腺组织病理损伤

胰腺组织HE评分结果显示,假手术组胰腺组织形态均正常,结构清晰,HE评分较低。与假手术组相比,SAP模型组胰腺组织腺体结构紊乱,存在坏死细胞,腺泡细胞水肿明显,腺体间隙及血管周围存在红细胞渗出,HE评分显著升高(P<0.000 1)。与SAP模型组相比,乌司他丁组和MSCs治疗组胰腺组织腺体结构轻微紊乱,腺泡轻微水肿,其坏死、出血均减轻,HE评分显著降低(P<0.05)。且与乌司他丁组相比,MSCs治疗组胰腺组织结构紊乱较少,腺泡水肿减轻,坏死、出血程度减轻,HE评分降低(P<0.05,图2、3)。

2.3　骨髓MSCs降低SAP血清淀粉酶、脂肪酶水平

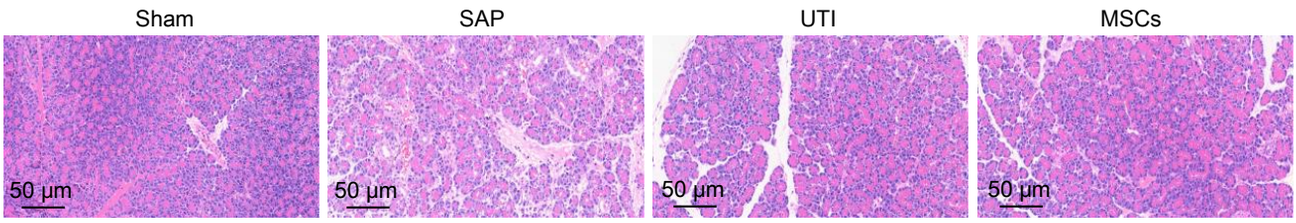
与假手术组相比,SAP模型组的淀粉酶、脂肪酶水平均明显升高(P<0.000 1)。乌司他丁组和MSCs治疗组血清淀粉酶、脂肪酶水平较SAP模型组相比,均显著降低(P<0.05);且与乌司他丁组相比,MSCs治疗组血清淀粉酶、脂肪酶水平下降更为显著(P<0.01,图4)。



A: Morphology of 3rd generation bone marrow MSCs; B: Adipogenic differentiation of BM-MSCs and oil red O staining; C: Osteogenic differentiation of BM-MSCS and alizarin red staining; D: Flow cytometry analysis showing high expression of CD29 (99.22%) and low expression of CD45 (0.42%)

图1　骨髓MSCs三系分化鉴定结果

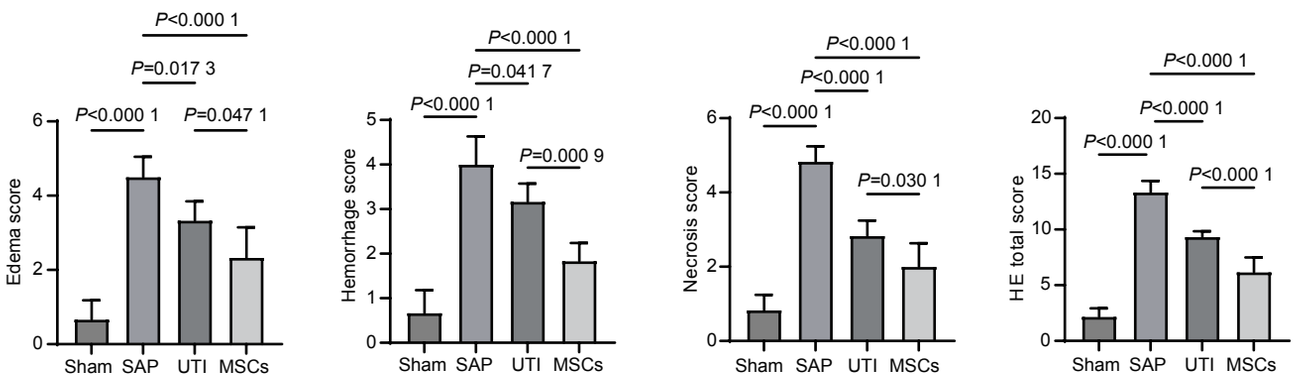
Figure 1　Identification of trilineage differentiation in bone marrow mesenchymal stem cells



UTI:Ulinastatin

图2 各组胰腺组织病理形态学改变

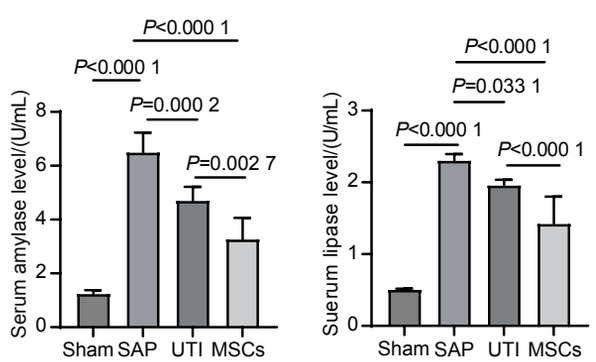
Figure 2 Histopathological changes of pancreatic tissues in each group



UTI:Ulinastatin

图3 各组大鼠胰腺组织病理学评分比较(n=6,$\bar{x}\pm s$)

Figure 3 Comparison of pathological scores in pancreatic tissues among experimental groups(n=6,$\bar{x}\pm s$)



UTI:Ulinastatin

图4 各组大鼠血清淀粉酶与脂肪酶水平比较(n=6,$\bar{x}\pm s$)

Figure 4 Comparison of serum amylase and lipase levels in rats from different groups(n=6,$\bar{x}\pm s$)

2.4 骨髓MSCs改善SAP炎症反应

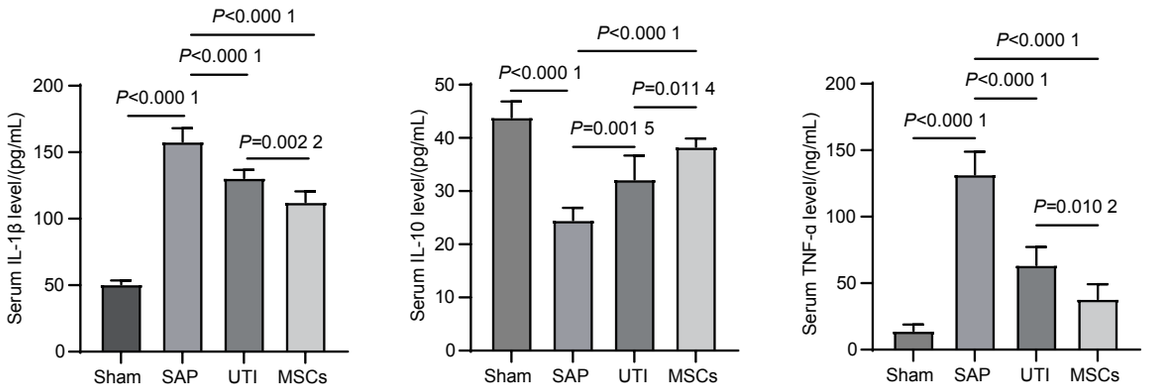
ELISA法检测血清IL-10、IL-1β、TNF-α炎症因子。结果显示,与假手术组相比,SAP模型组血清IL-1β、TNF-α水平均明显升高,IL-10水平明显降低(P<0.000 1)。与SAP模型组相比,乌司他丁组和MSCs治疗组血清IL-1β、TNF-α水平显著降低,IL-10水平明显升高(P<0.01);与乌司他丁组相比,MSCs治疗组血清IL-1β、TNF-α水平显著降低,IL-10水平升高(P<0.05,图5)。

2.5 TEM观察腺泡细胞的超微观形态特征

TEM结果显示,假手术组线粒体嵴正常,无肿胀;内质网排列紧密,无肿胀。与假手术组相比,SAP模型组腺泡细胞肿胀变大,线粒体肿胀,嵴结构不清晰或消失,核膜相对完整,内质网肿胀,排列紊乱,腔隙显著增宽、膨胀,部分呈碎片化断裂。与SAP模型组相比,乌司他丁组和MSCs治疗组腺泡细胞肿胀减小,线粒体肿胀减轻,线粒体内可见明显嵴结构;内质网排列轻度紊乱;且与乌司他丁组相比,MSCs治疗组线粒体和内质网受损程度减轻,线粒体嵴恢复明显,内质网腔隙减小,肿胀程度减轻(图6)。

2.6 骨髓MSCs有效减轻SAP大鼠胰腺组织焦亡相关蛋白表达水平

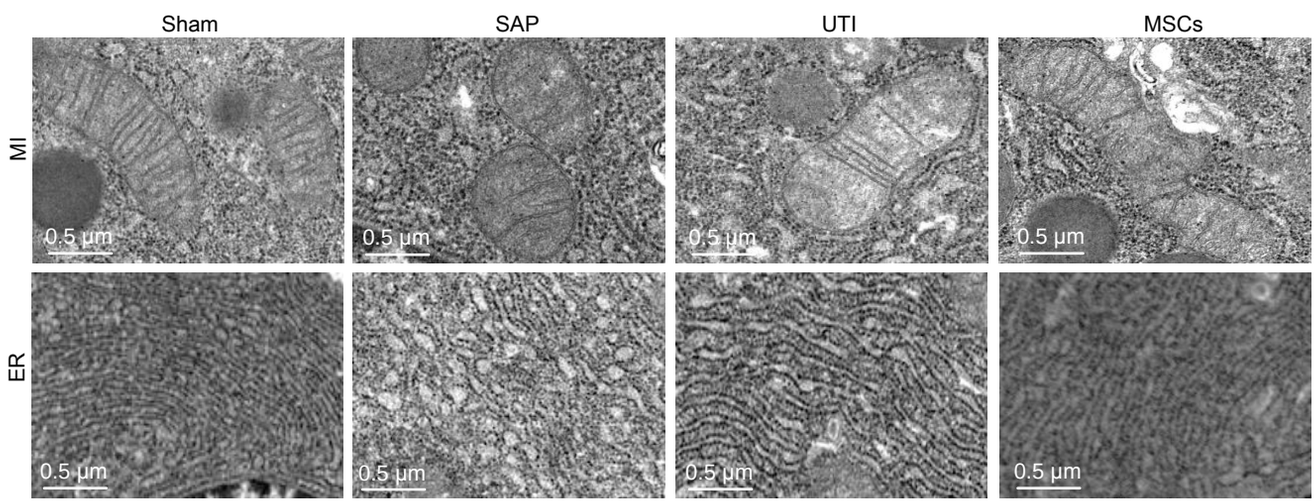
免疫荧光染色结果显示,与假手术组相比,SAP模型组中NLRP3、GSDMD、caspase-1蛋白表达显著升高(P<0.000 1);与SAP模型组相比,乌司他丁组和MSCs治疗组NLRP3、GSDMD、caspase-1蛋白表达明显降低(P<0.01);且较乌司他丁组相比,MSCs治疗组NLRP3、GSDMD、caspase-1蛋白表达显著下降(P<0.05,图7)。



UTI:Ulinastatin

图5 各组大鼠血清IL-1β、IL-10与TNF-α水平比较(n=6,$\bar{x}\pm s$)

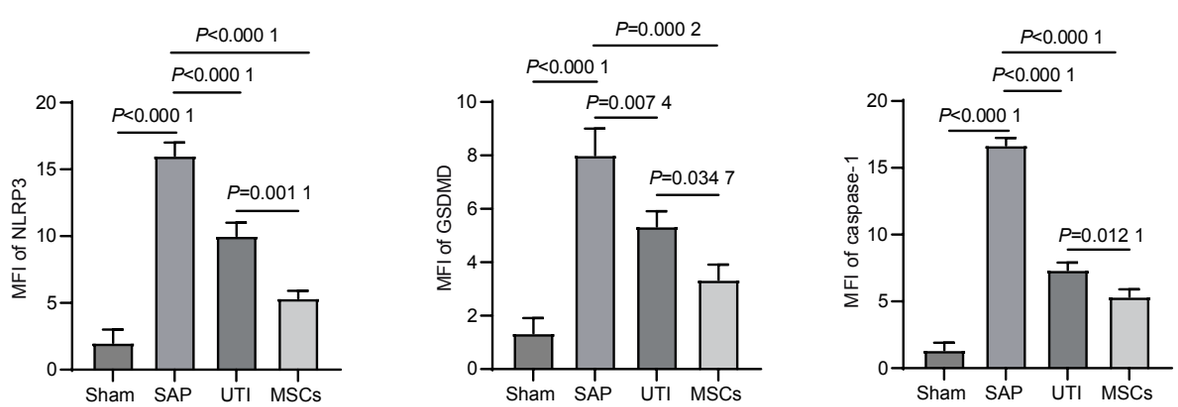
Figure 5 Comparison of serum levels of IL-1β, IL-10, and TNF-α in different experimental groups of rats(n=6,$\bar{x}\pm s$)



UTI:Ulinastatin;MI:Mitochondrial index; ER:Endoplasmic reticulum

图6 透射电镜下各组腺泡细胞的超微观形态特征

Figure 6 Ultrastructural characteristics of acinar cells in each group with transmission electron microscopy



MFI:Mean fluorescence intensity;UTI:Ulinastatin

图7 各组大鼠胰腺组织NLRP3、GSDMD与caspase-1蛋白免疫荧光定量分析(n=6,$\bar{x}\pm s$)

Figure 7 Quantitative immunofluorescence analysis of NLRP3, GSDMD, and caspase-1 protein expression in pancreatic tissues of rats in different groups(n=6,$\bar{x}\pm s$)

2.7 骨髓MSCs抑制SAP焦亡相关基因mRNA表达

与假手术组比较,SAP模型组胰腺组织NLRP3、GSDMD、caspase-1 mRNA相对表达量均显著升高(P<0.000 1);与模型组相比,乌司他丁组和MSCs治疗组大鼠胰腺组织3种基因相对表达水平显著降低(P<0.000 1);且与乌司他丁组相比,MSCs治疗组基因表达水平下降更为明显(P<0.01,图8)。

2.8 骨髓MSCs抑制NLRP3/GSDMD/IL-1β焦亡通路减轻SAP胰腺损伤

与假手术组相比,SAP模型组胰腺组织焦亡相关蛋白NLRP3、caspase-1、cleaved caspase-1、GSDMD、GSDMD-N、IL-1β蛋白相对表达量均显著升高(P<0.000 1)。与SAP模型组相比,乌司他丁组和MSCs治疗组胰腺组织NLRP3、caspase-1、cleaved caspase-1、GSDMD、GSDMD-N、IL-1β蛋白相对表达量均显著降低(P<0.000 1);且与乌司他丁组相比,MSCs治疗组蛋白表达水平下降更为明显(P<0.01,图9)。

3 讨论

SAP是一种严重的胃肠道急性疾病,其病理机制为胆汁、十二指肠液等消化液返流于胰管,激活胰管内的消化酶原,活化的消化酶自我消化胰腺组织,导致大量IL-1β、TNF-α、IL-6等促炎因子被释放,诱发炎症反应;重症情况下,炎症可向全身扩散,引起多器官功能障碍甚至衰竭,最终威胁患者生命[14-15]。SAP的治疗难点在于其可迅速引发全身系统性炎症反应综合征(systemic inflammatory response syndrome,SIRS),该过程中多器官损伤会进一步激活炎症反应的瀑布链式级联放大效应,目前临床上缺乏有效阻断这一病理环节的治疗方法[15-16]。

本研究采用逆行胰胆管注射牛磺胆酸钠构建大鼠SAP模型,SAP模型组胰腺组织结构紊乱、水肿明显,腺体间隙及血管周围存在红细胞渗出,血清淀粉酶、脂肪酶、IL-1β、TNF-α水平明显升高,抗炎因子IL-10水平明显下降。而乌司他丁组和MSCs治疗组上述指标均明显改善,且MSCs治疗后改善更为显著。TEM结果显示,与假手术组相比,SAP模型组腺泡细胞肿胀变大,线粒体肿胀,嵴结构消失,内质网肿胀,腔隙显著增宽、膨胀,部分呈碎片化断裂;而乌司他丁和MSCs治疗后腺泡细胞内线粒体肿胀减轻,嵴结构恢复,内质网肿胀减轻,腔隙变窄,受损程度减轻;且MSCs治疗组上述指标改善程度明显优于乌司他丁组。表明MSCs较乌司他丁具有更好缓解腺泡细胞损伤的作用。此外,本研究检测胰腺组织NLRP3/GSDMD/IL-1β焦亡信号通路相关基因和蛋白表达水平,与假手术组相比,SAP模型组胰腺组织焦亡相关基因NLRP3、caspase-1、GSDMD mRNA水平升高,胰腺组织焦亡相关蛋白NLRP3、caspase-1、cleaved caspase-1、GSDMD、GSDMD-N、IL-1β的蛋白相对表达量显著上升;乌司他丁和MSCs治疗后,上述指标基因水平和蛋白表达量均显著下降,且MSCs治疗组改善程度更为显著。证实MSCs可在一定程度上抑制NLRP3激活、caspase-1活化、GSDMD切割,具有明显抑制腺泡细胞焦亡改善SAP的作用。

焦亡是一种重要的自然免疫反应,在对外源性和内源性危险信号的反应中起着重要作用[17-18]。NLRP3/GSDMD/IL-1β通路是触发焦亡的主要通路[18],

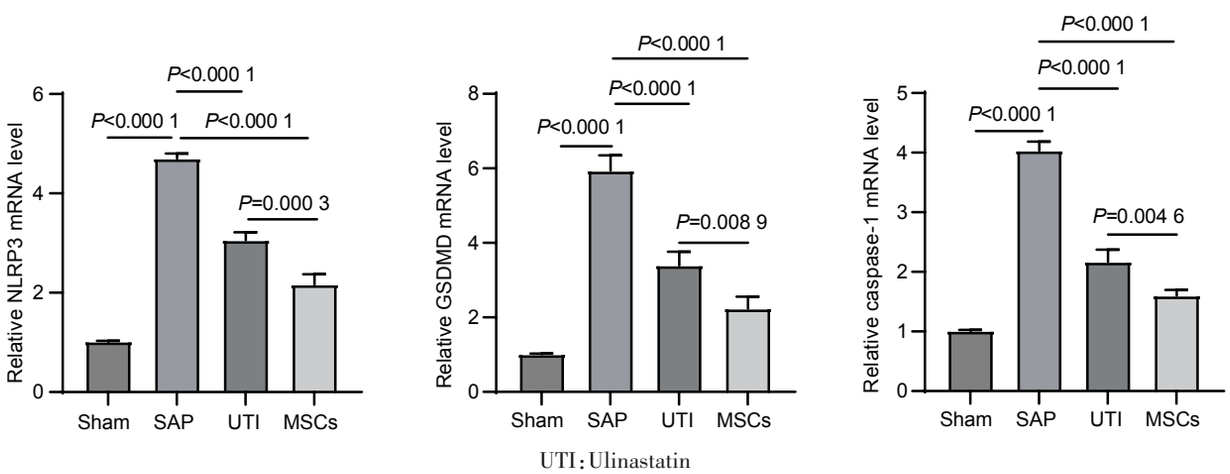
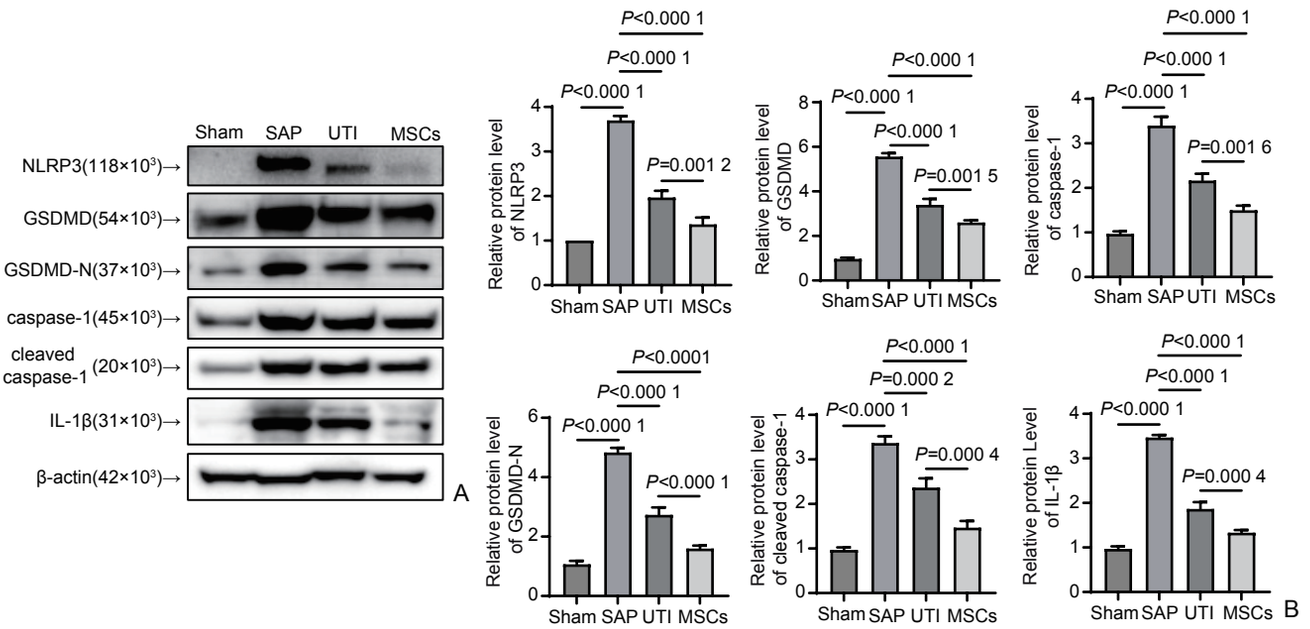


图8 各组NLRP3、GSDMD、caspase-1 mRNA相对表达量比较(n=6,$\bar{x}\pm s$)

Figure 8 Comparison of relative expression levels of NLRP3, GSDMD, and caspase-1 mRNA across experimental groups(n=6,$\bar{x}\pm s$)



A: Representative Western blotting images of pyroptosis-associated proteins in pancreatic tissues across different groups; B: Quantitative analysis of relative pyroptosis-associated protein expression levels in pancreatic tissues across different groups. UTI: Ulinastatin

图9 各组大鼠胰腺组织焦亡通路相关蛋白表达水平比较(n=6,$\bar{x}\pm s$)

Figure 9 Comparison of pyroptosis-associated protein expression levels in pancreatic tissues of rats among experimental groups(n=6,$\bar{x}\pm s$)

通常包括NLRP3、GSDMD、caspase-1、IL-1β 4种关键基因。细胞焦亡是一种程序性、炎症性的细胞死亡方式,主要由炎症小体激活caspase-1或caspase-4/5/11所引发[19]。其核心特征是细胞膜上形成孔洞,导致细胞肿胀、破裂,并释放大量的促炎细胞因子和内容物,从而招募免疫细胞、加剧炎症反应[20]。已有的研究表明,焦亡与SAP密不可分[21]。LYU等[13]研究发现在SAP期间,腺泡细胞体内的NLRP3炎症小体可通过氧化应激、NF-κB活化等刺激被激活,活化的NLRP3经细胞内一系列信号传导引发GSDMD、caspase-1活化,使pro IL-1β、pro IL-18剪切为IL-1β、IL-18,释放大量促炎因子,近一步加剧SAP炎症反应,扩大炎症范围。在小鼠急性胰腺炎模型中,腺泡焦亡水平在24 h达到顶峰,之后随时间延长而下降,与炎症水平变化并行[21-22]。NLRP3炎症小体的活化会介导IL-1β、IL-18等炎性细胞因子的过度释放,在SAP的发生发展中起着关键作用,因此,抑制NLRP3炎症小体的激活有望成为治疗SAP的潜在干预策略。

MSCs作为一种抗炎、促进组织修复及低免疫原性的多能干细胞,在急性胰腺及胃肠道疾病等研究中备受关注[23]。目前已经明确,MSCs通过免疫调节、炎症抑制、促进血管修复新生等发挥作用[23-25]。在腹腔注射雨蛙素、逆行胰胆管注射牛黄胆酸钠构建的急性胰腺炎动物模型研究中均发现MSCs通过抑制炎症反应、减轻胰腺组织水肿而发挥保护作用[26-28]。KIM等[29]发现用犬脂肪组织中提取的MSCs治疗大鼠急性胰腺炎,能降低血清淀粉酶、脂肪酶水平,降低促炎因子IL-1β、IL-6、IL-12、IL-17、IFN-γ mRNA的表达水平,提高抗炎因子IL-10、IL-4 mRNA表达水平。HU等[30]用骨髓MSCs治疗SAP,发现使用低氧诱导的骨髓MSCs能降低P-P65、P65的蛋白表达水平,减轻胰腺损伤,且低氧状态下MSCs抑制炎症的作用更好,胰腺组织HE染色评分更低,胰腺组织水肿、出血、坏死更少。研究表明MSCs既能够迁移至病灶,也能通过抑制炎症、调控免疫及旁分泌等方式改善SAP[31-32]。本研究从细胞焦亡的角度,证实了SAP大鼠胰腺组织存在细胞焦亡,进一步探索了NLRP3/GSDMD/IL-1β信号通路可能在MSCs改善胰腺损伤及细胞焦亡中的作用机制,为MSCs临床应用治疗SAP提供了一定的理论依据。

乌司他丁是一种广谱的胰酶抑制剂,能直接、有效地抑制多种水解酶的活性,是一种常见治疗急性胰腺炎的临床药物[33]。乌司他丁能显著抑制单核/巨噬细胞、中性粒细胞等过度活化,减少多种核心炎症介质的生成和释放;且具有一定的免疫调节功能,不仅

能抑制过度的炎症反应,还能通过调节T淋巴细胞亚群(如$CD4^+/CD8^+$比例)和单核细胞功能,帮助恢复机体的免疫平衡,降低继发感染风险。然而,关于其在抑制腺泡细胞焦亡改善重症急性胰腺炎中的研究较少。本研究证实乌司他丁能抑制焦亡改善大鼠重症急性胰腺炎。

综上所述,MSCs能减轻SAP大鼠的炎症反应,且与临床常用药物乌司他丁相比,MSCs显示出更好的治疗效果,其作用机制与抑制NLRP3/GSDMD/IL-1β通路,抑制SAP腺泡细胞焦亡有关。本研究尽管取得了一定的发现,但仍存在若干局限性,如:①本实验通过逆行胰胆管注射牛磺胆酸构建的大鼠SAP模型与临床SAP疾病存在差异,其发病机制和病情严重程度可能存在不同;②骨髓间充质干细胞治疗的进一步潜在机制仍需深入探讨,如骨髓间充质干细胞是如何介导调控焦亡因子抑制焦亡,这有待于在后续的细胞实验中进一步完善。但这些结果为MSCs可通过抑制腺泡细胞焦亡治疗SAP发挥保护胰腺组织作用提供了重要证据,有望能推动干细胞临床治疗的发展。

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