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·基础研究·

柴胡皂苷D载药囊泡缓解小鼠牙周炎的作用研究

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【摘要】　目的　制备装载柴胡皂苷D（SSD）的人脐带间充质干细胞（hUC-MSCs）来源的细胞外囊泡（EVs），并在小鼠牙周炎模型中评估其疗效，为牙周炎治疗提供新策略。**方法**　本研究通过单位医学伦理委员会审批与实验动物伦理委员会审批。取对数生长期的hUC-MSCs接种于96孔板，加入不同浓度的SSD药物处理24 h，采用CCK-8检测hUC-MSCs细胞活力，筛选最佳SSD作用浓度；流式细胞术分析不同浓度SSD对hUC-MSCs凋亡的影响及SSD载药率；利用纳米颗粒跟踪分析（NTA）和透射电子显微镜（TEM）观察hUC-MSCs-EVs和hUC-MSCs-SSD-EVs的形态与粒径分布。C57BL/6J小鼠动物实验分为对照组、牙周炎模型组（PD）、PD+SSD组（2 mg/kg）、PD+hUC-MSCs-EVs组（10 μg/μL）和PD+hUC-MSCs-SSD-EVs组（10 μg/μL），每组6只，每周给药3次，持续4周。采用显微CT（Micro-CT）和苏木精-伊红染色（HE）染色评估小鼠牙周炎模型牙槽骨吸收情况；实时荧光定量PCR（RT-qPCR）检测小鼠牙周炎模型牙周组织炎症因子表达水平；免疫荧光（IF）技术观察小鼠牙周炎模型牙周组织巨噬细胞M1/M2极化和Runt相关转录因子2（RUNX2）蛋白表达变化。**结果**　CCK-8结果显示，10 μmol/L SSD对hUC-MSCs细胞活力无明显影响（$P>0.05$）；流式细胞术证实SSD成功装载入囊泡，载药率为11.40 %；TEM和NTA结果显示hUC-MSCs-SSD-EVs呈圆形或类圆形膜性结构，直径为200~300 nm；Micro-CT和HE染色显示，与PD组相比，PD+hUC-MSCs-SSD-EVs组釉牙骨质界至牙槽嵴顶的距离（CEJ-ABC）减小（$P<0.01$），牙周炎模型区牙槽骨骨体积分数（BV/TV）和骨小梁数量（Tb.N）增加（$P<0.01$）；RT-qPCR和免疫荧光结果表明，hUC-MSCs-SSD-EVs可下调牙周炎组织中促炎细胞因子白细胞介素-1β、白细胞介素-6、肿瘤坏死因子-α、白细胞介素-12、白细胞介素-22的表达（$P<0.01$），上调抗炎因子白介素-10表达（$P<0.01$），促进M1型巨噬细胞向M2型转变，并增加RUNX2蛋白的表达（$P<0.01$）。**结论**　hUC-MSCs-SSD-EVs可有效缓解小鼠上颌牙周炎症反应，减少牙槽骨吸收，为牙周炎的治疗提供了新思路。

【关键词】　牙周炎；　间充质干细胞；　细胞外囊泡；　柴胡皂苷D；　载药囊泡；　巨噬细胞极化；　牙槽骨吸收；　炎症浸润；　细胞因子；　Runt相关转录因子2
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Study on the effect of saikosaponin D drug-loaded vesicles on alleviating periodontitis in mice

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【Abstract】　Objective　To explore new therapeutic strategies for periodontitis, this study prepared extracellular

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vesicles (EVs) derived from human umbilical cord mesenchymal stem cells (hUC-MSCs) loaded with saikosaponin D (SSD) and evaluated their efficacy in a mouse model of periodontitis. Methods　This study was approved by the Medical Ethics Committee and the Laboratory Animal Ethics Committee of the unit. hUC-MSCs at logarithmic growth phase were seeded into 96 well plates and then treated with various concentrations of SSD for 24 hours. The CCK-8 assay was used to determine the SSD concentration that preserves hUC-MSC viability. Flow cytometry (FCM) assessed SSD-induced apoptosis in hUC-MSCs and determined drug loading and loading efficiency. Nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) characterized the morphology and size of hUC-MSCs-EVs and hUC-MSCs-SSD-EVs. C57BL/6J mice were divided into five groups with six mice per group: control group, periodontitis model group (PD), PD+SSD group (2 mg/kg), PD+hUC-MSCs-EVs group (10 μg/μL) and PD+hUC-MSCs-SSD-EVs group (10 μg/μL). Administration was performed 3 times a week for 4 consecutive weeks. Micro-CT and hematoxylin-eosin (HE) staining evaluated alveolar bone resorption in periodontal tissues of a mouse model of periodontitis. RT-qPCR measured inflammatory cytokine expression in periodontal tissues of a mouse model of periodontitis. Immunofluorescence analyzed macrophage M1/M2 polarization and runt-related transcription factor 2 (RUNX2) protein expression in periodontal tissues of a mouse model of periodontitis. Results　The CCK-8 assay showed that 10 μmol/L SSD had little effect on hUC-MSCs viability (P>0.05). FCM confirmed the successful loading of SSD into EVs with a loading efficiency of 11.40%. TEM and NTA revealed that hUC-MSCs-SSD-EVs presented round or oval membranous structures with diameters ranging from 200 to 300 nm. Micro CT and HE staining showed that, compared with the PD group, the distance from cementoenamel junction to alveolar bone crest (CEJ-ABC) was markedly shortened (P<0.01).The alveolar bone of periodontitis-model　bone volume fraction (BV/TV) and trabecular number (Tb.N) were significantly elevated in the PD+hUC-MSCs-SSD-EVs group (P<0.01). RT-qPCR and IF results showed that hUC-MSCs-SSD-EVs in periodontal tissues markedly downregulated the expression of pro-inflammatory cytokines including interleukin-1β, interleukin-6 , tumor necrosis factor- α, interleukin-12 and interleukin-22 (P<0.01), upregulated the expression of the anti-inflammatory factor interleukin-10 (P<0.01), promoted the polarization of macrophages from the M1 phenotype to the M2 phenotype, and elevated the protein expression level of RUNX2 (P<0.01). Conclusion　hUC-MSC-SSD-EVs effectively alleviated inflammatory responses in a mouse model of maxillary periodontitis and reduced alveolar bone loss, providing a new idea for the treatment of periodontitis.

【Key words】 periodontitis; mesenchymal stem cells; extracellular vesicles; saikosaponin D; drug-loaded vesicles; macrophage polarization; alveolar bone resorption; inflammatory infiltration; cytokines; runt-related transcription factor 2

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牙周炎全球患病率约为11%[1]，该病是一种以严重炎症和免疫反应为特征的慢性疾病，最终可导致牙周牙槽骨和牙周韧带不可逆转性破坏[2]。使用根面平整术和全身性抗生素治疗虽能改善牙周炎，但无法实现已缺失牙周组织的良好再生[3-4]。因此，亟须开发一种微量而高效的治疗药物，为牙周炎疾病预防和牙周组织再生治疗带来新希望。

细胞外囊泡（extracellular vesicles，EVs）是一类由磷脂双分子层包裹的膜性小体，直径为纳米级别，携带蛋白质、核酸、脂质等多种生物活性分子，在细胞间通讯中发挥关键的载体作用[5-6]。来源于间充质干细胞的EVs（mesenchymal stem cells-derived EVs，MSC-EVs）在促进受损组织修复与再生方面展现出良好潜力[7]，并可诱导巨噬细胞从M1型向M2型极化，同时调控T细胞亚型平衡，从而发挥免疫调节作用[8]。然而，MSC-EVs的产量较低，且其内容物组成具有高度动态性，易受细胞来源、培养条件和生物合成途径等因素影响，增加了其在临床应用中实现标准化与可重复性的难度[9-10]。尽管如此，EVs凭借其体积微小、可继承母细胞分子特征且能被靶细胞稳定摄取等优势，仍具有重要的研究与应用价值[11]。研究表明，MSCs经牛磺酰脱氧胆碱酸[10]、过表达α-1抗胰蛋白酶[12]、化学药物、炎症因子等预处理后，其EVs的治疗潜力可被进一步增强[13]。因此，MSC-EVs能够继承母细胞经外源处理所获得的生物学特性，并放大其治疗效应，成为疾病治疗中具有前景的新策略。人脐带间充质干细胞（human umbilical cord MSCs，hUC-MSCs）在具备其他MSCs共性的同时，因其增殖能力强、免疫原性低、分泌谱丰富、来

源纯净安全、分化潜能优越及采集便捷无创等特点而受到广泛关注。然而，hUC-MSCs-EVs是否同样能够继承母细胞的增强特性并表现出优化的治疗效能，尚待系统研究。此外，其在牙周炎治疗中的应用潜力，尤其是其载药效应，仍需进一步验证。

柴胡皂苷D（saikosaponin D，SSD）是一类天然三萜皂苷化合物[14]，也是柴胡中主要的生物活性成分，具有抗炎[15]、抗氧化、抗凋亡、抗纤维化及抗癌等多种药理作用[16]，使其成为多种疾病治疗的候选药物[17]。然而，SSD水溶性差、口服生物利用度低，胃肠道吸收不佳，其体内药代动力学存在争议[18-19]，严重制约了其药效的充分发挥。EVs作为天然的通讯和递送载体，有望克服SSD的上述缺陷，实现对SSD的有效装载并发挥其治疗作用。然而，hUC-MSCs-EVs是否能够负载SSD，以及该载药体系在牙周炎治疗中的应用，目前尚缺乏相关研究。本研究拟以hUC-MSCs包载SSD后提取其EVs，通过局部给药的方式处理小鼠结扎诱导的牙周炎模型，探究装载SSD的囊泡在缓解牙周炎症、减少牙槽骨吸收方面的作用，以期为临床开发新型治疗药物及实现牙周炎的精准再生治疗提供新思路。

1　材料和方法

1.1　主要试剂与仪器

柴胡皂苷D（20874-52-6，Nanjing Qiushi，中国）；Cy5.5-SSD（Q-W0505523，QIYUEBIO，中国）；快速脱钙液（PVB-3002，PhenoVisionbio，中国）；磷酸盐缓冲液（phosphate buffer solution，PBS）（10010023，Thermo Fisher，美国）及α-最低必需培养基（alpha minimal essential medium，α-MEM）（12000063，Thermo Fisher，美国）；胎牛血清（13011-8611，TIANHANG，中国）；苏木素（3801616，Leica，德国）；伊红（3801619，Leica，德国）；一次性组织刀片（819，Leica，德国）；RNA反转试剂（R026B，Takara，日本）；RNA定量试剂（RR820A，Takara，日本）；组织包埋剂（MM1501-118ML，Tissue-Tek，日本）；二甲基亚砜（methyl sulfoxide，DMSO）（D103277，aladdin，中国）；RNA分离试剂盒（RE-03014，FOREGENE，中国）；戊巴比妥钠（P3761，Sigma-Aldrich，德国）；CCK-8检测试剂（DY40201，DEEYEEBIO，中国）；PKH67（DY50232，DEEYEEBIO，中国）；一抗：小鼠抗RUNX2（Runt-related transcription factor 2，RUNX2）（1∶300，sc-390351，Santa，美国）；大鼠抗F4/80（F4/80 antigen，F4/80）（1∶100，14-4801-85，Thermo Fisher，美国）；兔抗诱导型一氧化氮合酶（inducible nitric oxide synthase，iNOS）（1∶150，A3774，ABclonal，中国）；兔抗CD206（macrophage mannose receptor，CD206）（1∶400，GB113497-100，Servicebio，中国）；二抗：Cy3-山羊抗大鼠二抗（H+L）（1∶300，33308ES60，Yeasen，中国）；488抗兔二抗（1∶500，RGAR002，Proteintech，中国）；594抗鼠二抗（1∶500，RGAM004，Proteintech，中国）。

高速冷冻离心机（Centrifuge 5425，Eppendorf，德国）；纳米粒度及zeta电位分析仪（Litesizer™ 100，Anton Paar，奥地利）；多功能酶标仪（Thermo Fisher，Varioskan LUX，美国）；透射电镜（FEI Talos F200X，Thermo Fisher，美国）；共聚焦显微镜（Nikon-Eclipse-Ti，Nikon，日本）；流式细胞仪（Beckman CytoFLEX，Beckman，美国）；小动物显微CT（Micro-CT）成像仪（IVIS Spectrum CT，PerkinElmer，美国）；3D重建分析软件（VGSTUDIO MAX 2023.1，Volume Graphics，德国）；全自动多功能染色机（HistoCore CHROMAX ST，Leica，德国）；手动轮转式切片机（HistoCore BIOCUT，Leica，德国）；冷冻切片机（CM1860，Leica，德国）；荧光定量PCR仪（CFX96，Bio-Rad，美国）。

1.2　实验方法

1.2.1　hUC-MSCs细胞培养　本研究遵循《赫尔辛基宣言》准则，并通过单位医学伦理委员会审批（审批号：KY20252585-C-1）。hUC-MSCs由本团队前期分离，并获得研究参与者书面知情同意。hUC-MSCs在含10%胎牛血清的α-MEM中扩增培养。培养条件为37 ℃、5% CO_2、95% O_2，2~3 d进行传代培养。细胞学实验均进行3次独立的生物学重复实验。

1.2.2　CCK-8法检测hUC-MSCs细胞活力　取对数生长期的hUC-MSCs，以每孔3 000个接种于96孔板，每组6个复孔，培养过夜后，加入10 μmol/L SSD和20 μmol/L SSD药物处理24 h。每孔加入新鲜100 μL工作液（含有10 μL CCK-8溶液），避免产生气泡，孵育1 h后，用酶标仪测定450 nm波长处的光密度（optical density，OD）值。

1.2.3　流式细胞术检测hUC-MSCs细胞凋亡及载药比例　取对数生长期的hUC-MSCs，接种于培养板中，每组设3个复孔。待细胞贴壁后，处理组加

入10 μmol/L SSD药物处理24 h。收集各组细胞，经胰酶消化后，用200 μL PBS缓冲液重悬细胞沉淀，每管样品中加入5 μL Annexin V，10 μL PI染料，室温避光孵育20 min，PBS洗涤重悬后，流式上机检测hUC-MSCs细胞凋亡。荧光标记Cy5.5-SSD处理联合PKH67染料染色，用来检测hUC-MSCs细胞载药比例。方法同凋亡检测。

1.2.4　EVs提取与表征　收集正常和10 μmol/L SSD药物处理后的细胞上清，每组3个复孔，采用梯度离心法提取EVs。细胞上清经过800×g离心10 min、2 000×g离心10 min去除沉淀；16 800×g离心30 min收集沉淀即为细胞外囊泡。采用纳米颗粒跟踪分析技术（nanoparticle tracking analysis，NTA）和透射电子显微镜（transmission electron microscope，TEM）分析样品的颗粒尺寸分布及细胞外囊泡结构特征。

1.2.5　流式细胞术分析hUC-MSCs-SSD-EVs载药及载药比例　hUC-MSCs用10 μmol/L Cy5.5-SSD药物处理24 h后，按1.2.4方法提取EVs，每组3个复孔，通过流式细胞术检测hUC-MSCs-SSD-EVs载药成功。联合PKH67染料标记后，用来检测hUC-MSCs-SSD-EVs载药比例。

1.2.6　实验动物　本研究遵循ARRIVE指南2.0。实验经陕西中医药大学实验动物伦理委员会批准（批号：SUCMDL20250801002），动物实验方案按伦理委员会批准的方案执行（未进行注册）。根据预实验结果确定样本量，选取。健康SPF级6～8周龄雄性C57BL/6J小鼠30只，体质量20～25 g，无口腔炎症、无全身感染、无外伤及先天发育异常的小鼠，不符合该标准的小鼠予以排除。小鼠由空军军医大学实验动物中心提供，生产许可证编号：SCXK（陕）2024-002。动物饲养于12 h光照/12 h黑暗循环环境，温度（22±2）℃，相对湿度50%±10%，每笼5只，自由摄食饮水，适应性饲养1周。

1.2.7　小鼠实验性牙周炎模型的建立及分组给药　随机数字表法选取24只小鼠，小鼠使用1 %戊巴比妥钠麻醉，仰卧位放置于自制鼠板并用自制开口器将小鼠口腔撑开，眼科显微镊夹持5-0丝线分别从小鼠右上颌第二磨牙近中及远中的舌侧邻间隙穿过，再将缝合线两端于小鼠颊侧间隙拉出并打三重结[20-21]，剪去多余线头，待小鼠苏醒放回笼中，每3 d检查丝线的结扎情况，如发现丝线脱落及时重新结扎，持续4周即可造模成功。实验期间无非实验因素感染或外伤导致排除小鼠，最终24只小鼠建模成功。采用随机数字表法将小鼠分为4组（每组6只）：牙周炎组（PD组）：丝线结扎右上颌第二磨牙；PD+SSD组：丝线结扎右上颌第二磨牙并局部注射SSD；PD+hUC MSCs-EVs组：丝线结扎右上颌第二磨牙并局部注射hUC MSCs-EVs；PD+hUC MSCs-SSD-EVs组：丝线结扎右上颌第二磨牙并局部注射hUC MSCs-SSD-EVs。剩余6只小鼠为正常对照组（control组）：未进行手术，注射PBS。造模结束后开始给药：SSD（2 mg/kg）、hUC-MSCs-EVs（10 μg/μL）或hUC-MSCs-SSD-EVs（10 μg/μL），每周3次，局部注射于腭侧牙龈，每只5 μL，5 min内完成。对照组注射等体积PBS。给药从造模成功后第1天开始，持续4周（至实验结束）。实验结束取材前禁食12 h。采用双盲法评估牙周炎治疗效果。

1.2.8　小鼠右上颌骨组织显微CT扫描评价牙槽骨吸收　实验处理4周后，1 %戊巴比妥钠液经腹腔注射麻醉过量处死小鼠。分离右侧上颌骨，4 %多聚甲醛溶液固定48 h，显微CT（micro computed tomography，Micro-CT）上机扫描，分辨率为10 μm，电压50 kV，电流为80 μA，数据导出格式为dcm。VG Studio MAX软件进行3D重建，输出小鼠右侧上颌骨三维和二维图片，并测量右上颌骨颊侧第二磨牙釉牙骨质界到牙槽嵴顶的距离（cemento-enamel junction to alveolar bone crest distance，CEJ-ABC）、右上第二磨牙牙槽骨的骨体积分数（bone volume/tissue volume，BV/TV）、骨小梁数量（trabecular number，Tb.N）。

1.2.9　苏木素-伊红（hematoxylin eosin staining，HE）染色评估牙槽骨吸收及炎症浸润　取小鼠右侧上颌第二磨牙牙槽骨进行HE染色。4 %多聚甲醛溶液固定24 h，快速脱钙液4 ℃脱钙7 d，至1 mL注射器针头可以轻松穿过骨组织完成脱钙；冲水6 h后牙槽骨标本置于脱水机内梯度乙醇进行脱水与浸蜡，将组织修整后包埋，制备5 μm组织切片，常规脱蜡后将切片放入苏木精中染色、冲洗、盐酸乙醇分化、冲洗、稀氨水返蓝、伊红染色，脱水至透明后用中性树脂封片，即可显微镜下观察。

1.2.10　免疫荧光染色评估巨噬细胞极化及RUNX2蛋白表达　小鼠右侧上颌第二磨牙牙槽骨进行免疫荧光染色。4 %多聚甲醛溶液固定24 h，快速脱钙液4℃脱钙；30 %蔗糖溶液脱水24 h，OCT包埋，制备5 μm组织切片，PBS缓冲液洗涤以去除OCT、透膜、封闭、一抗孵育F4/80（1∶100）、iNOS

（1：150）、CD206（1：400）、RUNX2（1：300）抗体、洗涤、二抗（抗大鼠Cy3，1：300；抗兔488，1：500；抗鼠594，1：500）孵育、洗涤，4′，6-二脒基-2-苯基吲哚（4′，6-diamidino-2-phenylindole，DAPI）封片，即可共聚焦显微镜下观察。

1.2.11　RT-qPCR检测牙周组织炎症细胞因子表达　取小鼠右侧上颌第二磨牙牙周组织进行RT-qPCR。每组随机取3个样本，加入TRIzol裂解液和钢珠匀浆，冰上裂解10 min后加入1/5体积氯仿剧烈震荡，静置5 min后4 ℃，12 000×g离心15 min，RNA富集在上清，加入等体积异丙醇后，静置5 min后二次相同条件离心，RNA于沉淀中，75 %酒精洗涤RNA沉淀并晾干，进行浓度测定，A_{260}/A_{280}在1.9~2.0即认为RNA无杂质。随后按试剂盒说明书进行逆转录以及实时定量PCR反应，定量白细胞介素-1β（interleukin-1 beta，IL-1β）、白细胞介素-10（interleukin-10，IL-10）、白细胞介素-6（interleukin-6，IL-6）、白细胞介素-12（interleukin-12，IL-12）、白细胞介素-22（interleukin-22，IL-22）和肿瘤坏死因子-α（tumor necrosis factor-α，TNF-α）表达，引物序列如下表1。

表1　引物序列信息

Table 1　Primer sequence information

Gene name	Gene sequence
IL-1β	5′-GCAACTGTTCCTGAACTCAACT-3′
	5′-ATCTTTTGGGGTCCGTCAACT-3′
IL-6	5′-TAGTCCTTCCTACCCCAATTTCC-3′
	5′-TTGGTCCTTAGCCACTCCTTC-3′
IL-10	5′-GCTCTTACTGACTGGCATGAG-3′
	5′-CGCAGCTCTAGGAGCATGTG-3′
IL-12	5′-CAATCACGCTACCTCCTCTTTT-3′
	5′-CAGCAGTGCAGGAATAATGTTTC-3′
IL-22	5′-ATGAGTTTTTCCCTTATGGGGAC-3′
	5′-GCTGGAAGTTGGACACCTCAA-3′
TNF-α	5′-CCCTCACACTCAGATCATCTTCT-3′
	5′-GCTACGACGTGGGCTACAG-3′
β-Actin	5′-AACAGTCCGCCTAGAAGCAC-3′
	5′-CGTTGACATCCGTAAAGACC-3′

IL-1β: interleukin-1 beta; IL-6: interleukin-6; IL-10: interleukin-10; IL-12: interleukin-12; IL-22: interleukin-22; TNF-α: tumor necrosis factor-α

1.3　统计学分析

n值代表独立实验次数或每组单个小鼠数量，数据采用Graphpad Prism 10.0进行统计分析，计量资料以均值±标准差（$\bar{x}\pm s$）表示，经Shapiro-Wilk检验数据符合正态性，两组间比较采用独立样本t检验，多组间比较采用单因素方差分析（one-way ANOVA），组间比较采用Tukey检验，P<0.05表示差异有统计学意义。

2　结　果

2.1　柴胡皂苷D对hUC-MSCs细胞活力及凋亡的影响

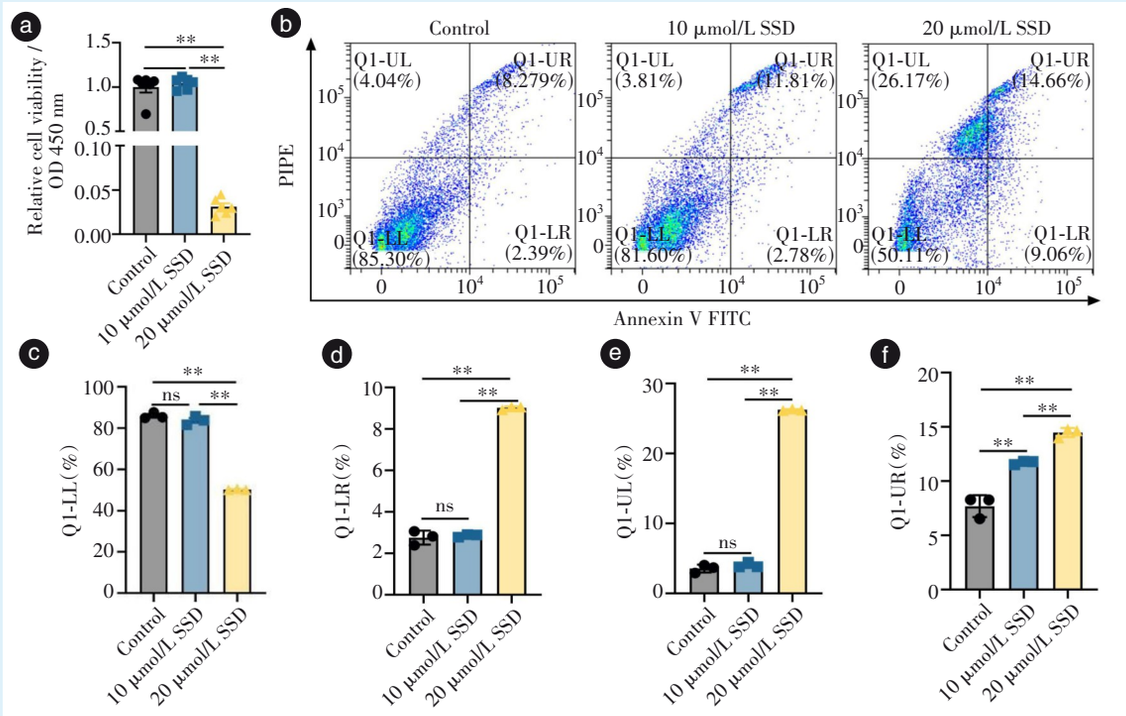
CCK-8结果显示（图1a），10 μmol/L SSD对hUC-MSCs细胞活力几乎无影响，而20 μmol/L SSD对hUC-MSCs细胞的抑制率高达97%（P<0.01）。流式细胞术检测结果（图1b-1f）进一步表明，20 μmol/L SSD对hUC-MSCs细胞活力有影响（P<0.01）。具体而言，Q1-LL象限（活细胞）比例降至约50%（P<0.01）；Q1-LR象限（早期凋亡细胞）比例为9.06%，显著高于对照组（2.39 %）和10 μmol/L SSD组（2.78 %）（P < 0.01）；Q1-UL象限（坏死或机械损伤细胞）比例为26.17 %（P < 0.01）；Q1-UR象限（晚期凋亡细胞）比例为14.66 %（P < 0.01）。综上，20 μmol/L SSD对hUC-MSCs造成的损伤（包括早期凋亡、坏死/机械损伤及晚期凋亡）均明显重于10 μmol/L SSD，而10 μmol/L SSD的细胞毒性较轻。结合CCK-8与流式细胞术结果，本研究确定10 μmol/L浓度的SSD为对hUC-MSCs细胞安全的作用剂量，并以此浓度进行后续hUC-MSCs来源EVs的载药研究。

2.2　hUC-MSCs-SSD-EVs载药、纳米颗粒跟踪分析技术和透射电子显微镜表征

Cy5.5-SSD和PKH67双重荧光标记结果显示（图2a、2b），hUC-MSCs经SSD处理24 h后，细胞及其分泌的EVs均成功装载SSD，hUC-MSCs的载药率为69.70 %，hUC-MSCs-EVs的载药率为11.40 %。为进一步验证EVs成功装载了Cy5.5-SSD，分别采用Cy5.5-SSD与未标记SSD处理hUC-MSCs，结果显示hUC-MSCs-Cy5.5-SSD-EVs的荧光峰值显著高于hUC-MSCs- SSD-EVs（图2c）；TEM和NTA检测评估SSD对EVs结构的影响，TEM结果显示（图2d），EVs呈圆形或近似圆形的膜包被结构，SSD处理未改变其形态；NTA检测进一步表明，hUC-MSCs-EVs和hUC-MSCs-SSD-EVs的粒径均分布在200~300 nm范围内（图2e）。

2.3　PD+hUC-MSCs-SSD-EVs对小鼠牙槽骨吸收及炎症浸润的影响

Micro-CT结果显示（图3a、3b），PD+hUC-MSCs-



a: CCK-8 assay measuring hUC-MSC viability after treatment with 10 and 20 μmol/L SSD. The 10 μmol/L SSD treatment had little effect on hUC-MSC viability. n = 3 biologically independent replicates. b: representative FCM images of apoptosis in hUC-MSCs after treatment with 10 and 20 μmol/L SSD from 3 biologically independent replicates. The 10 μmol/L SSD treatment had little effect on hUC-MSC apoptosis. The Y-axis represents PI labeled in the PE channel, and the X-axis represents Annexin labeled in the FITC channel. c-f: apoptosis analysis in hUC-MSCs treated with 10 and 20 μmol/L SSD. n = 3 biologically independent replicates. Statistical analysis was conducted using one-way ANOVA with post-hoc Tukey test for pairwise comparisons between control, 10 μmol/L SSD and 20 μmol/L SSD groups. All data were expressed as mean ± standard deviation (SD). $^{**}P < 0.01$; ns: non-significant. SSD: saikosaponin D; hUC-MSCs: human umbilical cord mesenchymal stem cells; FCM: flow cytometry; FITC: fluorescein isothiocyanate; Q1-LL: quadrant 1, lower left; Q1-LR: quadrant 1, lower right; Q1-UL: quadrant 1, upper left; Q1-UR: quadrant 1, upper right

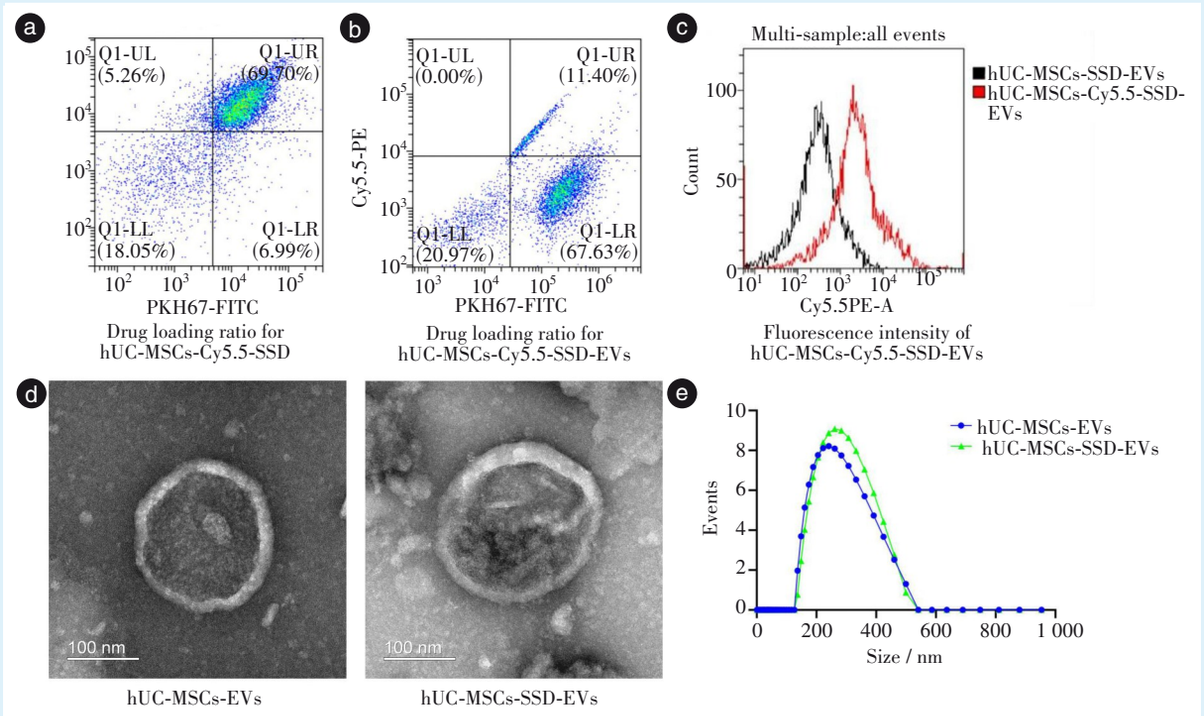
Figure 1　CCK-8/FCM analysis of the effects of different concentrations of SSD on hUC-MSCs

图1　CCK-8/FCM检测不同浓度SSD对hUC-MSCs细胞的影响

SSD-EVs较PD+SSD和PD+hUC-MSCs-EVs能显著缓解小鼠牙槽骨吸收。定量分析表明：PD组小鼠第二磨牙CEJ-ABC距离显著增大，PD+SSD和PD+hUC-MSCs-EVs处理后该距离减小（P<0.01），而PD+hUC-MSCs-SSD-EVs组的减小效果更为明显（P<0.01）；BV/TV（图3c）和Tb.N（图3d）在PD组降低（P<0.01），与PD组相比，PD+SSD与PD+hUC-MSCs-EVs处理组可缓解牙槽骨吸收（P<0.05），而PD+hUC-MSCs-SSD-EVs组能更好地保留牙槽骨组织（P<0.01）。HE染色结果（图3e）显示，与对照组相比，PD组牙龈乳头形态破坏，牙龈上皮层出现变性、坏死，表现为细胞形态不规则、核固缩；牙周韧带排列紊乱，纤维组织排列紊乱、稀疏；炎症细胞浸润增多，牙槽骨高度下降，吸收增多，PD+hUC-MSCs-SSD-EVs较PD+SSD和PD+hUC-MSCs-EVs单独有效作用基础上，能显著缓解小鼠牙周组织病变。

2.4　PD+hUC-MSCs-SSD-EVs对小鼠牙周组织中巨噬细胞极化及其下游细胞因子表达的影响

免疫荧光和RT-qPCR结果（图4）显示，与对照组相比，PD组牙周组织中M1型巨噬细胞标志物iNOS表达增加（P<0.01），M2巨噬细胞标志物CD206表达减少（P<0.01），说明巨噬细胞内环境比例失衡，下游炎性细胞因子IL-1β、IL-6、TNF-α、IL-12、IL-22表达增加（P<0.01或P<0.05），抗炎性细胞因子IL-10减少（P<0.01）。PD+SSD和PD+hUC-MSCs-EVs处理能缓解牙周组织M1/M2巨噬细胞失衡及其下游促炎和抗炎细胞因子释放，PD+hUC-MSCs-SSD-EVs载药囊泡较PD+SSD和PD+hUC-MSCs-EVs处理能显著促进M1型巨噬细胞向M2型巨噬细胞转化，



a-b: FCM detection of loading in hUC-MSCs and hUC-MSCs-EVs after treatment with 10 μmol/L SSD. SSD loading was successful. The Y-axis represents Cy5.5-SSD labeled in the PE channel, and the X-axis represents PKH67 labeled in the FITC channel. c: fluorescence intensity of Cy5.5-SSD loading in hUC-MSCs-Cy5.5-SSD-EVs; hUC-MSCs-SSD-EVs (black) and hUC-MSCs-Cy5.5-SSD-EVs (red) show distinct fluorescence expression peaks. d: TEM of the structures of hUC-MSCs-EVs and hUC-MSCs-SSD-EVs after 10 μmol/L SSD treatment. Loading did not alter the morphology of the hUC-MSCs-SSD-EVs. a–d: representative images from 3 biologically independent replicates. e: NTA analysis the particle sizes of hUC-MSCs-EVs and hUC-MSCs-SSD-EVs after 10 μmol/L SSD treatment. *n* = 3 biologically independent replicates. The particle size of hUC-MSCs-SSD-EVs did not change after loading. hUC-MSCs-Cy5.5-SSD: Cy5.5-fluorescently labeled SSD-treated hUC-MSCs; hUC-MSCs-Cy5.5-SSD-EVs: drug-loaded EVs derived from SSD-treated hUC-MSCs after Cy5.5 labeling. hUC-MSCs-EVs: EVs from normal hUC-MSCs; hUC-MSCs-SSD-EVs: SSD-loaded EVs derived from SSD-treated hUC-MSCs. FCM: flow cytometry; SSD: saikosaponin D; hUC-MSCs: human umbilical cord mesenchymal stem cells; EVs: extracellular vesicles; NTA: nanoparticle tracking analysis; FITC: fluorescein isothiocyanate; TEM: transmission electron microscope

Figure 2　Drug loading of hUC-MSCs-SSD-EVs and characterization by TEM and NTA

图2　hUC-MSCs-SSD-EVs载药、纳米颗粒跟踪分析技术和透射电子显微镜表征

并显著降低促炎细胞因子的表达，促进抗炎细胞因子IL-10的表达（P<0.01）。

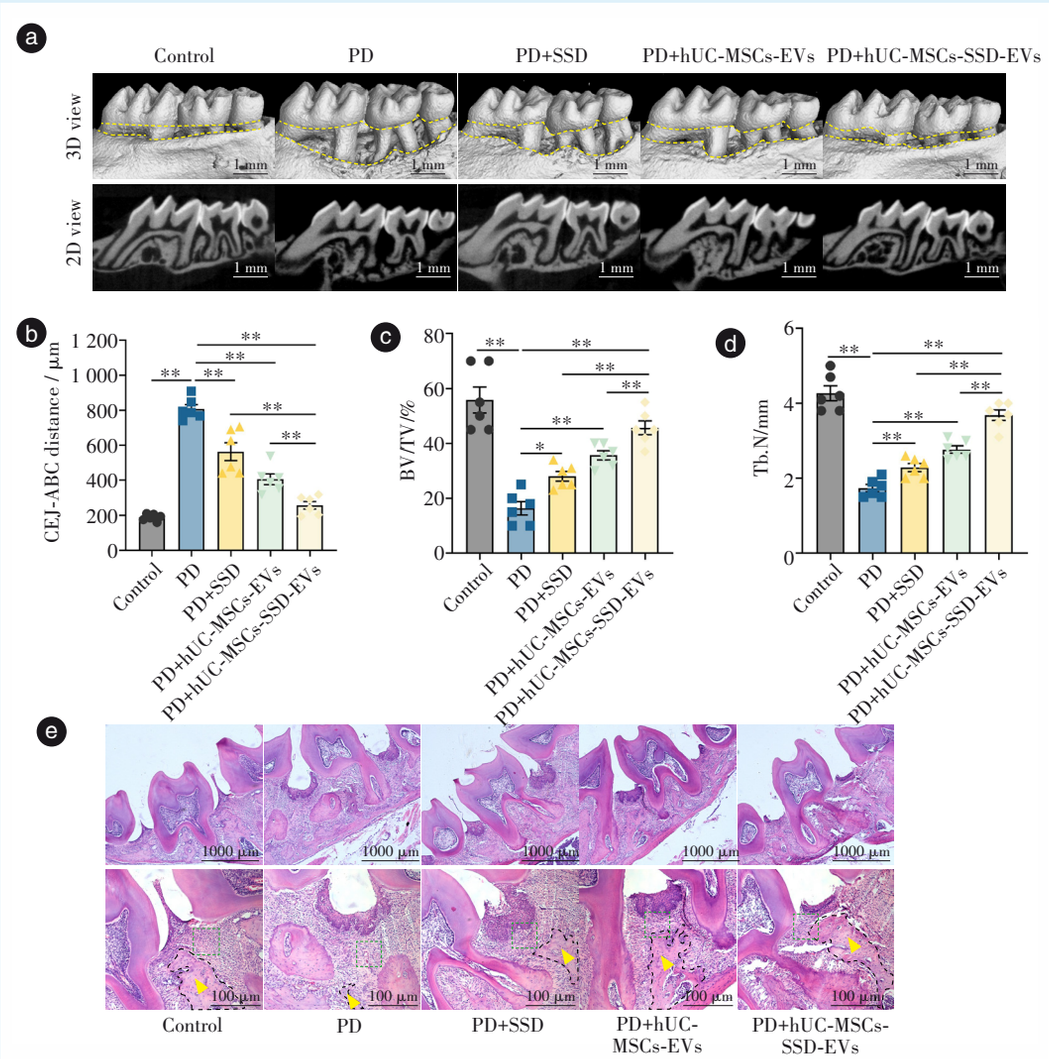
2.5　PD+hUC-MSCs-SSD-EVs对小鼠牙周组织中RUNX2表达的影响

免疫荧光检测结果（图5）显示，与对照组相比，PD组牙周组织中RUNX2蛋白表达显著降低（P<0.01），与PD组相比，PD+SSD或PD+hUC-MSCs-EVs组，RUNX2蛋白表达升高（P<0.01），PD+hUC-MSCs-SSD-EVs处理组的RUNX2蛋白表达提升效果更为显著（P<0.01）。

上述结果与前期观察到的小鼠牙槽骨吸收程度及牙周组织中促炎因子表达变化趋势一致，进一步表明hUC-MSCs-SSD-EVs可发挥显著的保护作用，更有效地保留小鼠牙槽骨组织。

3　讨　论

牙周炎是一种复杂的口腔多微生物疾病，其免疫激活后释放的炎症介质可破坏牙周组织并最终引起骨吸收[22]，导致患者牙齿脱落、咀嚼功能障碍、营养失衡、审美受损、言语改变等，进而降低整体生活质量[23-25]。目前，牙周炎的治疗方法主要包括龈上洁治、龈下刮治和根面平整术，以及依照医嘱使用甲硝唑、四环素、阿莫西林、罗红霉素等进行抗感染治疗[3, 26-27]。然而，牙周洁治与牙根清创仅能清洁牙周表面，难以完全清除已侵入牙周组织并诱发炎症级联反应的致病菌。抗生素虽然可

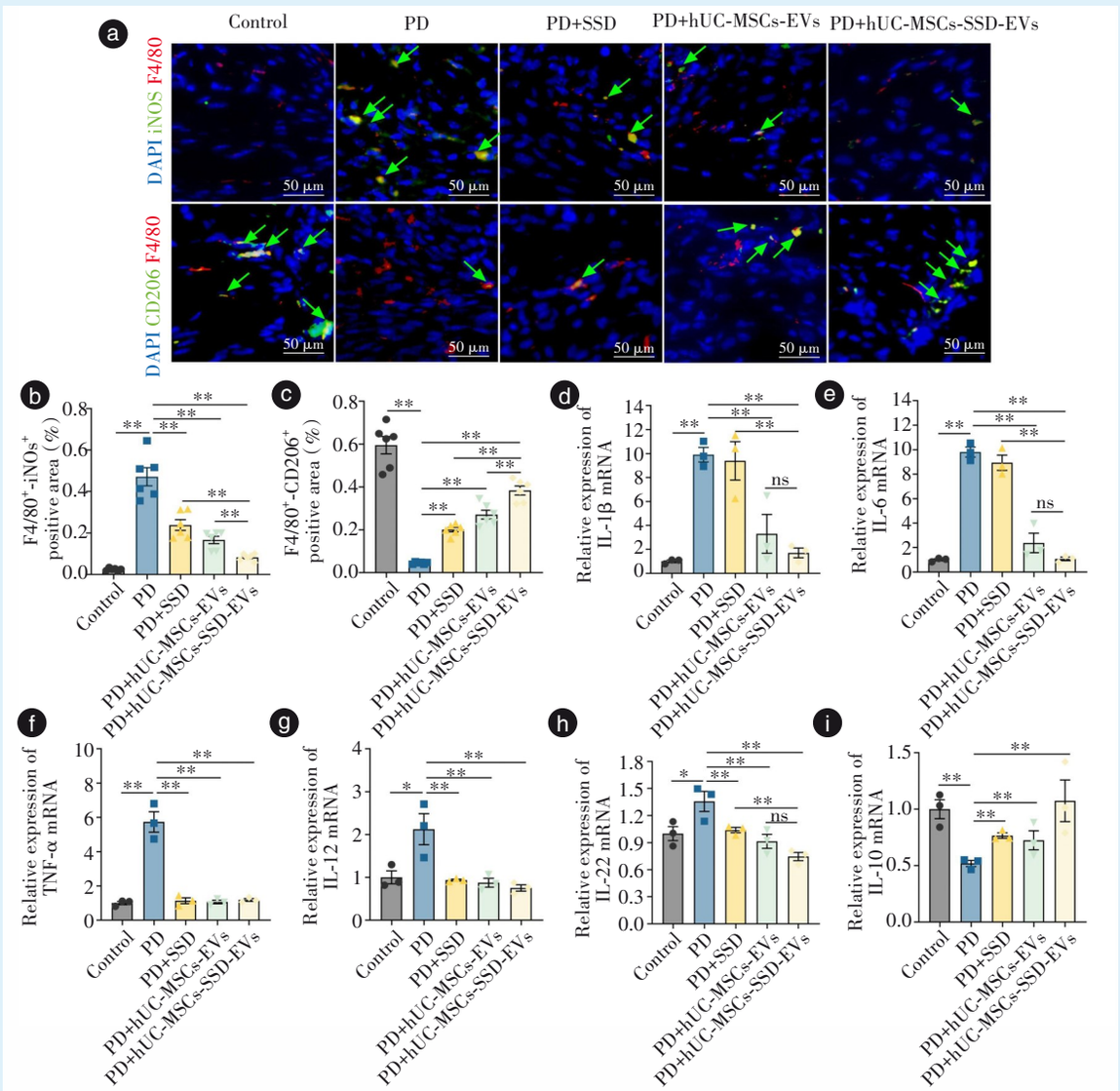


a: representative micro-CT 2D and 3D images from 6 mice per group of the maxillary alveolar bone with the Control, PD, PD + SSD, PD + hUC-MSCs-EVs, and PD + hUC-MSCs-SSD-EVs. The therapeutic efficacy of hUC-MSCs-SSD-EVs was greater than that of both SSD alone and hUC-MSCs-EVs. b-d: quantitative analysis of CEJ-ABC, BV/TV and Tb.N for the Control, PD, PD + SSD, PD + hUC-MSCs-EVs, and PD + hUC-MSCs-SSD-EVs. n = 6 mice (6 biologically independent replicates) CEJ-ABC: distance from the cementoenamel junction to the alveolar crest; BV/TV: bone volume fraction; Tb.N: trabecular number. e: representative HE staining analysis from 6 mice per group of alveolar bone resorption and inflammatory infiltration with the Control, PD, PD + SSD, PD + hUC-MSCs-EVs, and PD + hUC-MSCs-SSD-EVs. Treatment with PD + hUC-MSCs-SSD-EVs preserved more alveolar bone than treatment with PD + SSD alone or with PD + hUC-MSCs-EVs. Inflammatory cell infiltration (green box) decreased alveolar bone height with increased resorption (yellow arrow). Statistical analysis for two-group comparison (Control vs PD) was performed via two-tailed independent Student's t-test. Multi-group comparisons among PD, PD+SSD, PD+hUC-MSCs-EVs and PD+hUC-MSCs-SSD-EVs groups were conducted using one-way ANOVA with post-hoc Tukey test for all pairwise comparisons. All data were expressed as mean ± standard deviation (SD). $^{**}P < 0.01$, $^{*}P < 0.05$. Control: normal mouse maxillary alveolar bone; PD: ligature-induced periodontitis in mice; PD + SSD: ligature-induced mice treated with SSD; PD + hUC-MSCs-EVs: ligature-induced mice treated with hUC-MSCs-EVs; PD + hUC-MSCs-SSD-EVs: ligature-induced mice treated with hUC-MSCs-SSD-EVs; SSD: saikosaponin D; HE: hematoxylin and eosin; hUC-MSCs: human umbilical cord mesenchymal stem cells; EVs: extracellular vesicles

Figure 3　Micro-CT and HE staining analysis of SSD drug-loaded vesicles on alveolar bone resorption and inflammatory infiltration in mice

图3　Micro-CT和HE染色分析SSD载药囊泡对小鼠牙槽骨吸收及炎症浸润的影响

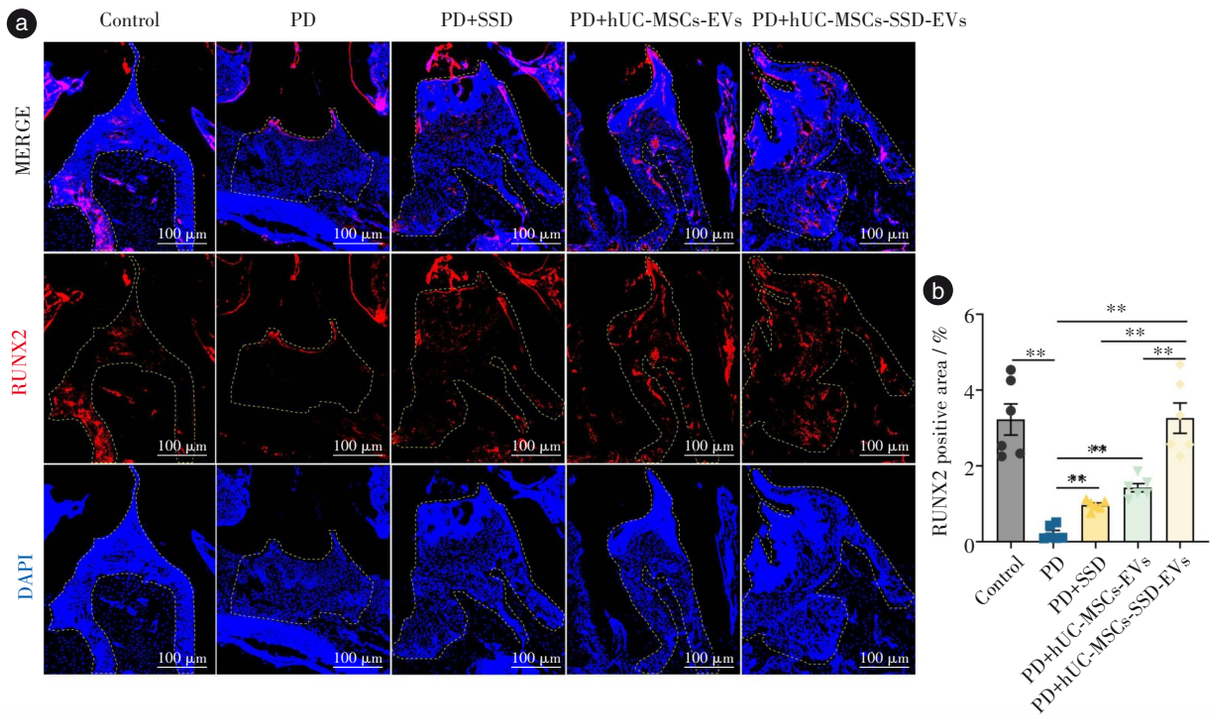
抑制病原菌，但需长期持续使用，易引起肠道菌群失调，并可能增加口腔微生物群中与牙周炎相关病原体的比例[1, 28-29]。MSC-EVs可继承母细胞的生物活性物质，具备与MSCs相似的生物学功能，



a: representative images of immunofluorescence (IF) detection for 6 mice per group of M1/M2 macrophage polarization in mouse periodontal tissue with the Control, PD, PD + SSD, PD + hUC-MSCs-EVs, and PD + hUC-MSCs-SSD-EVs. PD + hUC-MSCs-SSD-EVs reduced M1 macrophage polarization relative to PD + SSD alone and PD + hUC-MSCs-EVs, and promoted the retention of M2 macrophages. M1 phenotype: $F4/80^+$ (red)-$iNOS^+$ (green); M2 phenotype: $F4/80^+$ (red)-$CD206^+$ (green); DAPI (blue). b-c: Image J quantitative analysis of M1/M2 polarization phenotypes in mouse periodontal tissue with the Control, PD, PD + SSD, PD+hUC-MSCs-EVs, and PD + hUC-MSCs-SSD-EVs, n = 6 mice (6 biologically independent replicates). d-i: RT-qPCR quantitative analysis of the inflammatory cytokines IL-1β, IL-6, TNF-α, IL-12, IL-22, and IL-10 in mouse periodontal tissue after the above treatments. Treatment with hUC-MSCs-SSD-EVs reduced the expression of pro-inflammatory cytokines and promoted the expression of anti-inflammatory cytokines. n = 3 mice (3 biologically independent replicates). Statistical analysis for two-group comparison (Control vs PD) was performed via two-tailed independent Student's t-test. Multi-group comparisons among PD, PD+SSD, PD+hUC-MSCs-EVs and PD+hUC-MSCs-SSD-EVs groups were conducted using one-way ANOVA with post-hoc Tukey test for all pairwise comparisons. All data were expressed as mean ± standard deviation (SD). $^{**}P < 0.01$, $^{*}P < 0.05$, ns: non-significant. Control: normal mouse maxillary alveolar bone; PD: ligature-induced periodontitis in mice; PD + SSD: ligature-induced mice treated with SSD; PD+hUC-MSCs-EVs: ligature-induced mice treated with hUC-MSCs-EVs; PD+hUC-MSCs-SSD-EVs: ligature-induced mice treated with hUC-MSCs-SSD-EVs. CD206: macrophage mannose receptor; iNOS: inducible nitric oxide synthase; F4/80 is also known as epidermal growth factor-like module-containing mucin-like receptor-1 (EMR1); DAPI: 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride, also known as DAPI dihydrochloride; IL-1β: interleukin-1 beta; IL-6: interleukin-6; IL-10: interleukin-10; IL-12: interleukin-12; IL-22: interleukin-22; TNF-α: tumor necrosis factor-α; SSD: saikosaponin D; hUC-MSCs: human umbilical cord mesenchymal stem cells; EVs: extracellular vesicles

Figure 4　Immunofluorescence and RT-qPCR analysis of the effect of SSD drug-loaded vesicles on macrophage polarization in mouse periodontal tissue and its downstream cytokine expression

图4　免疫荧光法和RT-qPCR检测SSD载药囊泡对小鼠牙周组织巨噬细胞极化及其下游细胞因子表达的影响



a: representative images of immunofluorescence (IF) detection for 6 mice per group of RUNX2 protein expression in mouse periodontal tissue with the Control, PD, PD + SSD, PD + hUC-MSCs-EVs, and PD + hUC-MSCs-SSD-EVs. Treatment with hUC-MSCs-SSD-EVs promoted RUNX2 protein expression to a greater extent than SSD alone or hUC-MSCs-EV treatment. RUNX2 is red, DAPI is blue. b: Image J quantitative analysis of RUNX2 protein expression in mouse periodontal tissue after the above treatments; n = 6 mice (6 biologically independent replicates). Statistical analysis for two-group comparison (Control vs PD) was performed via two-tailed independent Student's t-test. Multi-group comparisons among PD, PD+SSD, PD+hUC-MSCs-EVs and PD+hUC-MSCs-SSD-EVs groups were conducted using one-way ANOVA with post-hoc Tukey test for all pairwise comparisons. All data were expressed as mean ± standard deviation (SD). $^{**}P < 0.01$. Control: normal mouse maxillary alveolar bone; PD: ligature-induced periodontitis in mice; PD + SSD: ligature-induced mice treated with SSD; PD + hUC-MSCs-EVs: ligature-induced mice treated with hUC-MSCs-EVs; PD + hUC-MSCs-SSD-EVs: ligature-induced mice treated with hUC-MSCs-SSD-EVs. RUNX2: Runt-related transcription factor 2; DAPI: 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride, also known as DAPI dihydrochloride

Figure 5　Immunofluorescence detection of RUNX2 expression in mouse periodontal tissue after SSD drug-loaded vesicle treatment

图5　免疫荧光法检测SSD载药囊泡对小鼠牙周组织中RUNX2表达的影响

正逐步发展为替代MSCs细胞治疗的一种无细胞策略[30]，并在牙周炎治疗中显示出潜在应用价值[31]。目前研究显示，MSC-EVs可通过降低活性氧的产生、减少炎症因子的释放、调控免疫反应、促进成骨与血管生成、调控细胞增殖与迁移、增强成纤维细胞黏附等多种机制来缓解牙周炎[31-34]。与上述研究一致，本研究发现，经hUC-MSCs及其EVs处理后，小鼠牙槽骨吸收、牙龈组织中巨噬细胞极化及其下游促炎细胞因子（IL-1β、IL-6、TNF-α、IL-12、IL-22）的表达异常，以及成骨分化关键调控因子RUNX2在牙周损伤后的表达下调均得到显著缓解。SSD是从中药柴胡中提取的萜类化合物，具有抗氧化、抗发热及抗炎等作用[35]。然而，由于SSD水溶性差，口服生物利用度低，其在牙周炎治疗中的应用受到限制，目前相关研究鲜有报道。本研究中，SSD处理同样可有效缓解小鼠牙周组织牙槽骨吸收、巨噬细胞极化、促炎因子表达异常及RUNX2表达下调等病理改变，提示SSD具备缓解牙周炎症的治疗潜力。

尽管hUC-MSCs-EVs与SSD单独应用均显示出一定的抗炎效果，但二者均存在应用局限。本研究利用hUC-MSCs-EVs高装载能力、低毒性、良好生物相容性与稳定性、免疫排斥反应轻微以及可继承母细胞生物活性物质等特点，通过体外自发装载方式将SSD负载于hUC-MSCs-EVs，旨在克服二者单独应用时的局限性，协同增强其治疗效能。

研究结果表明，hUC-MSCs-SSD-EVs具有良好的稳定性。TEM和NTA显示，hUC-MSCs-SSD-EVs呈圆形或近圆形的膜性结构，粒径为200~300 nm，SSD装载未改变MSC-EVs的形态与大小。在小鼠牙周炎疾病中，相较于hUC-MSCs-EVs或SSD单独处理，hUC-MSCs-SSD-EVs更能显著缓解小鼠牙槽骨吸收，抑制牙周组织巨噬细胞极化及其下游促炎细胞因子的表达，并改善RUNX2在牙周损伤后的表达降低。多项研究已尝试通过纳米技术策略克服SSD生物利用度不足的问题。研究发现，将巨噬细胞膜仿生药物递送系统（与T7肽杂交）涂覆于聚乳酸-羟基乙酸纳米颗粒表面并装载SSD，可改善其体内药代动力学行为与非特异性分布，降低严重副作用及系统毒性[36]；脂质体包被的SSD较游离SSD毒性更低，能显著提高小鼠存活率及生存时间，并在纤维化、炎症缓解以及肝组织修复方面表现出更优效果[37]。与上述纳米递送研究结果一致，本研究中SSD的作用呈现浓度依赖性。SSD可在hUC-MSCs中发生自发装载，装载率约为11.40%，所得hUC-MSCs-SSD-EVs稳定性良好，在小鼠牙周炎模型中能显著缓解牙槽骨吸收、牙周组织巨噬细胞极化及下游促炎细胞因子表达异常，并改善RUNX2表达水平。

RUNX2是编码521个氨基酸的蛋白[38]，在成骨细胞分化、骨形成和骨稳态维持中发挥着核心作用[39]。研究显示，在牙周炎大鼠模型中，RUNX2蛋白表达降低，而经乙醛脱氢酶2处理后，IL-6和TNF-α水平降低，RUNX2表达则升高[40]；此外，在小鼠牙周炎模型研究中，黄酮类化合物可逆转病理性骨吸收并促进牙周再生，其作用与抑制RUNX2表达下调相关[41]。本研究中，免疫荧光染色结果显示，小鼠牙周炎模型中RUNX2表达降低，而经hUC-MSCs-SSD-EVs治疗后，牙槽骨吸收显著缓解，RUNX2表达上调。以上结果进一步证实了RUNX2在小鼠成骨分化、骨形成及骨维持中的核心地位，且hUC-MSCs-SSD-EVs在维持RUNX2蛋白表达方面优于SSD或hUC-MSCs-EVs单用。然而，hUC-MSCs-SSD-EVs是否通过调控RUNX2发挥关键作用，仍需进一步的干预实验加以验证。

尽管本研究初步从SSD作用浓度、hUC-MSCs载药及验证、牙槽骨吸收评价、牙周组织巨噬细胞极化和炎症相关因子表达、RUNX2蛋白表达变化等方面初步验证了hUC-MSCs-SSD-EVs对小鼠牙周炎的缓解作用，具备一定创新性，但仍存在以下不足：第一，研究侧重于“现象观察”（如骨吸收、巨噬细胞极化和炎症因子及RUNX2表达），未对具体作用机制深入研究，缺乏关键信号通路或分子靶点的验证；第二，虽显示了治疗效应，但未提供载药囊泡的释放曲线等定量数据；第三，在牙槽骨吸收评价方面，仅检测了处理结束后第4周单一时间点，缺乏动态观察。因此，后续研究需进一步充分验证SSD载药囊泡对小鼠牙周炎的缓解作用。

综上所述，hUC-MSCs-SSD-EVs能缓解小鼠牙周组织的局部炎症环境，减少牙槽骨吸收，为牙周炎疾病的治疗提供了一种新的策略。

【Author contributions】 He GX performed the experiments, and wrote the article. Yang XY, Zhu YL, Wang QY, Hu TF performed the experiments, assisted the data analysis, and revised the article. Sui BD, Zhang S conceptualized the study and revised the article. All authors read and approved the final manuscript as submitted.

【Ethics Statement】 This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Medical Ethics Committee of our institution (Approval No. KY20252585 C 1), and written informed consent was obtained from the participant. The animal procedures were performed in compliance with the ARRIVE 2.0 guidelines and were approved by the Laboratory Animal Ethics Committee of Shaanxi University of Chinese Medicine (Approval No. SUCMDL20250801002).

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【Data availability statement】 The original contributions presented in this study are included in the article, with a supplementary table available online at 链接待补充. Further inquiries can be directed to the corresponding author upon reasonable request.

【Generative AI statement】 The authors declared that generative AI was not used in the creation of this manuscript.

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