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· 基础研究 ·

大鼠牙槽窝干细胞与牙周膜间充质干细胞增殖与成骨分化能力的比较

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【摘要】 目的 探讨大鼠牙槽窝干细胞(DSCs)与牙周膜间充质干细胞(PDLSCs)的增殖及成骨分化等方面能力的差异,为阐明2种来源间充质干细胞的功能特性提供实验依据。方法 获得单位实验动物福利伦理委员会的批准。将24只6~8周龄的SD雄性大鼠按拔牙术后0、1、3、7 d随机数字法分为4组,每组6只,通过拔除大鼠左侧磨牙的方式建立大鼠牙槽窝模型。HE染色观察拔牙后0、1、3、7 d的大鼠牙槽窝模型的组织形态变化,免疫组化法检测P75神经生长因子受体(P75NTR)的表达及分布;依据染色结果选取适宜时间点,采集牙槽窝的早期愈合组织,采用组织块法与酶消化法培养DSCs,并采用相同的细胞培养方法,从大鼠拔除的磨牙中提取PDLSCs。通过流式细胞术检测2种细胞(DSCs组和PDLSCs组)的表面标志物CD44、CD45、CD90和P75NTR,克隆形成检测和CCK-8法对比2组细胞在增殖和克隆形成能力上的差异;成脂诱导后通过油红O染色观察2种细胞脂滴形成情况;成骨诱导后,通过碱性磷酸酶(ALP)活性检测及茜素红染色(ARS),分别观察2种细胞ALP表达及矿化结节生成情况;进一步检测2种细胞成骨相关因子ALP和Runt相关转录因子2(RUNX2)的mRNA与蛋白表达水平,综合评价2种细胞的成骨分化潜能。结果 HE染色结果显示大鼠拔牙术后即刻和拔牙后1 d牙槽窝内充满红细胞,3 d牙槽窝内纤维结缔组织大量增生,7 d骨组织形成显著增多。免疫组化结果显示:大鼠拔牙术后即刻和1 d的牙槽窝仅在内壁处可见少量的P75NTR阳性表达,大鼠拔牙术后3 d,P75NTR分布于牙槽窝纤维结缔组织间,骨壁见少量着色;7 d该受体表达于骨小梁边缘的纤维结缔组织。依据染色结果选取了拔牙后3 d进行DSCs的培养。PDLSCs与DSCs在体外形态相似,2种细胞均高表达间充质干细胞相关标志物表型CD90、CD44以及P75NTR,低表达间充质干细胞的阴性表型CD45。CCK-8法结果显示DSCs增殖能力较PDLSCs强($P<0.05$),且克隆形成率更高($P<0.001$)。PDLSCs与DSCs在体外均具有成脂分化潜能,2种细胞胞浆内均可见空泡样结构;2种细胞都显示出成骨分化的潜能,DSCs组ALP蓝染面积及密度更高($P<0.001$),ARS染色结果显示2种细胞均形成了橙红色矿化结节,DSCs钙化结节形成更多($P<0.0001$),DSCs的ALP与RUNX2的mRNA和蛋白表达水平均高于PDLSCs(均 $P<0.05$)。结论 大鼠DSCs可体外稳定培养,其增殖、克隆形成及成骨分化能力均优于PDLSCs。

【关键词】 牙周膜间充质干细胞; 牙槽窝干细胞; P75神经生长因子受体; 成骨分化; 增殖; 克隆形成; Runt相关转录因子2; 碱性磷酸酶

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Comparison of the proliferation and osteogenic differentiation abilities of rat dental socket stem cells and periodontal ligament stem cells MENG Shengzi^{1,2}, CHEN Li³, TAN Jingyao³, YANG Kun¹. 1. Hospital of Stomatology, Zunyi Medical University, Zunyi 563006, China; 2. The Second Nanning People's Hospital, Nanning 530031,



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【Abstract】 Objective To investigate the differences in proliferation and osteogenic differentiation capacities between rat dental socket stem cells (DSCs) and periodontal ligament mesenchymal stem cells (PDLSCs), thereby providing a experimental basis for elucidating the functional characteristics of these two distinct sources of mesenchymal stem cells. **Methods** This study was approved by the Institutional Animal Care and Use Committee. Twenty-four 6- to 8-week-old male Sprague - Dawley rats were divided into four groups ($n = 6$ per group) based on the time points of 0, 1, 3, and 7 days post-extraction by the random number table method. A rat alveolar socket model was established by extracting the left maxillary molars. Hematoxylin and eosin (H&E) staining was performed to observe the histomorphological changes in the alveolar sockets at the specified time points. Immunohistochemistry was used to detect the expression and distribution of the p75 neurotrophin receptor (P75NTR). Based on the staining results, an optimal time point was selected to harvest early healing tissues from the alveolar sockets. DSCs were cultured using a combination of tissue block and enzymatic digestion methods. Concurrently, PDLSCs were isolated from the extracted molars using identical culture protocols. The surface markers CD44, CD45, CD90, and P75NTR were analyzed via flow cytometry. Cell proliferation and clonogenic potential were compared using the Cell Counting Kit-8 (CCK-8) assay and colony formation assays, respectively. After adipogenic induction, Oil Red O staining was conducted to observe lipid droplet formation. Following osteogenic induction, alkaline phosphatase (ALP) activity and alizarin red S (ARS) staining were utilized to evaluate ALP expression and mineralized nodule formation, respectively. Furthermore, the mRNA and protein expression levels of osteogenesis-related factors, including ALP and Runt-related transcription factor 2 (RUNX2), were quantified to comprehensively assess the osteogenic differentiation potential of both cell types. **Results** H&E staining revealed that the alveolar sockets were filled with erythrocytes 0 and 1 days post-extraction. By day 3, significant fibroblastic connective tissue proliferation was observed within the sockets, and bone formation increased markedly by day 7. Immunohistochemical analysis showed sparse P75NTR-positive staining limited to the inner socket wall 0 and 1 days post-extraction. On day 3, P75NTR was distributed throughout the fibrous connective tissue of the socket, with slight staining on the bone walls. By day 7, the receptor was expressed in the fibrous connective tissue at the edges of the trabeculae. Consequently, day 3 post-extraction was selected as the optimal time point for DSC isolation. Both PDLSCs and DSCs exhibited similar morphologies *in vitro*, characterized by high expression of mesenchymal stem cell markers (CD90, CD44) and P75NTR, alongside low expression of the hematopoietic marker CD45. CCK-8 assays demonstrated that the proliferation capacity of the DSCs was significantly higher than that of the PDLSCs ($P < 0.05$), accompanied by a greater colony formation rate ($P < 0.001$). Both cell types possessed adipogenic differentiation potential, as evidenced by vacuolar structures in the cytoplasm. Regarding osteogenesis, the DSCs exhibited a larger area and higher density of ALP staining ($P < 0.001$). ARS staining indicated that both cells formed orange-red mineralized nodules; however, the DSCs produced significantly more calcified nodules ($P < 0.0001$). Additionally, the mRNA and protein expression levels of ALP and RUNX2 were notably elevated in the DSCs compared with the PDLSCs (all $P < 0.05$). **Conclusion** Rat DSCs can be stably cultured *in vitro*. Compared with PDLSCs, DSCs exhibited superior capabilities in terms of proliferation, colony formation, and osteogenic differentiation.

【Key words】 periodontal ligament stem cells; dental socket stem cells; P75 neurotrophin receptor (P75NTR); osteogenic differentiation; proliferation; colony formation; Runt-related transcription factor 2; alkaline phosphatase

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作为牙周组织工程种子细胞的牙周膜间充质干细胞(periodontal ligament stem cells, PDLSCs),具有自我增殖和更新能力,有成骨^[1]、成脂^[2]等多向分化潜能。PDLSCs可整合生物活性因子与可降解支架材料,形成功能化复合体系^[3]。尽管PDLSCs的应用体系已较为成熟,其固有短板仍制约着临

床转化:牙周炎患者的PDLSCs易受炎症微环境抑制^[4];同时取材存在实操与伦理限制。因此,有必要开发取材微创、炎症耐受性佳的新型种子细胞。

牙槽窝作为机体天然的愈合微环境,其肉芽组织取材便捷、创伤小、细胞储备充足,且天然适配拔牙后牙槽窝的炎症愈合过程,成为极具潜力

的新型种子细胞来源地。2014年, Nakajima等^[5]首次成功从犬拔牙后牙槽窝中提取出牙槽窝干细胞(dental socket stem cells, DSCs), 相较于同种的骨髓间充质干细胞, 增殖及迁移能力都要更强, DSCs在体外还拥有多向分化能力, 在结合生物支架材料后, 可再生出完整的牙周组织结构。关于拔牙后牙槽窝来源间充质干细胞的成骨等分化相关潜能研究报道尚不多见, 生物学特性尚未阐明, 加之其细胞种群构成极为复杂, 该领域整体仍处于初步探索阶段, DSCs与PDLSCs在增殖、分化等关键功能上的差异也待考究。

P75神经营养因子受体(P75 neurotrophin receptor, P75NTR)作为低亲和力受体^[6], 通常可与神经生长因子、脑源性神经营养因子等结合, 可调节不同牙源性细胞群体的存活、凋亡、增殖和组织重塑等生物学过程^[7]。有学者在评估牙髓干细胞(dental pulp stem cells, DPSCs)的再生能力和细胞衰老相关特性时, 发现P75NTR的表达状态可以作为筛选高增殖能力以及多功能性DPSCs群体的评价指标^[8]。至于P75NTR在拔牙后牙槽窝的表达情况, 目前尚未见报道。

为阐明上述问题, 本研究以大鼠DSCs与PDLSCs为研究对象, 对比分析两种细胞的增殖能力与多向分化潜能, 同时观察P75NTR在拔牙后牙槽窝组织中的表达, 以期进一步阐明DSCs的应用价值, 也为牙周骨组织修复研究提供新的思路 and 参考。

1 材料和方法

1.1 实验动物

本研究动物实验遵守动物研究体内实验报告(The Animal Research: Reporting of In Vivo Experiment, ARRIVE)指南2.0(<http://arriveguideline.org>), 获得遵义医科大学实验动物福利伦理委员会批准(审批号: ZMU21-2303-197)。实验动物使用许可证号: SYXK(黔)2001-0004。SD雄性大鼠购买于遵义医科大学实验动物中心, 饲养于SPF级环境中, 6~8周龄, 体重均在200~300 g, 在室温21~26℃和相对湿度40%~60%的条件下, 采用室内光照与黑暗各12 h循环。经过适应性饲养7 d, 自由摄食饮水, 无口腔疾患, 健康状态良好。排除标准: 拔牙术中意外断根、术后感染、体重下降超过15%的SD大鼠。为控制混杂因素, 大鼠笼位在笼架上随机排列, 每周轮换位置以均衡光照和通风因素的

影响。

1.2 主要试剂

α -MEM (Gibco, 美国)、胎牛血清 (Gibco, 美国)、0.25%胰蛋白酶 (Gibco, 美国); 青霉素-链霉素-两性霉素B混合液 (Solarbio, 中国)、磷酸盐缓冲液 (Solarbio, 中国)、中性蛋白酶 II (Solarbio, 中国)、I型胶原酶 (Solarbio, 中国)、CCK-8试剂盒 (CA1210, Solarbio, 中国)、油红O染色试剂盒 (G1262, Solarbio, 中国)、结晶紫 (G1062, Solarbio, 中国)、BCA试剂盒 (PC0020, Solarbio, 中国)、10%EDTA脱钙液 (E1171, Solarbio, 中国)、地塞米松 (SD9520, Solarbio, 中国)、维生素C (SV8120, Solarbio, 中国)、 β -甘油磷酸钠 (Solarbio, 中国)、4%多聚甲醛组织细胞固定液 (Solarbio, 中国)、0.2%茜素红染色液 (Solarbio, 中国)、高效RIPA裂解液 (P0013B, 碧云天, 中国)、TBST缓冲液 (20 $\times$) (Solarbio, 中国)、ECL化学发光试剂盒 (Solarbio, 中国); 苏木素-伊红染色液 (Labcoms, 中国); 大鼠间充质基质细胞成脂诱导分化培养基试剂 (D3512, Solarbio, 中国); ALP检测试剂盒 (C3206, 碧云天, 中国)、SDS-PAGE电泳液 (P0015L, 碧云天, 中国); RNA-easy Isolation Reagent、HiScript III RT SuperMix for qPCR (+gDNA wiper) (R701-01, R323, Vazyme, 中国); SYBR Premix Ex Taq TM试剂盒 (DRR041A, Takara, 日本); PCR引物 (Sangon Biotech, 中国); PAGE凝胶快速制备试剂盒 (雅酶, 中国); PAGE蛋白上样缓冲液 (庄盟, 中国); Western快速电转液 (10 $\times$) (Bio-Rad, 美国); 脱脂奶粉 (博士德, 中国); 一抗 (兔抗) ALP (工作浓度 1:1 000)、Runt相关转录因子2 (runt-related transcription factor 2, RUNX2) (1:500)、P75NTR (免疫组化工作浓度 1:100, 流式检测工作浓度 1:60)、CD44 (1:500)、CD45 (1:200)、CD90 (1:500) (ab229126、ab192256、ab52987、ab316123、ab154885、ab307736 (Abcam, 美国); 羊抗兔二抗 (GB23303, 1:100, Servicebio, 中国), 羊抗兔二抗 (GB22303, 1:50, Servicebio, 中国); GAPDH (AF7021, 1:5 000, Affinity, 中国); 羊抗兔二抗 (70-GAR0072, 70-GAM0072, 1:1 0000, Multi sciences, 中国); 全波长酶标仪 (SpectraMax M3, Molecular Devices, 中国)、超微量分光光度仪、修复仪 (Thermo Scientific, 美国); 倒置显微镜 (BX53, Olympus, 日本); ABI荧光定量PCR仪 (ViiA 7Dx, Applied Biosystems, 英国)、垂直电泳槽、电转仪、凝胶成像系统 (Bio-Rad ChemiDoc MP, Bio-Rad, 美

国)、多功能染色机(莱卡,德国)。

1.3 大鼠牙槽窝模型建立与组织切片的制作

为观察拔牙术后大鼠牙槽窝的组织形态变化,随机数字法将24只大鼠按拔牙术后观察0、1、3、7 d分为4组,每组6只,样本量根据预实验确定,0 d组作为基线对照组。

各组大鼠同期开展手术,经腹腔注射1%戊巴比妥钠40 mg/kg麻醉后固定于手术台,使用开口器暴露并固定上下颌骨,术区常规消毒后,在止血钳与口腔探针辅助下,微创拔除大鼠左上颌3颗磨牙及左下颌3颗磨牙,局部缝合,压迫止血,术后常规饲养。

各组大鼠饲养至对应观察天数后处死取材:采用腹腔注射过量戊巴比妥钠150 mg/kg的方法麻醉,确认大鼠无疼痛反应后,颈椎脱臼法处死大鼠,并解剖出下颌骨,将下颌骨组织移至4%多聚甲醛组织固定液中进行固定,1 d后将组织放入10%EDTA脱钙液中进行脱钙,每脱钙3 d换1次新的脱钙液,约40 d后,若金属探针易刺入组织标本为脱钙完成标准。下颌骨标本经梯度脱水、石蜡包埋、切片,用于后续实验检测与分析。

所有手术及样本的采集均在遵义医科大学实验动物中心无菌操作室内完成。本研究24只大鼠全部完成实验,无大鼠因意外或并发症被排除。

1.4 大鼠下颌拔牙后牙槽窝组织切片HE染色

将石蜡切片依次放入二甲苯及不同浓度乙醇中反应后,纯水浸泡后苏木精染色5 min,冲洗后置入盐酸酒精分化液中5 s,冲洗后置入85%乙醇3 min,伊红染色5 min,经过梯度乙醇脱水后完成HE染色,显微镜下检查拔牙后不同天数的牙槽窝组织形态学变化,包括肉芽组织、新生骨的形成等,进行定性描述。

1.5 大鼠下颌拔牙后牙槽窝组织切片免疫组织化学染色

P75NTR免疫组织化学染色观察P75NTR在牙槽窝愈合过程中的表达部位和分布范围变化,进行定位描述。将石蜡切片依次放入二甲苯及不同浓度乙醇中反应后,冲洗后浸入柠檬酸盐缓冲液(pH 6.0),微波炉高火加热10 min,停火8 min后中高火加热10 min,PBS冲洗后放入3%过氧化氢中10 min,PBS再次冲洗后滴加正常山羊血清,20 min后滴加一抗:P75NTR(1:100),4℃过夜后PBS冲洗后加入二抗,37℃反应30 min,PBS再次冲洗,经过DAB显色,苏木素复染后成片。

1.6 大鼠牙周膜间充质干细胞和牙槽窝干细胞分离培养及表型鉴定

PDLSCs:拔除24只大鼠左上颌及左下颌全部磨牙后即刻放入含有2%三抗的 α -MEM中,在超净台内用PBS溶液反复冲洗,无菌刀片刮取牙周膜组织,加入2 mg/mL的I型胶原酶与2 mg/mL中性蛋白酶II的等比混合液消化,放于37℃的恒温水浴中等待50 min,使用等量完全培养基中和,其中完全培养基成分为 α -MEM、10%FBS、2%青霉素-链霉素-两性霉素B,离心后加入新的完全培养基重悬组织块,均匀铺于T25细胞瓶内,调节为5%CO₂的细胞孵育箱有助于组织块贴壁,待T25瓶内的细胞达到80%~85%的融合度,进行1:2的传代培养。

DSCs:刮取大鼠拔牙后牙槽窝模型建立3 d(6只大鼠左上和左下颌全部磨牙拔牙后牙槽窝)的早期愈合组织,即刻放入2%三抗混合液的 α -MEM中保存,用PBS冲洗,加入2 mg/mL的I型胶原酶与2 mg/mL中性蛋白酶II等比混合液消化,37℃恒温水浴条件下消化50 min,最后接种到新的T25细胞瓶,5%CO₂细胞孵育箱中培养。DSCs与PDLSCs的传代培养步骤相同。

流式细胞术鉴定:取用第3代的PDLSCs和DSCs,常规消化离心,用3%FBS的PBS重悬细胞,经过计数调整细胞浓度为 2×10^6 个/mL,分装于流式管内加入不同的流式抗体:CD44-FITC、CD45-Pacific Blue、P75NTR-PE、CD90-FITC,孵育30 min,加入3%FBS的PBS洗涤,1 000 r/min离心6 min,重复洗涤2次后,依次放入流式细胞仪进行检测。

1.7 大鼠牙周膜间充质干细胞和牙槽窝干细胞的细胞增殖和克隆形成能力检测

细胞增殖能力检测:取生长良好的第3代PDLSCs和DSCs,常规消化重悬后,经过细胞计数稀释后,PDLSCs和DSCs均以 3×10^3 个/孔的密度种入提前标记好的96孔板内,每组5个复孔,每孔完全培养基均为100 μ L。每24 h取出对应的96孔板,每孔加入10 μ L CCK-8,细胞孵箱内避光孵育2 h,酶标仪检测450 nm处的吸光度值,连续检测7 d。

克隆形成:取生长状况良好的第3代PDLSCs和DSCs,消化离心重悬后进行细胞计数,PDLSCs和DSCs都以每皿800个细胞的数量移植于直径10 cm的细胞培养皿中,每组3个重复培养皿,常规换液培养,12 d时,取出两组培养皿用PBS冲洗3次,加5 mL的4%多聚甲醛固定30 min,PBS冲洗3次,加入5 mL的1%结晶紫染色1 h,PBS冲洗3次,干燥

后观察染色,计算克隆形成率。克隆形成率=(克隆数/接种细胞数)×100%。

1.8 大鼠牙周膜间充质干细胞和牙槽窝干细胞多向分化能力验证

成脂诱导及染色:取第3代的PDLSCs和DSCs, PBS冲洗2次,细胞计数后以每孔 2×10^5 个细胞的密度种于6孔板内,每种细胞设3个复孔,按成脂分化诱导试剂盒说明书配制诱导液,48 h后观察待细胞融合率达到80%左右,更换完全培养基为成脂诱导培养基,按试剂说明书的提示换诱导液,诱导21 d后在显微镜下观察细胞已形成了充足的脂滴, PBS冲洗2~3次后,4%多聚甲醛固定30 min,配制油红O染色液,在室温下染色30 min,显微镜下拍照记录。

成骨诱导及染色:选取第3代的PDLSCs和DSCs,常规消化离心后,适当稀释,以 2×10^5 个/孔的密度接种于6孔板中,每种细胞设3个复孔,培养48 h后,达到80%左右的密度,更换为含10 mmol/L

β -甘油磷酸钠,50 μ g/mL维生素C, 1×10^{-8} mol/L地塞米松、1%青霉素-链霉素-两性霉素B混合液、10%FBS的 α -MEM培养基的成骨诱导液。配置ALP染色液,在细胞成骨诱导7 d时,每孔加入2 mL的4%多聚甲醛,固定30 min的时间,加入染色液染色30 min,纯水冲洗后即可显微镜观察染色结果。另外,成骨诱导21 d时,显微镜下可见散在的钙结节,经4%多聚甲醛固定30 min,每孔直接加入2 mL的0.2%ARS染色液,室温避光染色30 min,纯水冲洗后对PDLSCs和DSCs形成的红色矿化结节进行拍摄分析。

1.9 大鼠牙周膜间充质干细胞和牙槽窝干细胞成骨相关基因实时荧光定量PCR检测

两种细胞经上述步骤进行成骨诱导7 d,采用Trizol法提取细胞总RNA,通过反转录试剂盒将其逆转录为cDNA,以GAPDH作为内参基因,进行qRT-PCR检测,目标基因的相对表达量依据 $2^{-\Delta\Delta Ct}$ 法计算,引物序列信息见表1。

表1 基因引物序列

Table 1 Gene primer sequences

Genes	Forward (5'-3')	Reverse (5'-3')
RUNX2	GGA CTG GAC GAG GCA AGA CT	AGG CGG TCA GAG AAC AAA CT
ALP	CGG ACC ATT CCC ACG TCT TC	CAT TCT CTC GTT CAC CGC CC
GAPDH	TCT CTG CTC CTC CCT GTT CT	ATC CGT TCA CAC CGA CCT TC

RUNX2: runt-related transcription factor 2. ALP: alkaline phosphatase. GAPDH: glyceraldehyde-3-phosphate dehydrogenase

1.10 大鼠牙周膜间充质干细胞和牙槽窝干细胞成骨相关蛋白Western blot检测

两种细胞的成骨诱导步骤同1.7。取成骨诱导7 d的PDLSCs和DSCs,每孔内加入100 μ L由(RIPA:磷酸酶抑制剂:PMSF=100:1:1)组成的裂解液,4℃低温条件下裂解30 min,收集到15 mL的离心管中,离心10 min(4℃、12 000 r/min),收集蛋白后使用BCA法测定浓度,样品加入适量上样缓冲液,100℃煮5 min后蛋白质变性;制胶以备后续操作,在凝胶的两侧孔位处加入marker的量为2 μ L,根据蛋白浓度取10 μ L样品分别加入孔中,110 V电泳40 min,观察溴酚蓝逐渐分散至胶底部,结束电泳。按滤纸、PVDF膜、胶与滤纸的顺序逐层湿润,放入电转盒内,400 mA转膜30 min。转膜完成后用TBST洗3次,加入8%脱脂奶液,室温封闭2 h,结束后TBST洗PVDF膜3次,每次5~10 min。将膜放入配制好的ALP、RUNX2和GAPDH的一抗稀释液,一抗如下:GAPDH(1:5 000)、ALP(1:1 000)、

RUNX2(1:500),放入4℃冰箱,摇床使抗体孵育过夜。将PVDF膜用TBST洗3次,每次10 min。再将膜放入配制好的二抗稀释液,室温孵育2 h, TBST洗3次,每次10 min。膜干燥后,在膜上滴加ECL曝光工作液,使用凝胶成像系统显影检测蛋白条带,灰度值的分析用Image J软件进行。

1.11 统计学方法与可重复性

所有细胞实验生物学重复次数至少为3次。统计与计算在GraphPad Prism 9.0软件协助下进行。本研究所有定量数据均采用Shapiro-Wilk检验完成正态性验证,同时采用Levene检验进行方差齐性检验。经检验,本研究全部计量数据均符合正态分布且各组间方差齐,满足独立样本t检验与方差分析等参数检验方法的应用前提。CCK-8数据采用方差分析,对克隆形成检测、RT-PCR和Western blot的结果进行独立样本t检验, $P < 0.05$ 为差异有统计学意义。

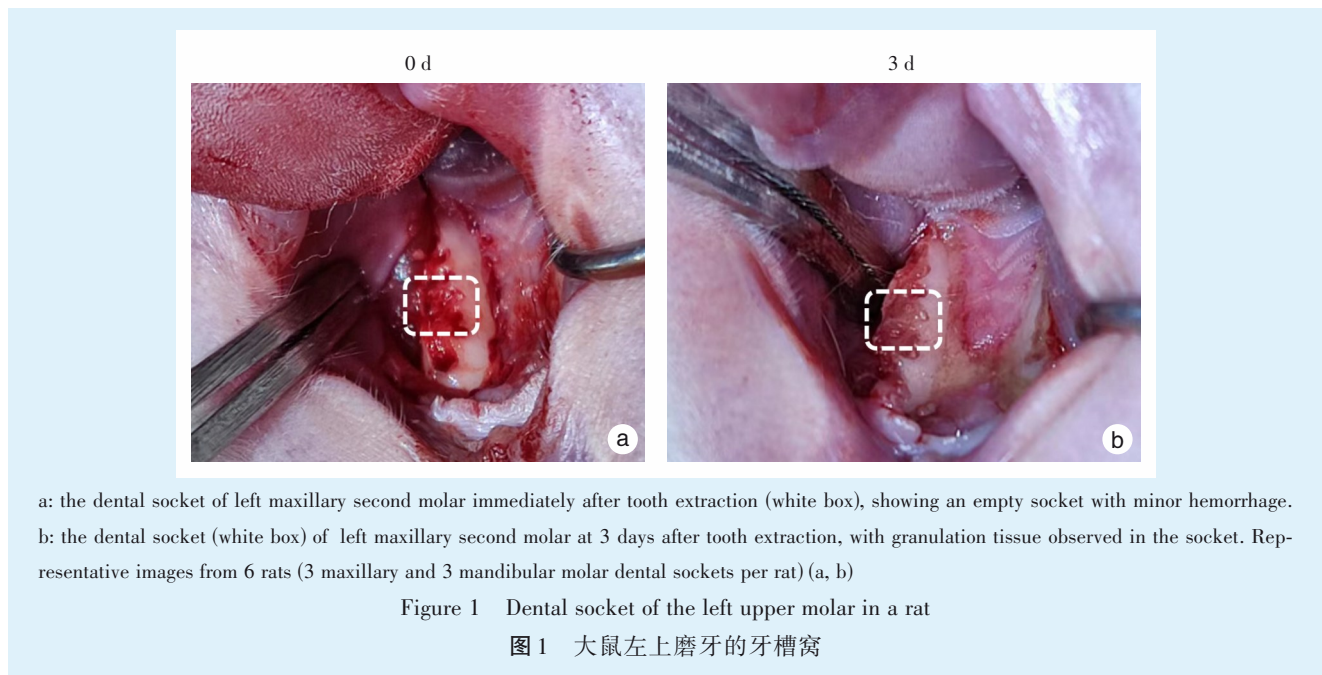
动物分组由不参与后续实验操作与评估的通信作者采用随机数字法完成,所有手术由同一操

作者实施,手术顺序按组随机化安排,实施者知晓分组情况。组织学评估采用单盲法:所有 HE 染色和免疫组化切片由通信作者重新编号后,交由两名不知晓分组信息的研究者独立进行组织学观察,分析完成后揭盲对应。

2 结果

2.1 大鼠拔牙后牙槽窝

大鼠磨牙拔牙后即刻,牙槽窝空虚,局部有出血,拔牙术后 3 d,可见牙槽窝内充满大量淡红色质地软的早期愈合组织(图 1)。



2.2 大鼠拔牙后牙槽窝组织切片 HE 染色观察

镜下观察显示,拔牙术后即刻和拔牙后 1 d,牙槽窝内充满红细胞;拔牙后 3 d,牙槽窝内有大量的纤维结缔组织;拔牙后 7 d,牙槽窝内可见初期的骨性愈合,主要有新生骨细胞和大量的破骨细胞,排列不规则,周围仍可见纤维结缔组织和大量破骨细胞(图 2)。

2.3 大鼠牙槽窝组织切片 P75 神经营养因子受体免疫组织化学染色

拔牙术后即刻和 1 d 时:仅在牙槽窝内壁处可见少量的 P75NTR 阳性表达;而拔牙后 3 d,表达区域发生了变化,即牙槽窝内壁有少量着色的同时,牙槽窝中心纤维结缔组织的周围有散在的阳性区域;拔牙术后 7 d, P75NTR 表达于新生骨松质组织周围的纤维结缔组织中(图 3)。

2.4 大鼠牙周膜间充质干细胞和牙槽窝干细胞的培养及鉴定

PDLSCs 原代细胞从组织块爬出,呈纺锤状,细胞胞浆饱满,逐渐增殖成簇;DSCs 原代细胞亦呈纺锤状,贴壁后逐渐聚集生长。流式细胞仪的鉴定结果明确表明,培养的 PDLSCs 和 DSCs 均高度表达

与间充质干细胞相关的标志性表型,包括 CD90、CD44 以及 P75NTR,而同时低表达间充质干细胞的阴性表型 CD45,说明 PDLSCs 和 DSCs 均源自于间充质干细胞(图 4)。

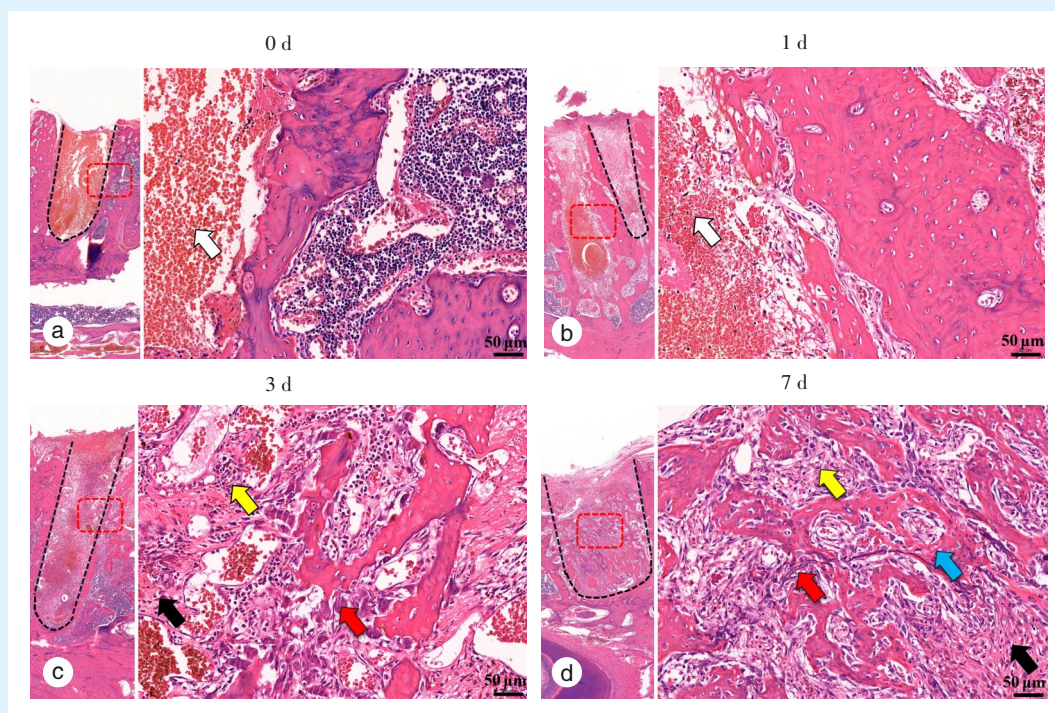
2.5 大鼠牙周膜间充质干细胞和牙槽窝干细胞的增殖能力及克隆形成能力对比

选用传代至第 3 代的 DSCs 和 PDLSCs,连续 7 d 检测增殖情况,结果显示,5 d ($P=0.000\ 2$)、6 d ($P=0.000\ 6$)、7 d ($P=0.031\ 6$) 时, DSCs 细胞数量均较 PDLSCs 多,差异有统计学意义(图 5)。

结晶紫染色结果可见,2 种细胞均可形成大小不等的细胞团簇, DSCs 和 PDLSCs 克隆形成率分别为 $29.65\%\pm 1.05\%$ 及 $19.21\%\pm 1.36\%$, DSCs 组形成的团簇数量更多,且面积更大,差异具有统计学意义 ($P<0.001$)(图 5)。

2.6 大鼠牙周膜间充质干细胞和牙槽窝干细胞的成脂分化能力检测

成脂诱导 21 d 后,显微镜下观察两种细胞的胞浆内均可见空泡样结构,经过油红 O 染色处理后,相较于 PDLSCs, DSCs 同样可进行成脂分化,能够稳定形成典型脂肪细胞特征性的红色脂滴,成



a: immediately after tooth extraction. A large accumulation of red blood cells was observed in the dental socket, with a small number of osteoclasts visible at the bone margin. b: one day after tooth extraction, showing similar histological features to those immediately post-operation. c: three days after tooth extraction, the amount of red blood cells in the dental socket decreased and was gradually replaced by fibrous connective tissue. d: seven days after tooth extraction. The formation of new bone cells was observed in the dental socket, with increased quantity but disordered arrangement, forming a large area of bony callus. Proliferation of fibrous connective tissue and many osteoclasts were noted around the callus. Black arrow: fibroblasts; white arrow: erythrocytes; blue arrow: osteoblasts; yellow arrow: fibrocytes; red arrow: osteoclasts; black dotted line: dental socket; red box: selected field for high power microscopic magnification. Representative HE staining images selected from histopathological sections of the left mandibular second molar alveolar sockets in six rats per group (a, b, c, d)

Figure 2 Histomorphology of a rat dental socket after tooth extraction

图2 大鼠拔牙后的牙槽窝组织形态

脂分化表型明确,结果证实,两种细胞都具有良好的成脂分化特性(图6)。

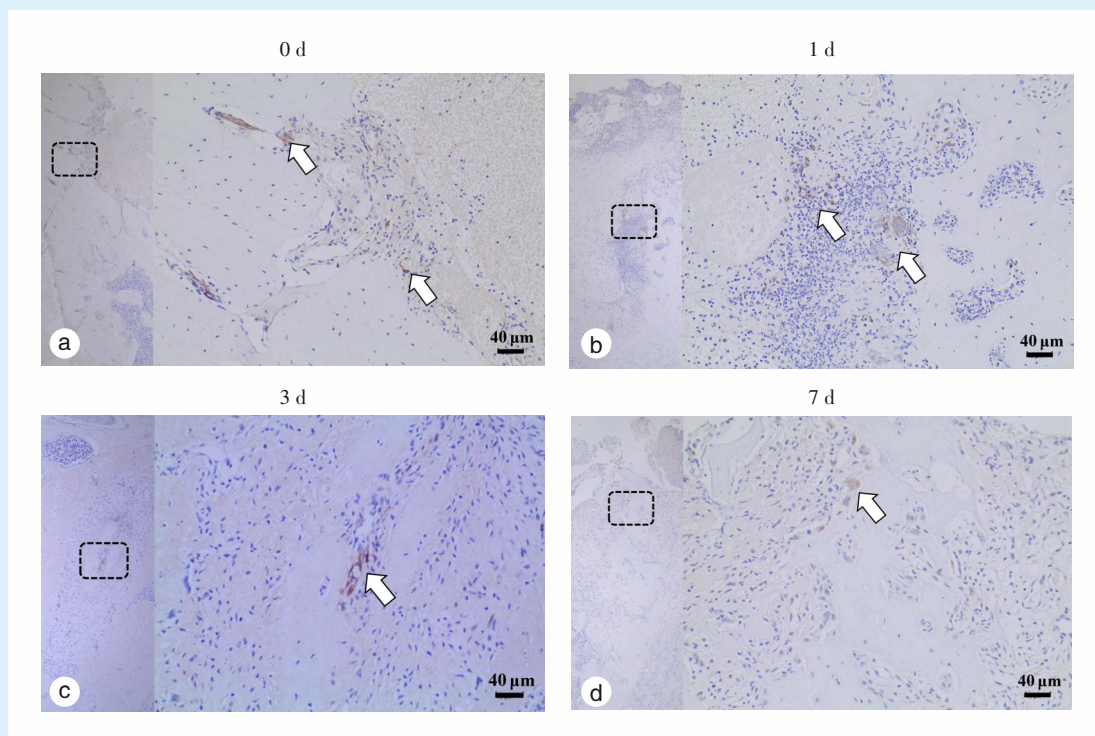
2.7 大鼠牙周膜间充质干细胞和牙槽窝干细胞的成骨分化能力的对比

成骨诱导7 d后,DSCs组较PDLSCs组ALP蓝染面积及密度高,差异具有统计学意义($P<0.001$);ARS染色结果显示,成骨诱导21 d后,2组细胞周围均形成了橙红色矿化结节,相比PDLSCs组,DSCs组的数量更多,2种细胞差异具有统计学意义($P<0.0001$)(图7)。qRT-PCR及Western blot结果显示,成骨诱导7 d后,2种细胞的成骨相关基因ALP、RUNX2 mRNA和蛋白表达均上调,DSCs高于PDLSCs,差异具有统计学意义(均 $P<0.05$)(图7)。上述结果说明,DSCs的成骨分化能力较PDLSCs强。

3 讨论

3.1 牙槽窝:具有潜力的间充质干细胞来源

慢性牙周炎会使牙周组织发生不可逆的破坏,显著影响着患者日常生活质量和全身健康的维持^[9]。如何修复牙周组织结构已成为如今口腔再生医学的重要命题。为克服当前牙周治疗技术的瓶颈,牙周组织工程技术作为创新再生策略,有望取代传统治疗模式,成为经临床验证的牙周再生解决方案^[10]。PDLSCs在体外能稳定扩增,目前已有大量临床应用转化研究,其能在炎性的细胞外基质刺激下快速的增殖^[11],PDLSCs可通过分泌外囊泡与内囊泡,介导PDLSCs增殖、抑制炎症因子释放,并显著增强PDLSCs的成骨分化潜能^[12],这可有助于维持干细胞移植后免疫环境的稳态。PDLSCs具有良好的组织相容性,在牙周缺损的动物模型中再生出新的牙周复合体^[13]。尽管如此,



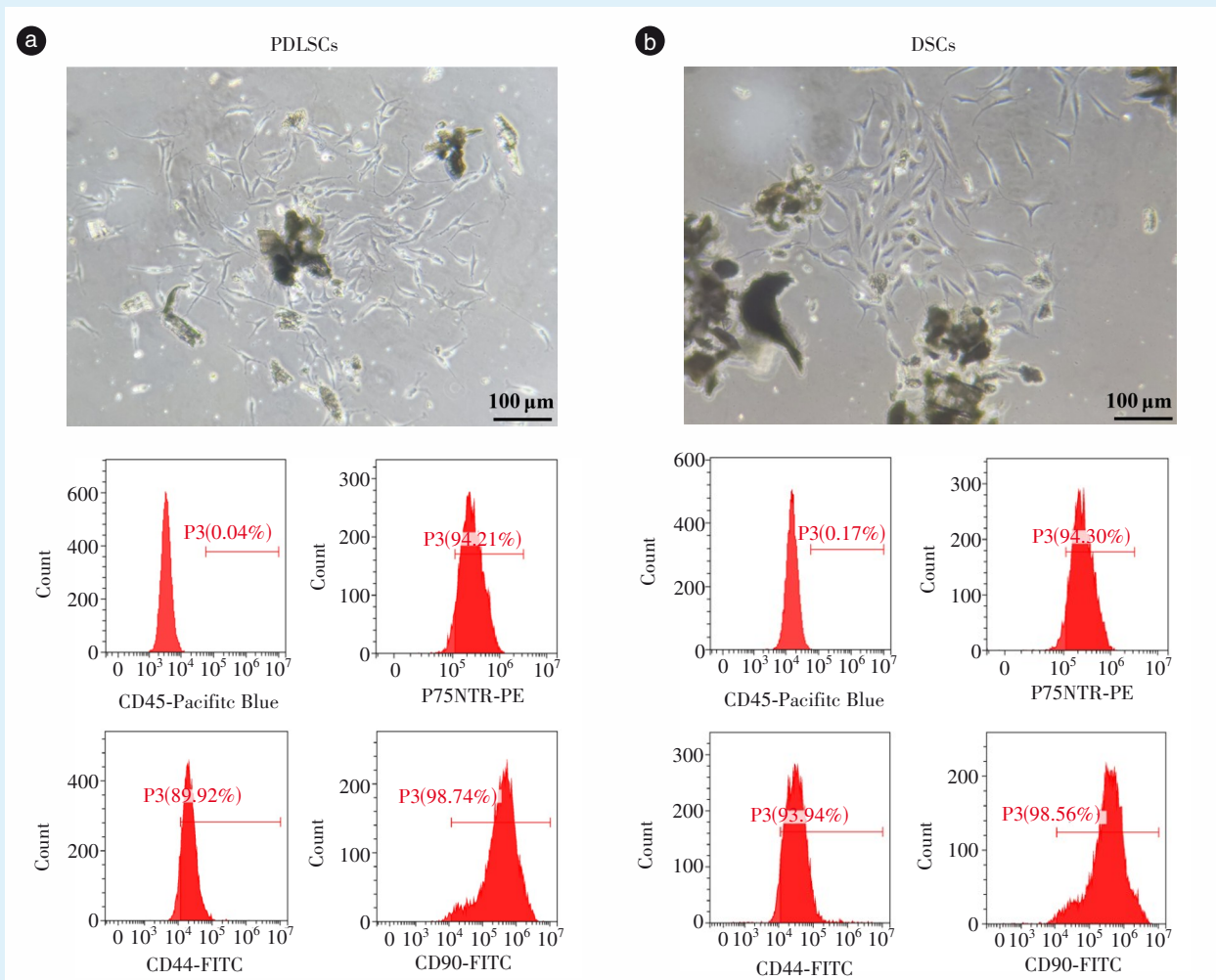
a: immediately after tooth extraction, slight P75NTR positivity was observed on the inner wall of the alveolar bone. b: one day after tooth extraction, mild P75NTR positivity was detected on the alveolar bone wall, with the positive region gradually extending toward the interior of the dental socket. c: three days after tooth extraction, scattered positive areas were present around the fibrous connective tissue in the center of the dental socket. d: seven days after tooth extraction, P75NTR was expressed in the fibrous connective tissue surrounding the newly formed cancellous bone. White arrow: regions with P75NTR expression; black box: selected field for high-power microscopic magnification. P75NTR: P75 neurotrophin receptor. Representative immunohistochemistry staining images selected from histopathological sections of the left mandibular second molar alveolar sockets in six rats (a, b, c, d)

Figure 3 P75NTR immunohistochemical staining of a rat dental socket after tooth extraction

图3 大鼠拔牙后的P75NTR免疫组织化学染色

在病理性炎症中引发的局部微环境失衡,可通过炎症介质、气体分子、自噬通路及牙周炎相关信号通路,诱导PDLSCs发生内源性力学适应性改变,使其生物学功能受到严重损伤^[14]。同时,PDLSCs还面临取材范围受限、细胞纯度不均、异质性偏大等现实应用局限。以往的研究从牙髓^[15]、牙龈^[16]、牙周膜^[17]等部位提取牙源性间充质干细胞,而组织生长活跃的牙槽窝或许也是较为理想的部位。牙齿脱落或被拔除后,伤口开始自然愈合,形成的血凝块由纤维蛋白桥稳定,经过机化过程后,会演变成富含合成细胞外基质和成纤维细胞的肉芽组织^[18],在M2巨噬细胞等炎症细胞的影响下^[19],逐步发展成为新骨附着的起始结构,包括临时基质和编织骨,这些新形成的组织富含未分化的间充质干细胞、纤维结缔组织基质以及尚未完全矿化的骨质^[20],以上共同构成了牙槽窝愈合过程中的关键组成部分。牙槽窝愈合过程是不断变化的,

除了动态且复杂的炎症条件外^[21],牙槽窝的早期愈合组织内含有如生长因子、骨形态发生蛋白等大量活性多肽分子^[22],基因与转录因子的表达受到精细调控,直接决定了归巢的间充质干细胞的命运走向。从细胞层面深入观察,有多种未分化的间充质细胞在这一阶段迁移至拔牙创口处,并经历分化过程,其中有骨细胞的增殖和分化,以及细胞外基质的合成和矿化,最终到骨形成和重塑^[23]。在牙槽窝愈合早期的组织环境中,很可能富集着数量和种类繁多的间充质干细胞^[24]。内源性干细胞向组织缺损区域的定向募集,构成激活自我修复能力的关键程序,从而愈合伤口^[25]。这些细胞的存在,为拔牙后牙槽窝区域的骨愈合过程注入了强大的动力,显著提升了其愈合效率与效果。即使是牙槽窝炎性肉芽组织,经自然转归后,其组织形态与细胞构成可趋近正常修复性肉芽组织,还可通过调控免疫应答与组织重塑平衡



a: representative images of PDLSCs migrated out from tissue explants and proliferated gradually. Flow cytometry results showed that PDLSCs highly expressed the mesenchymal stem cell phenotypic markers CD90, CD44 and P75NTR, with low expression of the negative marker CD45. PDLSCs were isolated from periodontal ligaments of the maxillary and mandibular first, second, and third molars of 24 rats immediately after tooth extraction. Flow cytometric analyses were performed from three biologically independent repeats. b: representative images of DSCs displayed a spindle-like morphology and proliferated in aggregated colonies after attaching to the culture surface. Flow cytometry results showed that DSCs highly expressed the mesenchymal stem cell phenotypic markers CD90, CD44 and P75NTR, with low expression of the negative marker CD45. DSCs were derived from early healing tissues of the alveolar sockets the maxillary and mandibular first, second, and third molars in 6 rats at 3 days post-extraction. Flow cytometric analyses were performed from three biologically independent repeats. P75NTR: P75 neurotrophin receptor. PDLSCs: periodontal ligament stem cells. DSCs: dental socket stem cells

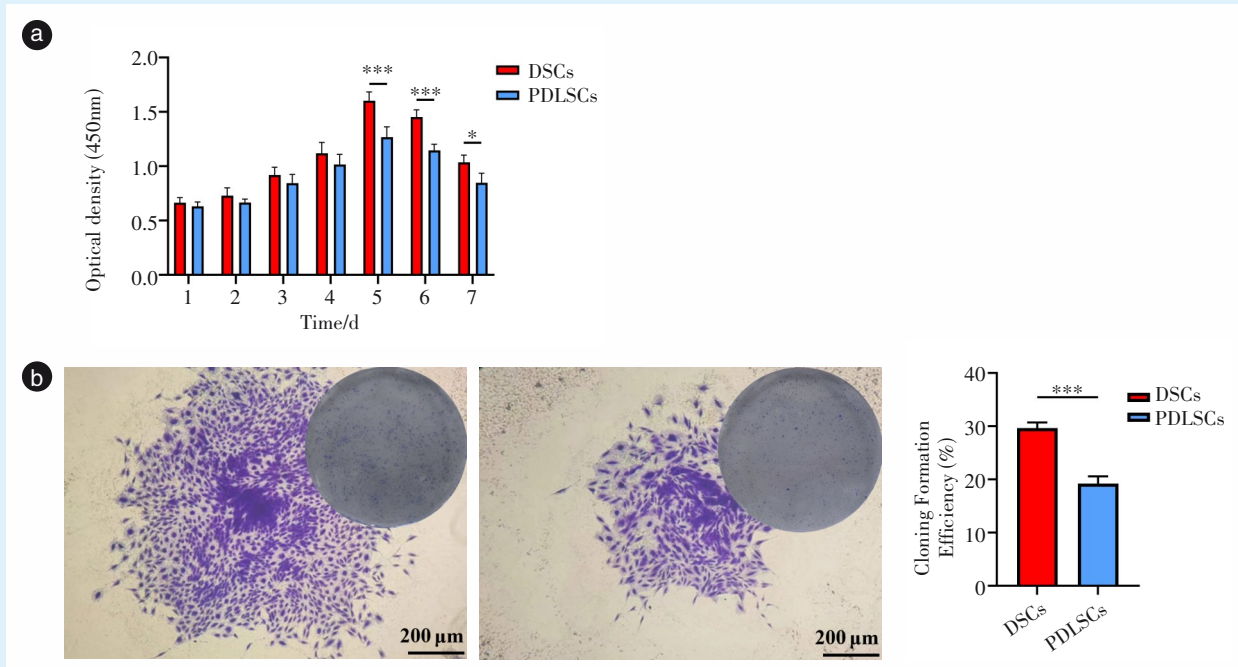
Figure 4 Culture and identification of DSCs and PDLSCs

图4 大鼠DSCs和PDLSCs的培养及鉴定

局部炎症并助力创口愈合^[26]。在临床中DSCs可取材自临床患者的智齿、正畸减数牙或病原牙的牙槽窝中获取,创伤小且取材便捷,安全性高,免疫原性低,不影响原位牙槽窝的愈合,实操性与伦理优势显著,具备较高的临床推广可行性。大鼠具有与人类相类似的固有口腔组织形态,是研究口腔再生医学和相关的病理机制等领域的常用动物载体^[27]。拔除大鼠磨牙后,可从其牙槽窝内获取足量的早期愈合组织。

3.2 牙槽窝干细胞与牙周膜间充质干细胞的生物学特性对比

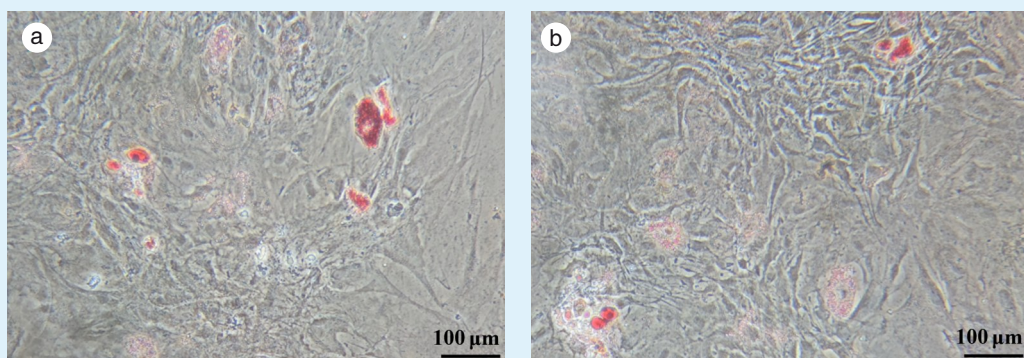
本研究在体外培养出大鼠的DSCs和PDLSCs,二者同样表达间充质干细胞相关的表型抗原,其中PDLSCs和DSCs的P75NTR表达率分别为94.21%和94.30%,反映二者均起源于颅神经嵴。相较于PDLSCs,DSCs有更强的细胞克隆形成能力和增殖能力。在多向分化能力检测方面,经过成脂诱导后PDLSCs和DSCs均可形成有红色脂滴,



a: the proliferative differences between DSCs and PDLSCs were determined using a CCK-8 assay. DSCs presented a higher cell quantity than PDLSCs at days 5, 6 and 7. $n = 3$ biologically independent repeats. Statistical analysis used analysis of variance, $*P < 0.05$, $***: P < 0.001$. b: representative gross and microscopic images of the colony formation assay in DSCs and PDLSCs, along with a comparison of colony formation rate between the two cell types. The DSC group formed a greater number of cell colonies with a larger colony area. $n = 3$ biologically independent repeats. Statistical analysis used t test, $***: P < 0.001$. PDLSCs: periodontal ligament stem cells. DSCs: dental socket stem cells. CCK-8: Cell Counting Kit-8

Figure 5 Comparison of the proliferation and colony-forming abilities of DSCs and PDLSCs

图5 DSCs和PDLSCs的增殖能力及克隆形成能力对比



a: representative image of Oil Red O staining in DSCs from 3 biologically independent repeats. b: representative image of Oil Red O staining in PDLSCs from 3 biologically independent repeats. Red-stained lipid droplets were observed in both cell groups. PDLSCs: periodontal ligament stem cells. DSCs: dental socket stem cells

Figure 6 Comparison of Oil Red O staining of DSCs and PDLSCs

图6 DSCs和PDLSCs的油红O染色情况比较

DSCs可与PDLSCs一样进行成脂分化,且能稳定形成脂肪细胞特征性脂滴,分化表型清晰典型,证实大鼠DSCs具备成脂分化能力,这一发现填补了DSCs分化特性研究的空白,有助于完善其生物学

功能研究体系。另外,经过成骨诱导后,在相同的时间内DSCs组比PDLSCs组形成的钙结节更多,ALP和RUNX2的蛋白及mRNA相对表达量均表现出同样的趋势,提示DSCs的成骨分化能力较

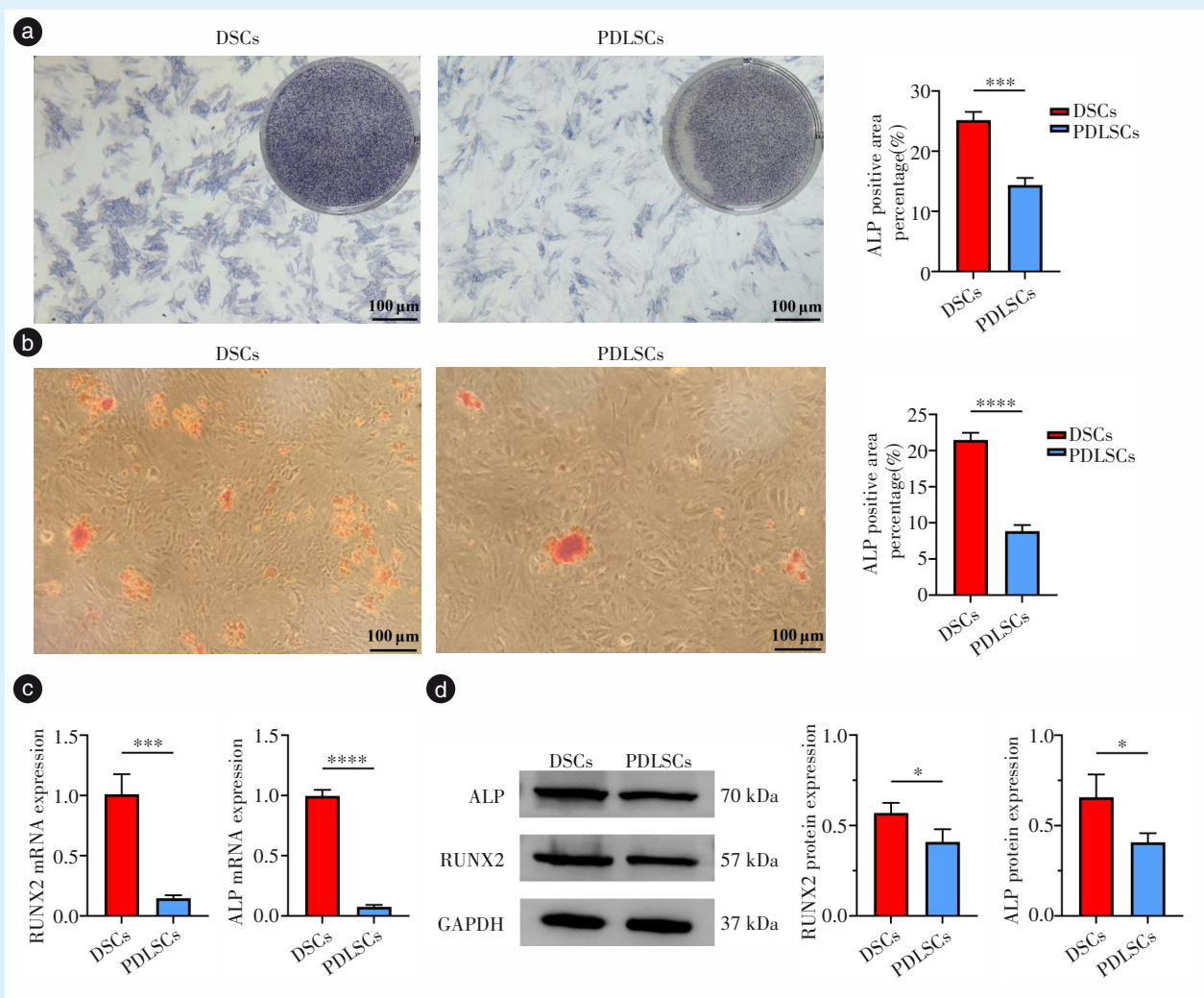


Figure 7 Comparison of the osteogenic differentiation abilities of DSCs and PDLSCs

图7 DSCs和PDLSCs的成骨分化能力对比

PDLSCs强。在前期研究中,课题组发现相较于P75NTR表达阴性和未分选的PDLSCs,P75NTR表达阳性的PDLSCs显示出更好的成骨分化能力,并发现其有较高的细胞外基质受体信号通路因子整合素 $\alpha 1$ 的表达,证明了P75NTR可能上调了整合素 $\alpha 1$ 的表达从而优化PDLSCs的成骨分化能

力^[28]。利用P75NTR分选的PDLSCs在体外表现出更高的成骨标志物同源转录因子DLX5(distal-less homeobox 5,DLX5)和RUNX2,以及成软骨标志物胶原蛋白II型 $\alpha 1$ 链和性别决定区Y框蛋白9的表达,说明P75NTR可能影响了PDLSCs的成骨分化^[29]。PDLSCs处于牙周膜的复合微环境中,需在

机械应力、炎症微环境等复杂条件下维持牙周膜的稳态与修复,保持了适度的增殖与成骨分化,而DSCs处于活跃的骨代谢微环境中,受牙槽窝内复杂信号网络的调控,据此课题组推测在包括P75NTR介导的下游信号调控下,DSCs的增殖与成骨分化能力得到了增强。

3.3 P75NTR介导的牙槽窝干细胞成骨分化机制

神经营养因子和P75NTR之间的交叉信号转导,可在牙胚发育初期的牙囊中发挥作用^[30],与此同时,该受体也深度参与颌颌面组织生长与发育阶段的细胞增殖、分化等核心生物学过程^[31]。P75NTR作为间充质干细胞的关键表面标志物,在协同促进干细胞保持未分化潜能、促进细胞迁移以及实现归巢等关键特性上扮演着至关重要的角色^[32]。利用P75NTR的影响机制,能选择性招募内源细胞^[33],影响着牙源性间充质干细胞再生潜能的发挥^[34]。Xu等^[35]的研究结果观察到额骨缺损部位出现了神经生长因子的显著上调现象,这一变化触发了表达P75NTR阳性的间充质干细胞向骨缺损区域的迁移,若干扰神经营养因子与P75NTR之间的信号转导过程,可能会阻碍间充质干细胞迁移至损伤部位,这种干扰还可能抑制具有抗炎作用的M2表型巨噬细胞的激活,这可能引发免疫反应的继发性减弱或钝化,对损伤修复过程产生不利影响。在牙髓成纤维细胞与成牙本质细胞中,神经营养因子、P75NTR及酪氨酸激酶受体均呈低水平表达;而在支配牙髓组织的神经纤维中则表现为显著高表达,龋坏或外伤牙损伤区周边成牙本质细胞的神经营养因子、酪氨酸激酶受体选择性上调,这一表达模式的改变提示神经营养因子与受体家族在牙体组织的修复进程中的密切关联^[36]。牙槽窝内密布着复杂的信号网络,可被多样化的免疫分子及细胞活性因子所激活,其中也包括神经营养因子及其特异性受体的相互作用^[37]。本实验首次捕捉到牙槽窝愈合初期P75NTR表达的动态变化特征,免疫组化结果显示,随着时间的推移,在拔牙后P75NTR表达区域趋向于从牙槽骨内逐步迁移至牙槽窝的中心,根据组织学观察结果,最终确定以拔牙术后3 d作为DSCs的分离提取时间。推测在牙槽窝缺损修复的启动期,P75NTR的表达构成关键分子信号,提升间充质干细胞迁移效率,并同步提升其向多谱系分化的潜能,进而促进了牙槽窝组织的修复与重塑过程。

3.4 总结与展望

综上所述,DSCs具有较强的体外增殖活性,同时具备优异的成骨分化潜能,而鉴于DSCs群体的高度异质性,其生物学特征及凋亡、微环境响应、多向分化等核心生物学行为的调控机制,尚需进一步深入探索。本研究初步揭示了P75NTR在大鼠牙槽窝愈合过程中的表达情况,为理解牙槽窝修复过程提供了不同角度的实验依据,而牙槽窝可作为可靠、易获取的间充质干细胞来源,拓宽了牙周组织工程种子细胞的选择范围,具有潜在临床转化价值。本研究比较大鼠DSCs与PDLSCs的核心生物学特性,DSCs具有更强的增殖与成骨分化能力;并提出以下假设:靶向调控P75NTR表达,或将成为优化DSCs募集、增强其再生潜能创新策略,进而构成牙槽窝愈合分子调控网络中再生修复的核心单元。鉴于当前实验的局限性,还需通过后续实验进行系统性验证该假设。

【Author contributions】 Meng SZ conducted the experiment implementation and manuscript writing, as well as the analysis of experimental indicators and data processing. Chen L and Tan JY were in charge of animal experiment implementation. Yang K was responsible for the experimental design, undertook the manuscript review and revision. All authors read and approved the final manuscript submitted.

【Ethics statement】 All animal procedures were carried out in compliance with the ARRIVE guidelines (<https://arriveguidelines.org>) and adhered to the guidelines for the care and use of laboratory animals issued by the China Animal Research Council. All animal experiments were approved by Laboratory Animal Welfare and Ethics Committee of Zunyi Medical University (Approval ID: ZMU21-2303-197).

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【Data availability statement】 The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author upon reasonable request.

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