不同培养代次对经血源子宫内膜干细胞生物学活性的影响

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摘要 该研究明确了不同培养代次经血源子宫内膜干细胞(menstrual blood derived endometrial stem cells, MenSCs)的生物学活性差异, 为深入研究MenSCs生物学特性及其潜在临床应用提供理论支持。该研究使用钙黄绿素AM(Calcein-AM)染色检测体外培养至第三代(passage 3, P3)、P9和P15代MenSCs的形态; β-半乳糖苷酶染色检测不同培养代次MenSCs的衰老程度; 活性氧试剂盒检测不同培养代次MenSCs中活性氧的变化; 随后利用MTT、流式细胞术及细胞活死染色在接触式共培养条件下检测P3、P9和P15代MenSCs对小鼠脾淋巴细胞活性、细胞周期、死亡情况以及脾脏淋巴细胞中$CD3^+$和$CD19^+$淋巴细胞比例的影响。结果表明, 随着培养代次的增加MenSCs细胞面积显著增大, 在培养至P15代时MenSCs开始出现大量丝状伪足。衰老程度及活性氧的含量也随培养代次的增加而显著升高; 随后与MenSCs接触式共培养体显著增加了小鼠脾淋巴细胞的活性, 降低淋巴细胞死亡率, 且随培养代次的增加, MenSCs对促进淋巴细胞存活、降低死亡的能力显著降低; 进一步细胞周期检测发现, MenSCs无刺激淋巴细胞增殖分裂活性, 但可显著降低淋巴细胞死亡及碎片化, 且培养代次的增加可显著降低MenSCs维持淋巴细胞存活的能力; 此外, 不同培养代次MenSCs在体外均对小鼠脾淋巴细胞中$CD3^+$和$CD19^+$细胞亚群百分比无显著性影响。综上, MenSCs随着体外培养时间和代次的增加, 出现明显的生物学活性降低等特征, 且对淋巴细胞活性的调节能力显著降低, 上述结果为临床应用中保障MenSCs质量、平衡细胞培养代次和细胞数量及保证稳定的MenSCs临床治疗效果提供理论支持。

关键词 经血源子宫内膜干细胞; 培养代次; 细胞衰老; 氧化损伤

The Effect of Subculture Passage on the Biological Characteristics of Menstrual Blood Derived Endometrial Stem Cells

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中国细胞生物学学报

Abstract The biological characteristics of MenSCs (menstrual blood derived endometrial stem cells) cultured *in vitro* at different passages (passage 3, 9 and 15) were examined, in order to provide support for further studies on the potential clinical applications of MenSCs. Therefore, in this study, the morphology of MenSCs was observed after Calcein-AM staining. The senescence of MenSCs was detected by β-galactosidase staining. The production of ROS (reactive oxygen species) in MenSCs was detected by ROS probe staining. The activity of mouse derived spleen lymphocytes co-cultured with MenSCs was determined by conventional MTT assay and Live/Dead staining, respectively. Additionally, both the cell cycle of mouse derived spleen lymphocytes co-cultured with MenSCs and the activation of $CD3^+$ and $CD19^+$ lymphocytes in mouse derived spleen lymphocytes co-cultured with MenSCs were analyzed by flow cytometry. Consequently, our results showed that no matter the cell size of MenSCs, or the degree of senescence and ROS production in MenSCs had significant increases as the subculture passage increased *in vitro*, also a large number of filamentous pseudopodia was observed in P15 MenSCs. Subsequently, the mouse derived spleen lymphocytes co-cultured with MenSCs exhibited a superior metabolic activity than the lymphocytes cultured alone, the following Live/Dead staining results also confirmed a lower death rate in the mouse derived spleen lymphocytes co-cultured with MenSCs, and P3 MenSCs exhibited the optimal viability-keeping capacity. Additionally, the further cell cycle examination showed no influence on the proliferation of mouse derived spleen lymphocytes, no matter co-cultured with or without MenSCs, but the percentage of debris in lymphocytes co-cultured with MenSCs had a significant decrease, which suggested MenSCs were capable of decreasing the death rate of co-cultured mouse derived spleen lymphocytes. Finally, there was no significant change in the percentage of $CD3^+$ and $CD19^+$ lymphocytes in mouse derived spleen lymphocytes, no matter co-cultured with or without MenSCs, which suggested the low immunogenicity of MenSCs. In summary, with the increased subculture passages *in vitro*, MenSCs not only exhibited a significant decrease in their viability, but also the MenSCs derived viability-keeping capacity for lymphocytes was significantly down-regulated. These results will contribute to balance the quality and quantity of MenSCs used in clinic, and then guarantees the therapeutic effect of MenSCs based therapies.

Keywords menstrual blood derived endometrial stem cells; subculture passage; cell senescence; oxidative damage

间充质干细胞不仅具有自我更新、多向分化等潜能, 同时具有较强的免疫调节等能力, 其组织来源包括骨髓、脂肪组织、牙髓及围产期组织(如脐带和胎盘)等, 然而上述组织来源间充质干细胞在其获取方式(具有侵入性)、来源丰富程度以及伦理等因素在临床应用上受到一定限制[1-3]。而经血源子宫内膜干细胞(menstrual blood derived endometrial stem cells, MenSCs)凭借其较强的增殖活性、无侵入性的样本获得方式、来源丰富及无伦理问题等优势, 在基础研究及临床应用中获得持续关注[4-6]。

细胞质量是干细胞治疗的核心, 而临床治疗中干细胞的使用数量亦对疾病的治疗效果影响较大, 而干细胞使用数量的差异也部分解释了现有基础及临床研究中所获得的不一致的甚至相互矛盾的实验结果[7-10]。众所周知, 原代细胞体外培养随着时间和代次的增加, 增殖过程中产生的突变诱发细胞产生明显的衰老特征[11-12]。而在保证细胞培养质量的前提下, 如何平衡和优化MenSCs的培养代次和细胞得率间的关系, 是促进MenSCs临床应用的重要因素。因此, 本研究选择在体外常规培养至第三代(passage 3, P3)、P9、P15代MenSCs为研究对象, 从形态、衰老、活性氧(reactive oxygen species, ROS)产生以及对淋巴细胞活性调节的影响等多个方面, 分析不同代次MenSCs生物学活性的差异, 以期明确体外长期传代培养对MenSCs生物学特性的改变, 进一步为临床应用中保障MenSCs质量、平衡细胞培养代次和细胞数量提供理论支持。

1 材料与方法

1.1 材料

本实验所使用的MenSCs均来自于年龄在20~35岁之间的健康女性志愿者(*n*=5), MenSCs的采集与

分离均在志愿者知情同意的情况下进行, 所涉及的MenSCs相关实验操作均符合新乡医学院伦理委员会要求。BALB/c雌性小鼠购自北京维通利华生物公司(许可证号SCXK(京)2012-0001), 研究中所涉及动物实验操作均获得新乡医学院动物保护委员会批准, 并按照中国动物保护协会要求进行操作。

1.2 试剂及仪器

DMEM高糖培养基、RPMI-1640培养基和MTT试剂购自Sigma公司; 胎牛血清(FBS)购自Gibco公司; 淋巴细胞分离液购自天津市灏洋生物技术有限公司; 活性氧试剂盒和细胞周期检测试剂盒购自上海碧云天生物技术有限公司; 细胞衰老β-半乳糖苷酶染色试剂盒购自北京索莱宝科技有限公司; 倒置荧光显微镜购自Nikon公司; 流式细胞仪购自Beckman coulter公司; 荧光酶标仪购自Molecular device公司。

1.3 细胞形态观察

MenSCs分离、培养及传代方法详见参考文献[5]。将P3、P9和P15代的MenSCs(n=5)接种于24孔板中(2×10^{4}个/孔), 在37 °C、5% CO_2培养箱中培养12 h后更换为DMEM无血清培养基, 继续培养48 h; 加入钙黄绿素AM(Calcein-AM)工作液(终浓度为1 μmol/L), 室温避光染色10 min; 磷酸盐缓冲液(PBS)洗涤2次; 荧光显微镜观察并拍照, 随机挑选10张图片后, 通过ImageJ软件分析细胞大小。

1.4 β-半乳糖苷酶染色

将P3、P9和P15代的MenSCs(n=5)接种于24孔板中(5×10^{4}个/孔), 在37 °C、5% CO_2条件下培养72 h; 弃去培养基后PBS洗涤2次, 加入1 mL β-半乳糖苷酶染色固定液, 室温固定15 min; PBS洗涤细胞3次, 每次3 min; 随后, 每孔加入1 mL β-半乳糖苷酶染色工作液(按照试剂盒说明书配制), 37 °C孵育过夜; 弃去染色液, 加入1 mL PBS, 光学显微镜观察并拍照, 随机挑选10张图片后, 统计同一视野中半乳糖苷酶染色阳性细胞数量与细胞总数, 计算半乳糖苷酶染色阳性细胞占所有细胞的百分比。

1.5 活性氧(ROS)检测

将P3、P9和P15代的MenSCs(n=5)接种于24孔板中(5×10^{4}个/孔), 每代细胞设置5个重复, 在37 °C、5% CO_2条件下培养48 h; 更换为DMEM无血清培养基后, 加入DCFH-DA探针(终浓度为10 μmol/L), 室温避光孵育30 min; 荧光酶标仪检测荧光强度, 同时在荧光显微镜下观察并拍照。

1.6 小鼠脾淋巴细胞的分离提取

无菌条件下取出小鼠脾脏置于含有RPMI培养基的培养皿中; 在70 μm孔径的滤网中进行研磨, 获取脾细胞悬液, 1 500 r/min离心5 min; 加入红细胞裂解液重悬细胞, 室温孵育3 min, 1 500 r/min离心5 min; PBS洗涤2次, 加入RPMI完全培养重选细胞并计数备用。

1.7 细胞活死染色

将P3、P9和P15代的MenSCs(n=5)接种于24孔板中(5×10^{4}个/孔), 在37 °C、5% CO_2培养箱中培养12 h; 弃去旧培养基后每孔加入2 mL RPMI-1640无血清培养基重悬的小鼠脾淋巴细胞(终浓度为5×10^{5}个/mL); 继续培养72 h后加入Calcein-AM(终浓度为1 μmol/L)和PI(终浓度为2 μmol/L), 室温避光孵育20 min, 荧光显微镜下观察并拍照, 随机挑选10张图片后, 计算同一视野中活死细胞数量, 并进一步计算细胞死亡率。

1.8 MTT

将P3、P9和P15代的MenSCs(n=5)接种于24孔板中(5×10^{4}个/孔), 在37 °C、5% CO_2培养箱中培养12 h; 弃去旧培养基后每孔加入2 mL RPMI-1640无血清培养基重悬的小鼠脾淋巴细胞(终浓度为5×10^{5}个/mL), 单独淋巴细胞组为对照组, 单独RPMI-1640无血清培养为空白组; 继续培养48 h后重悬淋巴细胞并转移至96孔板中, 每孔100 μL; 加入10 μL MTT工作液(浓度为5 mg/mL), 在37 °C、5% CO_2的培养箱中孵育4 h; 1 500 r/min离心10 min, 弃去上清后加入120 μL DMSO振荡混匀10 min; 常规MTT法计算细胞活性(Viability)=(实验组吸光度值–空白组吸光度值)/(对照组吸光度值–空白组吸光度值)。

1.9 细胞周期

将P3、P9和P15代的MenSCs(n=5)接种于24孔板中(5×10^{4}个/孔), 在37 °C、5% CO_2培养箱中培养12 h; 弃去旧培养基后每孔加入2 mL RPMI-1640无血清培养基重悬的小鼠脾淋巴细胞(终浓度为5×10^5 个/mL); 继续培养48 h后收集淋巴细胞, PBS洗涤2次, 加入预冷70%乙醇重悬, 4 °C过夜, 按试剂盒操作每个样品中加入5 μL RNA酶和10 μL PI, 室温避光孵育30 min后, 流式细胞仪检测细胞周期变化。

1.10 淋巴细胞亚群检测

将P3、P9和P15代的MenSCs(n=5)接种于24孔板

中(5×10^4个/孔), 在37 °C、5% CO_2培养箱中培养12 h; 弃去旧培养基后每孔加入2 mL RPMI-1640无血清培养基重悬的小鼠脾淋巴细胞(终浓度为5×10^5个/mL); 分别培养至12和24 h时收集淋巴细胞, PBS洗涤2次; 随后, 分别加入CD3-APC和CD19-PE流式抗体, 充分混匀后4 °C避光孵育30 min; 1500 r/min离心5 min, 弃上清, PBS清洗2次; 加入500 μL PBS重悬细胞, 流式细胞仪检测淋巴细胞亚型变化。

1.11 数据统计

实验数据为五位志愿者来源MenSCs开展上述实验所获得, 以$\bar{x}\pm s$形式表示, 采用SPSS 13.0统计软件包进行数据统计分析。各组间均值比较采用单因素ANOVA中的Dunnett检验, $P<0.05$表示差异有统计学意义。

2 结果

2.1 培养代次增加导致MenSCs细胞面积显著增加

P3、P9还是P15代MenSCs细胞均呈典型的成纤维样梭型结构, 如图1A所示, 但随着代次的增加细胞面积显著增加(图1B, $P<0.05$), 且P15代MenSCs细胞出现大量的丝状伪足。

2.2 培养代次增加明显促进MenSCs细胞衰老

将P3、P9和P15代的MenSCs细胞经β-半乳糖苷酶染色结果如图1C和图1D所示, 不同代次MenSCs均存在衰老细胞, 且随着培养时间和代次的增加, MenSCs中衰老细胞数量百分比显著增加(P15>P9>P3, $P<0.05$)。

2.3 培养代次增加明显促进MenSCs细胞ROS产生

将P3、P9和P15代的MenSCs细胞同步化处理后, 经DCFH-DA探针染色, 结果如图1E和图1F所示: MenSCs中ROS含量随培养代次增加显著性升高($P<0.05$)。

2.4 MenSCs细胞对淋巴细胞活性的维持随培养代次增加显著降低

淋巴细胞活死染色结果如图2A所示, 相较于无MenSCs细胞共培养组, 与P3和P9代MenSCs细胞接触式共培养的淋巴细胞凋亡数量显著减少(图2C, $P<0.05$)。随后的MTT结果与上述结果一致, 随着培养代次的增加, MenSCs细胞维持小鼠脾淋巴细胞的活性的能力显著降低(图2B, $P<0.05$)。进一步的细胞周期检测结果(图3)亦验证了上述实验结果: 尽管P3、P9和P15代MenSCs细胞对淋巴细胞周期无明显影响(G_1、G_2和S期均无统计学差异), 但细胞碎片比例显著降低($P<0.05$), 尤其是与P3和P9代MenSCs细胞接触式共培养24 h的淋巴细胞, 细胞碎片比例极显著降低($P<0.01$), 暗示MenSCs的存在可增加淋巴细胞活性。

2.5 MenSCs细胞对CD3$^+$和CD19$^+$淋巴细胞无明显活化作用

为检测MenSCs的免疫原性, 流式细胞术检测MenSCs与小鼠脾淋巴细胞接触式共培养后对其CD3$^+$和CD19$^+$淋巴细胞亚群的影响, 结果如图4所示, 与MenSCs接触式共培养的CD3$^+$和CD19$^+$淋巴细胞亚群无统计学差异, 暗示MenSCs具有较低的免疫原性。

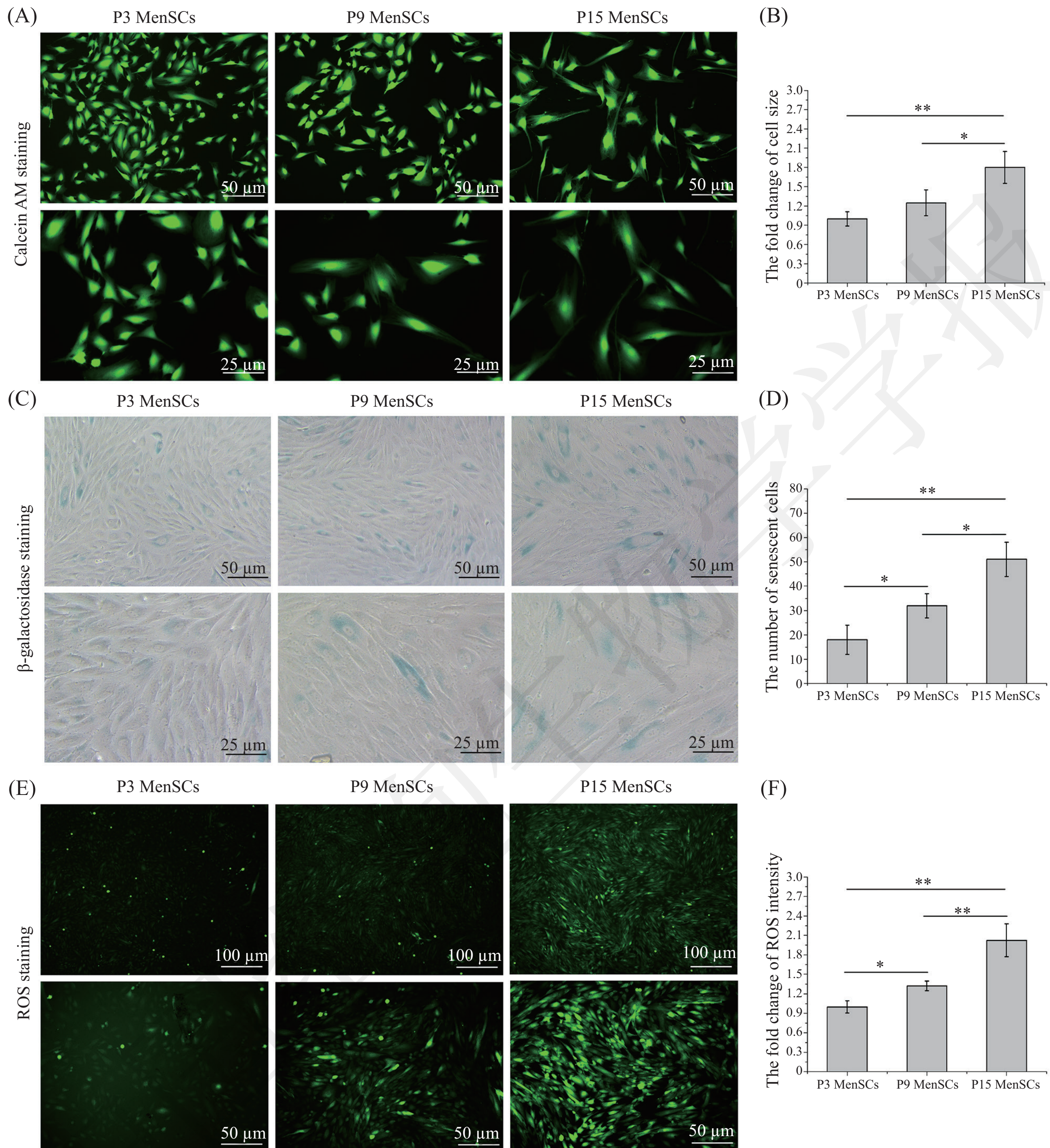
3 讨论

MenSCs是来源于女性子宫内膜基底层的间充质干细胞, 自从2007年一经报道便获得广泛关注。研究人员在对MenSCs研究时发现, 其具有较强的增殖活性, 且传至68代未发现核型畸变, 证明其遗传的稳定性[4-6,13]。随后, 本课题组前期在裸鼠实验中亦发现MenSCs无致瘤性, 为其临床应用的安全性提供保障[5]。目前, 已有报道表明, MenSCs在I型糖尿病、中风、子宫内膜修复、卵巢早衰及结肠炎等动物疾病模型中表展现出良好的治疗效果[14-16]。而进一步在临床研究中也已逐步开展MenSCs对宫腔黏连、多发性硬化症、心脏早衰及下肢缺血等疾病的改善和治疗研究[15,17-18]。

目前临床干细胞治疗中对于细胞数量没有明确定量, 常用数量为10^7~10^8个/人, 而动物实验结果表明不同细胞用量对疾病的改善具有明显差异[7-10]。通常为获得足够量的细胞, 势必会增加干细胞体外培养时间和传代次数, 而干细胞体外长时间的培养传代对细胞活性具有明显副作用, 进一步限制干细胞临床治疗的改善效果。因此, 在保证细胞培养质量的前提下, 如何平衡和优化MenSCs的培养代次和细胞得率间的关系, 是促进MenSCs在组织工程和再生医学应用中的重要因素。

本研究发现, 体外长期传代培养对MenSCs的细胞形态具有显著影响, 培养高代次的MenSCs细胞面积显著大于低代次MenSCs, 而细胞面积增大与细

中国细胞生物学学报



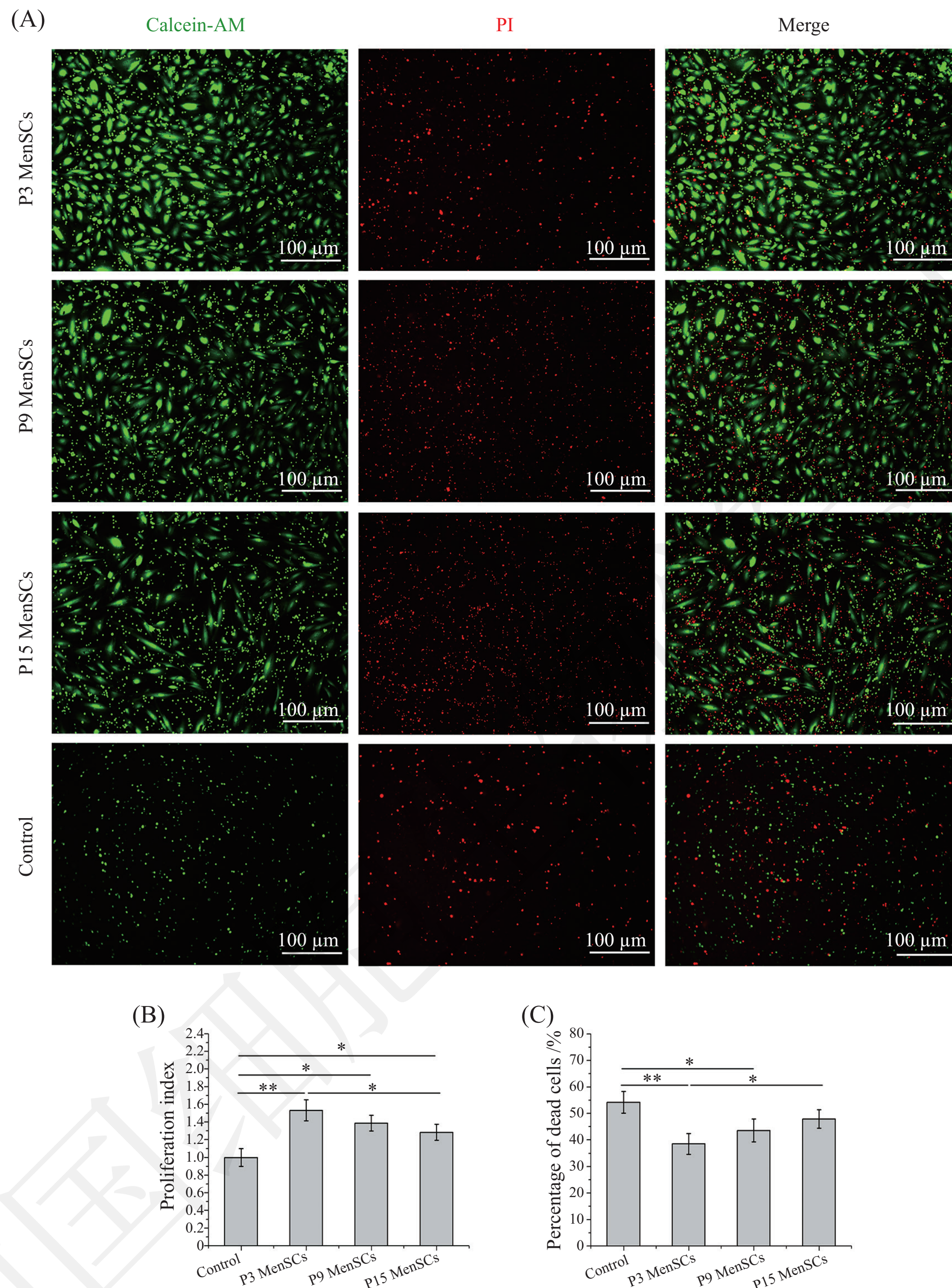
A、B: P3、P9和P15代MenSC经Calcein-AM染色后进行形态观察, ImageJ软件量化细胞大小; C、D: P3、P9和P15代MenSC经β-半乳糖苷酶染色后进行衰老细胞百分比量化分析; E、F: P3、P9和P15代MenSC经ROS染色后进行胞内ROS量化分析。*P<0.05, **P<0.01。

A,B: Calcein-AM staining was used to observe the morphology of MenSCs, then the size of P3, P9 and P15 MenSCs were quantified by imageJ; C,D: β-galactosidase staining was used to detect the senescence of MenSCs, and the senescence degree of P3, P9 and P15 MenSCs were quantified by calculating the positive cells; E,F: ROS staining was used to detect the production of ROS in MenSCs, and the fluorescence intensity was quantified by microplate reader. *P<0.05, **P<0.01.

图1 不同体外培养代次MenSCs生物学活性差异

Fig.1 The biological activity variations of MenSCs cultured at different passages *in vitro*

胞衰老程度呈正相关。随后, β-半乳糖苷酶染色也进一步验证了高代次MenSCs中存在更多衰老细胞, 而形态观察亦发现, P15代MenSCs细胞中丝状伪足明显多于P3和P9代的MenSCs。推测较长时间的体外培养和传代, 造成MenSCs中有害突变的增多, 端粒长度减少, 端粒酶活性降低, 且代谢废物在细胞中



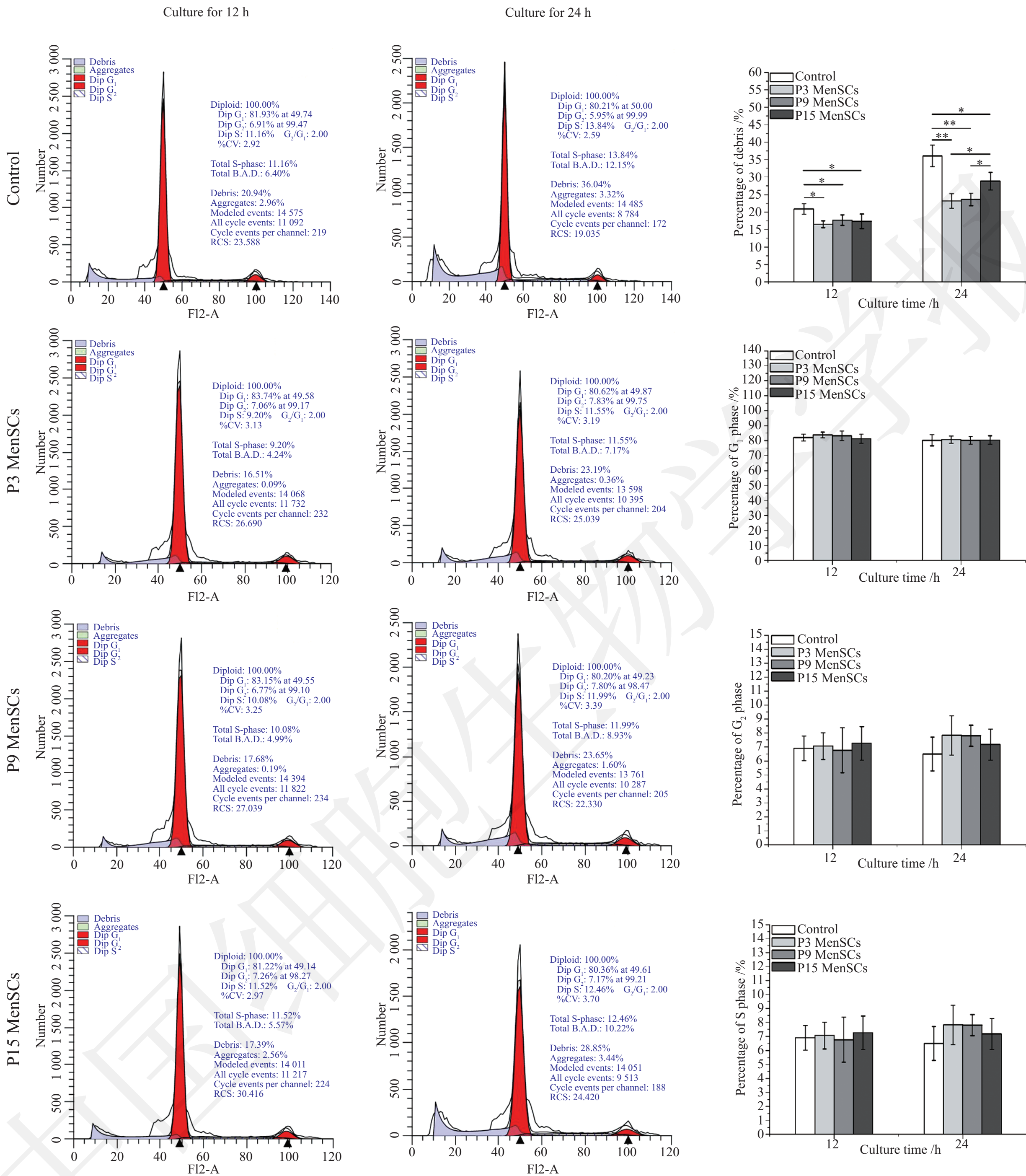
A、C: 细胞活死染色检测在接触式共培养条件下P3、P9和P15代MenSCs对小鼠脾淋巴细胞活性的影响, 并计算细胞的死亡率; B: MTT法检测在接触式共培养条件下P3、P9和P15代MenSCs对小鼠脾淋巴细胞代谢活性的影响。*P<0.05, **P<0.01。

A、C: live/dead staining was used to detect the viability of mouse derived spleen lymphocytes co-cultured with MenSCs, and calculating the death rate of lymphocytes; B: conventional MTT assay was used to examine the metabolic activity of mouse derived spleen lymphocytes co-cultured with MenSCs. *P<0.05, **P<0.01.

图2 MenSCs共培养促进小鼠脾淋巴细胞存活

Fig.2 MenSCs exhibited viability-keeping capacity for mouse derived spleen lymphocytes

不断累积, 最终造成细胞出现明显衰老。进一步, 已知间充质干细胞的主要产能方式为糖酵解而并非三羧酸循环, 而糖酵解过程不经过氧化呼吸链, 因此MenSCs细胞中ROS产生有限。但本研究检测发现, P15代MenSCs细胞中ROS显著多于P3和P9代MenSCs, 该结果暗示, MenSCs随着培养代次的增加其



*P<0.05, **P<0.01.

图3 MenSCs共培养对小鼠脾淋巴细胞增殖无明显影响, 但可显著抑制细胞碎片的产生

Fig.3 MenSCs had no effect on the proliferation of mouse derived spleen lymphocytes but can significantly decrease the percentage of debris in lymphocytes

线粒体功能开始逐步被激活或发生异常。而已有报道证实, 线粒体与干细胞分化及功能改变密切相关, 该实验结果也从部分解释了高代次MenSCs形态发生改变的原因。此外, 免疫调节作为干细胞发挥疾病治疗效果的重要手段, 已获得大量基础及临床研究验证[19-21]。而本研究进一步发现, MenSCs具有维持小鼠脾淋巴细胞活性的能力, 且低代次MenSCs细胞对淋巴细胞活性的维持效果最佳, 该结果表明,

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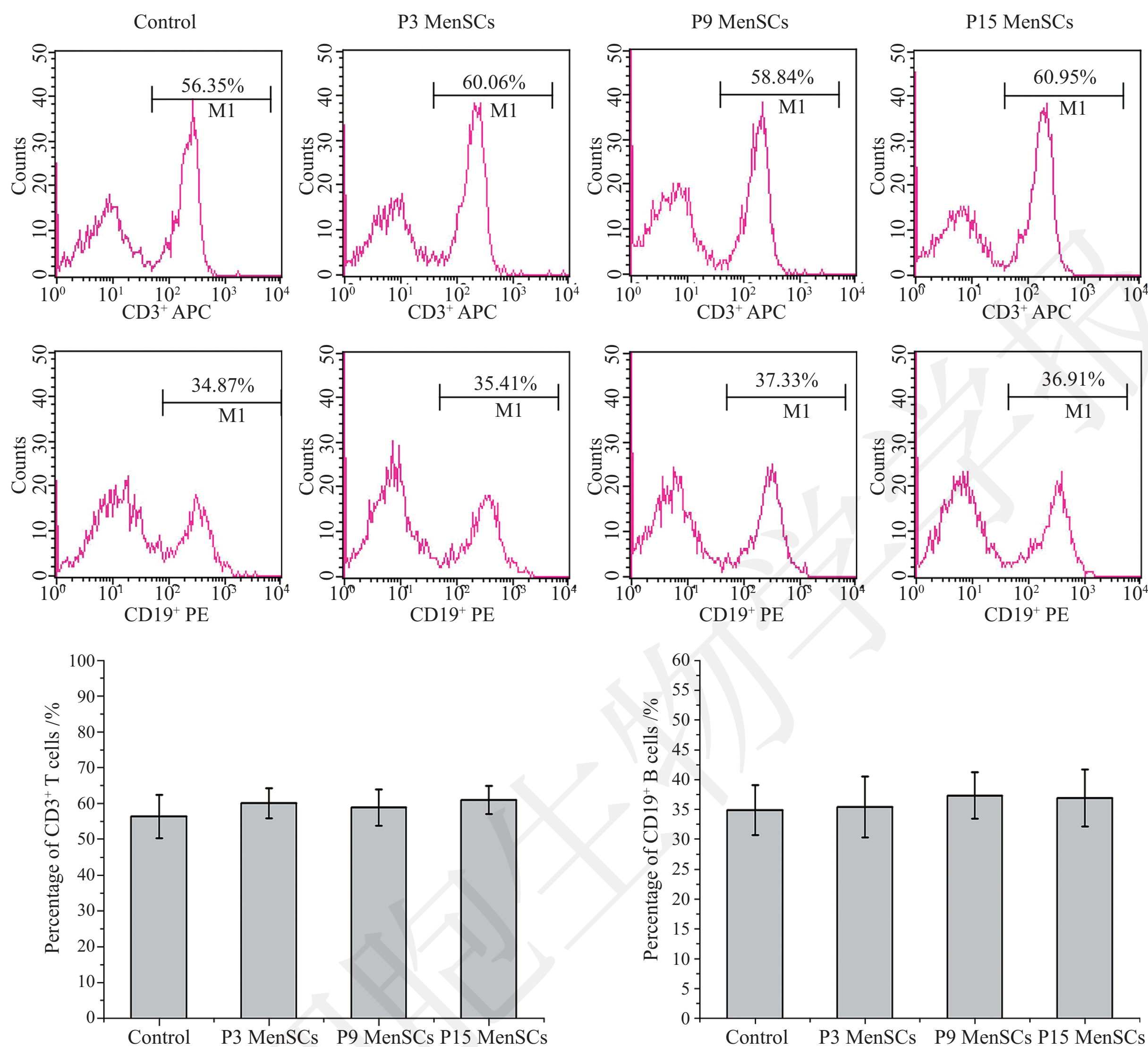

图4 MenSCs共培养对小鼠脾脏CD3⁺和CD19⁺淋巴细胞无明显活化作用

Fig.4 MenSCs had no significant increase of $CD3^+$ and $CD19^+$ lymphocytes in mouse derived spleen lymphocytes

MenSCs可通过分泌活性因子等间接作用或通过接触等直接作用调节淋巴细胞活性, 为其临床中通过调节患者免疫系统从而达到对疾病的改善目的提供理论支持。另外, 通过与脾淋巴细胞接触式共培养, MenSCs对脾淋巴细胞中T淋巴细胞和B淋巴细胞百分比均无明显影响, 暗示了其较低的免疫原性, 进一步为其在临床应用中的安全性提供保障。

综上所述, MenSCs随体外培养时间和传代次数的增加, 出现明显的生物学活性降低等特征, 且对淋巴细胞的调节能力显著下降, 因此为提供高质量MenSCs, 应选择低代次MenSCs, 建议使用P3至P6代MenSCs, 保证细胞数量的同时保障细胞质量, 最终确保稳定的临床治疗效果。

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