间充质干细胞长期传代培养条件的研究

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摘要 间充质干细胞(mesenchymal stem cells, MSCs)是存在于成体组织间质部分的多能前体细胞, 在体外具有自我更新增殖及向成脂、成骨、成软骨分化的潜能, 在组织工程和细胞治疗方面具有广阔的应用前景。MSCs在体外长期培养获得足够数量的细胞是MSCs应用的一个重要因素。然而, 目前还没有建立MSCs长期传代培养的最适培养体系。该文分别从培养体系中的基础培养基、血清和生长因子对于MSCs细胞长期传代培养的影响进行了论述, 旨在为建立MSCs体外长期传代生长的最适培养体系提供理论依据。

关键词 间充质干细胞; 长期传代; 培养体系; 基础培养基; 血清; 生长因子

Research Progress in Culture Condition of Mesenchymal Stem Cells during Long-term Subculture

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Abstract Mesenchymal stem cells (MSCs) are multipotent precursors derived from the stromal fraction of adult tissues. MSCs possess the capacity of self-renewal and multipotency to differentiate into adipocytes, osteoblasts and chondrocytes, which give rise to promising application in tissue engineering and cell therapy. The long-term subculture to harvest high numbers of MSCs is one of the most crucial factors for the application. However, optimal culture system has not been established so far. This paper reviewed the effect of basal medium, serum and growth factor of culture system on MSCs during the long-term subculture respectively, aiming to provide theoretical basis to establish optimal culture system for long-term subculture of MSCs.

Keywords mesenchymal stem cells; long-term subculture; culture system; basal medium; serum; growth factor

间充质干细胞(mesenchymal stem cells, MSCs)是来源于中胚层非造血系的成体干细胞。MSCs最初是从骨髓中分离得到的[1], 但是随着研究的深入, 人体的其他组织中也分离出了MSCs, 包括脂肪、肌肉、脐带血、外周血、肺和心脏等[2]。国际细胞治疗协会(international society for cellular therapy, ISCT)对于MSCs的定义和性质作了统一的规定: (1)MSCs在标准培养条件下能够贴壁生长; (2)95%以上细胞能够表达CD90、CD105和CD73, 但是不超过2%的细胞能够表达CD45、CD34、CD14、CD19和II型HLA等表面抗原; (3)MSCs具有向成脂、成骨和成软骨分化的多向分化能力[3]。MSCs作为成体来源

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干细胞, 相比于胚胎干细胞(embryonic stem cells, ESCs)和诱导多能干细胞(induced pluripotent stem cells, iPSCs), 具有低免疫原性和不存在伦理问题的优点, 有望应用于组织工程和再生医学[4-5]。

在体外培养MSCs时, 为了保持细胞的生长和功能特性, 常常模拟细胞的体内微环境, 提供适宜的温度、pH值、湿度、培养基质以及营养充足的培养基等[6]。培养基的选择对于细胞的体外培养尤为重要, 特别是在MSCs体外长期传代培养条件下, 培养体系中的基础培养基、血清浓度和培养添加物等因素都会影响MSCs的功能特性。基础培养基为细胞提供生长所需的氨基酸、维生素、无机盐以及特殊的代谢物(包括碱基、核苷和脂类等物质), 能够满足细胞对营养物质的基本需求[7]。血清是细胞体外增殖分化的决定性物质, 是细胞生长和维持所必需的条件。但是, 血清中也含有少量的抑制细胞增殖和非生长调节因子, 会对细胞的功能产生负面影响[8]。细胞因子是最重要的培养添加物之一, 在培养基中额外添加生长因子能够有效促进细胞增殖和维持细胞在体外的功能[9-10]。本文将对MSCs体外长期传代培养的培养体系中基础培养基、血清和生长因子对MSCs的影响进行论述, 旨在为建立MSCs体外长期传代生长的最适培养体系提供理论依据。

1 基础培养基对MSCs生长的影响

MSCs生长培养的基础培养基有Dulbecco改良的伊格尔培养基(Dulbecco's modified Eagle's medium, DMEM)、DMEM/F12和α-MEM, 这几种基础培养基都含有细胞生长必需的营养成分, 但是在营养物质的种类和浓度上有所不同。

DMEM是细胞培养中最常用的培养基, 含有大量的氨基酸、维生素、无机盐、微量元素、核苷、糖类和脂类等营养物质[7]。根据DMEM中葡萄糖的浓度不同, 可分为高糖DMEM(DMEM-HG, 葡萄糖含量为4.5 g/L)和低糖DMEM(DMEM-LG, 葡萄糖含量为1 g/L)。DMEM中营养成分浓度高, 但是营养成分组成较少, 如缺少丙氨酸、天冬氨酸、谷氨酸和脯氨酸等氨基酸以及生物素和维生素B_{12}等维生素[7]。DMEM/F12是Barnes等[11]将F12和DMEM按1:1混合而成的培养基。F12是多种营养素混合的培养基, 里面含有低浓度的氨基酸、维生素和无机盐, 弥补了DMEM营养成分组成较少的缺点。α-MEM与DMEM/F12的主要营养成分大致相似, 但是α-MEM中的营养成分浓度要低于DMEM/F12。此外, α-MEM相比DMEM/F12添加了碱基和腺苷类物质, 但是缺少Cu、Fe和Zn等微量元素[7]。

在用于细胞培养时, 这几种基础培养基的适用范围也不大一样, 特别是在细胞体外长期培养的条件下, 基础培养基对细胞的影响会逐渐显现出来。通常情况下, DMEM-LG用于绝大多数细胞系和部分原代细胞的生长传代培养; DMEM-HG用于生长代谢较快的细胞, 此外常用于配制细胞分化培养基; 而DMEM/F12经常被用作细胞的原代培养, 同时可作为无血清培养体系的基础培养基[12]; α-MEM常被用来培养对营养成分要求较高的细胞, 许多MSCs的培养就是使用α-MEM作为基础培养基[13-14]。

培养基中葡萄糖的浓度对MSCs的生长特性有很大的影响。低浓度葡萄糖的培养基有利于MSCs的体外生长增殖[15-17], 而高浓度的葡萄糖能够促进MSCs活性氧(reactive oxygen species, ROS)的产生, 激活PKCβ信号通路, 诱导MSCs的成脂分化[18]。Dhanasekaran等[17]发现, 人骨髓间充质干细胞(bone marrow mesenchymal stem cells, BMSCs)在DMEM-LG和α-MEM(葡萄糖含量1 g/L)培养基中长期培养之后仍能保持细胞的分化和免疫表型特征。Pal等[16]研究了不同的基础培养基对人BMSCs长期培养下细胞表型和功能的影响, BMSCs在含有10% FBS的DMEM-HG培养基中培养至第5代时, 细胞变成扁平的叶状, 胞质中有颗粒形成, 细胞生长停止。而DMEM-LG和DMEM/F12(葡萄糖含量3.15 g/L)培养的BMSCs均可传代至25代。

另外, 基础培养基中的营养物质的组成越全面, 对细胞的生长和功能特性维持越有利。Pal等[16]研究发现, 人BMSCs在DMEM-LG和DMEM/F12培养基中可生长至25代。然而, 在DMEM-LG培养基中, 细胞传至10代后形态开始拉长, 出现多角颗粒, 并且群体倍增时间延长; 而在DMEM/F12培养基中, 细胞一直保持正常的形态, 并且在培养至25代后还能保持正常的形态和成脂成骨分化能力。Sotiropoulou等[15]研究报道, 在α-MEM中添加性质更加稳定、不易被分解的谷氨酰胺替代物, 能够更加有利于MSCs的生长。相比于DMEM, DMEM/F12和α-MEM中由于增加了氨基酸、维生素和无机盐等其他的营养物质种类, 使得培养基的营养物质组成更加丰富和全

面, 更有利于保持MSCs的生长和功能特性。

因此, DMEM-LG、DMEM/F12和α-MEM均可作为MSCs培养的基础培养基。但是, 最适的基础培养基可能还与MSCs的组织来源有关。Marappagounder等[19]研究发现, DMEM-LG是人脂肪干细胞(adipose stem cells, ASCs)长期传代培养的最适培养基, 其次是α-MEM, 而DMEM/F12对人ASCs的培养效果最差。DMEM/F12是脐带血间充质干细胞(umbilical cord blood-derived mesenchymal stem cells, UCB-MSCs)长期传代培养的最适培养基[20]。鉴于MSCs组织特异性的研究较少, 针对不同组织来源的MSCs选择适合的基础培养基还需要更深入的探索。

2 血清对MSCs生长的影响

血清的主要成分如表1所示[6-7], 为细胞的生长提供激素、转运蛋白、矿物质、微量元素、脂类、黏附分子和生长因子等营养物质[6]。另外, 血清适用性非常广, 对于绝大多数人和动物细胞都有效。国内外文献中, MSCs培养所使用的血清一般都是胎牛血清(fetal bovine serum, FBS或者fetal calf serum, FCS)[21]。绝大多数研究中, MSCs培养时血清浓度范围为10%~20%[22-24]。Yoon等[23]通过单细胞分离培养人BMSCs, 在含有17% FBS的培养基中细胞能够长期生长繁殖至群体倍增数(population doubling, PD)达140, 并且在PD为120时能够保持细胞的端粒长度和多向分化能力。Izadpanah等[13,25]用含20% FBS的培养基对人BMSCs和ASCs进行长期传代培养, BMSCs和ASCs分别培养至20代和30代。

低浓度血清对细胞的生长传代有很大的影响。Pal等[16]研究表明, 在含2%低浓度血清的培养基中, 细胞在传代至第5代时, 出现细胞生长停滞、表面抗原降低等不利影响, 这是因为当血清浓度过低时, 细胞营养供应不足, 细胞生长受阻。在正常的血清浓度范围内, 细胞的生长是随着血清浓度的提高而加快的, Peister等[26]的研究结果发现, 细胞在含20%血清的培养基中的生长速度是含5%血清培养基的5倍。但血清浓度过高时, 由于血清中含有大量促进细胞分化的因子, 可能会使细胞自发分化, 不利于MSCs干性的维持[8]。而含10% FBS的培养基作为标准的培养基, 被广泛地用于MSCs的体外培养[16,22,27-28]。

血清对细胞的生长促进作用毋庸置疑。但是, 培养基中添加血清也有不利的影响。首先, 血清的组成成分还没有被完全鉴定出来, 而那些未知的成分对细胞特性的影响也还不确定[29]。其次, 血清的质量会随着血清的种类和批次的不同而参差不齐,

表1 血清的主要组成成分(根据参考文献[6-7]修改)

Table 1 Major components of serum (modified from references [6-7])

成分 Component	名称和浓度范围 Name and concentration range
Serum proteins & transport proteins	Albumin (20~50 mg/mL), globulins (1~15 mg/mL), protease inhibitor: α1-antitrypsin, α2-macroglobulin (0.5~2.5 mg/mL), transferrin (2~4 mg/mL), transcortin, α1-lipoprotein, β1-lipoprotein
Attachment & spreading factors	Fibronectin (1~10 μg/mL), laminin, serum spreading factor
Growth factors & cytokines	Epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF), endothelial cell growth factor (ECGF), platelet~derived growth factor (PDGF), insulin-like growth factor (IGF), interleukins, interferons, transforming growth factor (TGF) (1~100 ng/mL)
Hormones	Insulin (1~100 ng/mL), glucagon, corticosteroids, vasopressin, parathyroid hormone, thyroid hormones (100 nmol/L), growth hormone, prostaglandins
Nutrients & metabolites	Triglycerides, phospholipids (0.7~3.0 mg/mL), cholesterol (10 μmol/L), ethanolamine, phosphatidylethanolamine;
	Vitamin A (10~100 ng/mL), B1, B12, C, E, pyridoxalphosphate, folic acid (5~20 ng/mL), biotin, nicotinic acid;
	Glucose (1.0~2.0 mg/mL), galactose, fructose, mannose, ribose, glycolytic metabolites
Minerals & nonprotein nitrogens	Urea, purines/pyrimidin, polyamines, creatinine, amino acids; Selenium, iron, zinc, Cu, Co, Cr, I, F, Mn, Mo, V, Ni, Sn

不同品牌的血清质量千差万别[30]。此外, 血清中可能含有内毒素、血红素和一些生长抑制物, 不利于细胞的生长[7]。特别是在MSCs体外长期传代培养的过程中, 血清对细胞的不利影响会逐渐积累并放大, 最终导致MSCs细胞形态改变、生长停滞、细胞衰老、分化功能缺失等问题[27,31]。因此, 无血清培养体系越来越多地被应用于MSCs的培养, 特别是化学成分明确的无血清培养体系。利用成分构成更加简单明确的血清替代物(serum replacement, SR)来代替血清, 也能够有效地维持MSCs的生长和功能特性。

Battula等[32]用含血清替代物的ESCs培养体系培养MSCs, 发现MSCs的增殖活性是含血清培养基的4~5倍, 而且在此培养条件下, 细胞能够向三个胚层的细胞分化。另外, 商品化的无血清培养基也越来越多地用于MSCs的培养, 在保证MSCs体外高效增殖能力的同时, 还能够保持细胞的表面抗原和分化功能特性[33]。Meuleman等[24]用含有2%血清替代物的无血清培养基培养人BMSCs, 相比于含15% FBS的α-MEM培养基, MSCs在无血清培养基中具有更强的增殖能力。

3 生长因子对MSCs的影响

生长因子是由细胞分泌的多肽类物质, 能够促进细胞生长增殖[7]。碱性成纤维细胞生长因子(basic fibroblast growth factor, bFGF)、血小板生长因子(platelet-derived growth factor, PDGF)、表皮生长因子(epidermal growth factor, EGF)、转化生长因子β(transforming growth factor β, TGFβ)和胰岛素样生长因子-1(insulin-like growth factor-1, IGF-1)等常用的细胞因子, 均对MSCs的生长和增殖有促进作用[5]。

bFGF能够有效地促进MSCs的生长, 是常用的细胞生长因子。bFGF能够通过ERK1/2信号通路促进细胞的自我更新和保持干细胞的多能性[34]。EGF能够增强MSCs的迁移和增殖, 保持细胞的分化潜能[35-36]。PDGF能够通过JNK活化和JAK3/STAT1通路来刺激MSCs的生长[37-38], 而且能够通过PDGF-β激活Akt和ERK通路来促进MSCs的增殖和抑制细胞的分化[39]。IGF-1通过其受体激活胞内的PI3K/Akt信号通路, 促进MSCs的迁移[40]。

生长因子可单独添加到培养基中促进MSCs的生长增殖, 也可以共同作用来维持细胞的功能。Ya-

表2 MSCs长期传代培养的条件体系

Table 2 Long-term subculture condition system of MSCs

MSCs类型 MSCs type	培养条件 Culture condition	细胞特性 Cell characteristics	参考文献 Reference
hBMSCs	DMEM-LG, 10% FBS	Cells didn't differentiate spontaneously with normal chromosomal karyotype telomerase activity in 20 passage	[22]
hBMSCs	DMEM-LG, 17% FBS, 10 ng/mL EGF, 10 ng/mL PDGF and 1 000 U/mL LIF	PD 140, MSCs maintained telomerase activity and multipotent ability during PD 120	[23]
mBMSCs	α-MEM, 10% FBS, 4 ng/mL bFGF	MSCs could maintain proliferation and multipotency when cells successive culture for 120 d	[41]
hUCB-MSCs	MesenCult-XF serum-free medium	MSCs could culture for passage 25. The apoptosis and chromosomal karyotype of passage 20 cells showed no significant difference to P3 cells. Cells maintained multipotency	[48]
hBMSCs, hASCs	α-MEM, 20% FBS, 1% L-glutaminate	MSCs could culture for PD 180~210. The proliferation and telomerase activity decreased in the late period	[25]
hBMSCs	DMEM, 10% FBS, 2 mmol/L L-glutaminate	Cells didn't present malignant transformation at passage 25	[28]
hBMSCs	DMEM/F12, 10% FBS	Cells maintained normal phenotype, chromosomal karyotype, differentiation property at passage 25	[16]
hUCB-MSCs	DMEM/F12, 10% FBS, 2 mmol/L L-glutaminate	Cells maintained normal phenotype and chromosomal karyotype, but proliferation decreased at passage 20	[20]
hBMSCs	α-MEM, 10% FBS	Cells could be passaged at 20 and maintained normal phenotype, chromosomal karyotype and proliferation ability	[17]

LIF代表白血病抑制因子(leukemia inhibitor factor); 前缀h和m分别代表人源和鼠源。

LIF represents leukemia inhibitor factor; prefix h and m respectively represent human and mouse.

machika等[41]研究发现, 小鼠BMSCs在含有bFGF的培养基中连续培养至24代仍保持高效的增殖活性和多向分化能力。Hebert等[42]发现, 1~10 ng/mL的EGF或者bFGF均能促进长期冻存BMSCs的生长增殖并保持成脂分化能力; 此外, 低浓度的EGF和bFGF联合作用具有叠加效果。Ng等[43]通过全局基因表达分析筛选出与MSCs生长分化相关的3个信号通路: TGFβ、PDGF和FGF信号通路。这3个信号通路对于MSCs的生长是必需的, 当信号通路被阻断时, MSCs更倾向于细胞的分化。

在培养基中添加生长因子能够降低血清使用浓度。在无血清培养体系中, 生长因子的使用是必不可少的。美国菌种保存中心(American type culture collection, ATCC)建立了UCD-MSCs和ASCs细胞系, 这两种干细胞系所用的生长维持培养基是低血清培养基: 2% FBS、5 ng/mL bFGF、5 ng/mL aFGF、5 ng/mL EGF和2.4 mmol/L L-丙氨酰-L-谷氨酰胺。Gronthos等[44]分离的BMSCs在含有10 ng/mL EGF和10 ng/mL PDGF的无血清培养基中能够生长、增殖至PD为40。表2为近年来MSCs长期传代培养条件体系的研究。

除了基础培养基、血清和生长因子对MSCs的生长培养有重要影响之外, 细胞的传代密度也是一个很重要的因素。多项研究表明, 低密度接种条件更有利于细胞增殖和细胞干性的维持[15,45-46]。MSCs高密度接种时会产生密度依赖性生长抑制, 细胞群体倍增数会低于低密度接种的细胞[47]。Sotiropoulou等[15]分别以50, 100, 250, 500, 1 000, 5 000细胞/cm^2的密度进行培养发现, 50和100细胞/cm^2的细胞生长最快。Neuhuber等[47]以200细胞/cm^2的密度培养发现, 处于指数生长期的MSCs进行传代, 最有利于细胞的生长。但是原代分离的MSCs在长期培养条件下传代密度太低, 可能会引起细胞增殖缓慢、细胞生长所需的时间较长甚至会出现细胞生长停滞的现象。ATCC对UCD-MSCs和ASCs培养中建议的传代密度是5 000细胞/cm^2。

4 结语与展望

在过去的几十年中, 成体组织MSCs的功能特性逐渐被揭示出来。由于其高效的增殖活性和多向分化潜能, MSCs有望替代ESCs应用于细胞治疗和组织工程中。然而, MSCs体外长期培养体系的研究还远不如ESCs成熟, 建立最适培养体系还需要更深入和全面的探索。首先, 目前MSCs培养使用的含血清的培养体系, 对于MSCs的体外长期培养的功能特性有不利影响[25,27], 因此需要寻找适合MSCs的无血清培养基。其次, 不同组织来源的MSCs存在不同的生物学特性[49-53], 其最适培养条件也可能存在差异, 因此针对不同组织来源的MSCs需要研究出不同的最适培养基。另外, 动物个体年龄、细胞传代密度、细胞的冻存等因素均能够影响MSCs体外生长状态[15,45-46,54], 因此, MSCs的分离和培养还需在此基础上进行逐步优化。总而言之, 对于MSCs体外长期传代培养条件体系的标准化和统一化, 还需要从多方面去研究。而这种培养体系的建立, 将大大加快和拓宽干细胞治疗和组织工程的临床应用。

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