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【文献综述】

角膜内皮细胞培养的研究进展△

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Research advances in corneal endothelial cells culture

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【Key words】 corneal endothelial cells;cell culture;*in vitro*

【Abstract】 Corneal endothelial dysfunction can be treated by keratoplasty. Because of extremely limited sources of donor cornea, cultured corneal endothelial cells (CEC) *in vitro* can be proliferated and applied in CEC transplantation and researches of tissue engineering cornea which brings new hope to corneal endothelial dysfunction. Currently, the most commonly used method to isolate CEC is stripping Descemet's membrane with endothelium from donor cornea and enzyme digestion. Commonly used culture medium consists of basal medium and different growth factors which can promote the proliferation of CEC to some extent. Cultured CEC after several passages are difficult to remain the normal phenotypes and cell morphology. Recently, the immortalizing cell line was established by gene transfection. The advancement in cell cycle, signal pathway and ion channel of CEC will promote the progression of cell culture *in vitro*. This article reviews the research advances in corneal endothelial cells culture *in vitro*.

【关键词】 角膜内皮细胞;细胞培养;体外

【摘要】 角膜内皮功能失代偿引起的失明可通过角膜移植来治疗。人们通过体外培养角膜内皮细胞并使其增殖,进行角膜内皮细胞移植和角膜组织工程学研究。目前常用的分离角膜内皮细胞的方法是从供体上撕除后弹力层和角膜内皮层,利用酶消化作用使角膜内皮细胞分离。常用的培养基为基础培养基加各种生长因子。这些生长因子在一定程度上促进细胞增殖,但培养的角膜内皮细胞长期传代后仍难以维持正常的细胞形态和功能。基因转染技术建立了永生化角膜内皮细胞系,有关细胞周期、信号通路和离子通道的研究成果也促进了角膜内皮细胞体外培养的研究进展。本文综述了角膜内皮细胞体外培养各个环节的最近进展。

角膜维持透明的关键在于角膜内皮细胞(corneal endothelial cells,CEC)拥有正常的屏障功能和泵功能。成年人的CEC不具备有丝分裂的能力,损伤修复的方式是正常细胞移行和代偿。当受到各种物理化学因素的严重损伤导致细胞密度小于300~500 mm^{-2}时,会出现角膜内皮功能失代偿、角膜水肿混浊,甚至失明。常规的治疗方法是穿透性角膜移植和角膜内皮移植,但由于角膜供体来源受限,且有限的角膜供体中内皮细胞质量严重影响治疗的效果。因此体外培养CEC并促其增殖,用于CEC移植或作为组织工程学角膜构建的种子细胞,一直是研究的热点。本文就CEC体外培养的研究进展综述如下。

1　种子细胞的提取

体外培养CEC首先要提取种子细胞,方法包括机械刮除法、组织贴块法、揭膜法、揭膜-消化两步法[1-10]。目前最常用的方法是揭膜-消化两步法,即在显微镜下从供体角膜周边到中央部完整撕除后弹力层和角膜内皮层,然后利用胶原酶、分散酶或胰蛋白酶/EDTA等[4-8]进行消化,在显微镜下观察到细胞变圆、间隙变大时加入血清终止消化,经过漂洗、离心后获得种子细胞进行接种培养。该法优点是效率较高,缺点是揭膜时需要熟练的操作技巧,且可能会有角膜基质细胞混入。由于不同的供体角膜后弹力层厚度有差异,特别薄或粘连很紧的后弹力层很难揭下,甚至可能出现部分撕裂[9]。因此有学者将揭膜的方法进行改进,利用类似于后弹力层角膜内皮移植术中使用的真空吸

引器进行分离,可以提高分离效率和降低操作难度[3-4]。

2 CEC 的纯化

在种子细胞提取过程中,可能会混入基质层的角膜细胞,可以采用以下方式进行纯化。(1)Engelmann 等[11]使用不含有 L-缬氨酸的选择性培养基,添加 D-缬氨酸进行培养,在培养过程中去除角膜成纤维细胞。(2)磁性细胞分选术:该法的基本原理是在磁性微球或磁纳米颗粒表面修饰具有生物活性的抗体,在外加磁场的作用下利用抗体和细胞的特异性结合,快速有效地将细胞分离,包括阳性分选和负性耗尽两种方法。Peh 等[12]利用抗成纤维细胞抗体磁珠标记的负性消耗法将混入 CEC 的大部分成纤维细胞耗尽,可以增加细胞的获取量,分离效率较高。(3)应用转化生长因子受体抑制剂:Okumura 等[13]应用一种选择性 TGF-β 受体抑制剂 SB431542 可以清除成纤维细胞,促进培养的 CEC 形成接触抑制单层细胞。

3 CEC 的鉴定

3.1 细胞形态学 用相差显微镜或电子显微镜进行观察,并进行茜素红染色,细胞呈现六边形是原代 CEC 的典型表现,培养的 CEC 多呈现多边形外观。

3.2 标志性蛋白和表面分子的检测 CEC 缺乏特异性标志物,通常采用多种标志物联合检测来鉴定。(1)细胞标志物:包括胶原Ⅷ α1、内皮细胞特异性底物神经元特异性烯醇化酶、Ⅳ型胶原 α-1 和 α-2、基底膜蛋白聚糖(perlecan)。(2)CEC 功能蛋白:CEC 具有紧密的细胞连接和泵功能,可以通过一些细胞连接和通道的关键性蛋白检测来确定 CEC 的功能,包括水通道蛋白、Na^+-K^+-ATP 酶和间隙连接蛋白 43 等,目前最常用的是水通道蛋白和 Na^+-K^+-ATP 酶。Cheong 等[14]认为 GPC4(Glypican-4)和 CD-200 是可以将 CEC 和基质成纤维细胞相鉴别的细胞表面标志物。

3.3 基因水平鉴定 通过 PCR 检测总 DNA、RT-PCR 检测 mRNA 鉴定是否 CEC。Ⅳ型胶原是内皮源性的特异性基因,检测 K3 和 K12 可排除角膜上皮细胞的污染。Chng 等[15]认为 SLC4A11、COL8A2、CYYR1 这组基因是鉴别 CEC 的可靠的基因标记。

4 培养基的选择

CEC 生长需要的培养基由基础培养基加不同的营养物质和生长因子混合而成,不同的实验室有不同配方的培养基[4,8,16-22]。近年来报道的基础培养基包括:DEME、OptiMEM-1、DMEM/F12、Ham's F12/M199、CEM。添加的营养成分包括:胎牛血清、胰岛素、转铁蛋白、硒、抗生素等。内皮素-1 加入培养基可促进 CEC 增殖,而氯通道阻断剂 5-硝基-2(3-苯丙胺)苯甲酸对内皮素-1 有浓度依赖性抑制作用[23]。Li 等[8]发现用于上皮细胞培养的 SHEM 培养基在 CEC 培养中也能使 CEC 有效扩展。来源于鼠细胞的 NIH-3T3 的条件培养基(NIH-3T3-CM)培养 CEC 效率较高,但存在异种抗原干扰的问题[24]。

Peh 等[2]比较了四种不同配方的培养液(M1:DEME、M2:OptiMEM-1、M3:DMEM/F12、M4:Ham's F12/M199),发现 M2 和 M4 能显著促进细胞增殖,并能促使细胞中 Na^+-K^+-ATP 酶和水通道蛋白特异性膜蛋白的表达,而 M1 和 M3 培养的细胞分别只能在第一代和第三代以内保持增殖能力。CEC 在 M2 和 M4 中培养时三代以内可以维持特殊的细胞形态[25]。

在培养基中加入细胞外基质成分可促进体外培养 CEC 黏附和增殖。这些成分包括胶原、硫酸软骨素、层粘连蛋白、纤连蛋白等人工基质,以及骨髓间充质干细胞[3,8-9,26-27]、鼠胚胎干细胞、牛 CEC 分泌的细胞外基质[27-28],羊膜[22]及羊水[29]。Choi 等[30]发现壳聚糖包被培养板亦能促进增殖。

5 影响体外培养的 CEC 增殖的因素

5.1 供体的条件 供体的年龄、全身健康状况、死亡原因、CEC 细胞密度、供体死亡到保存的时间和供体角膜的保存期都会影响 CEC 的培养[27-33]。

由于 CEC 具有随年龄变化的特征,年龄越大 CEC 细胞密度降低越明显。来自人类胚胎和 20 岁以下供体的 CEC 体外更容易长期培养,且年轻供体的 CEC 相比高龄供体来源的 CEC 反应更迅速,进入细胞周期时间更短,具有有丝分裂象的细胞数量更多[29,31-32]。

5.2 细胞来源的解剖位置 大多数学者认为与来自中央的 CEC 相比,来自周边角膜的 CEC 保留着更高的复制潜能,是分离 CEC 进行体外培养的最佳解剖位置[7,33]。而 Konomi 等[34]则认为中央与周边角膜取材的 CEC 无论是细胞形态还是增殖能力都没有明显差别。

5.3 接种的细胞密度 Peh 等[35]将第二代 CEC 按照 2500 cm^{-2}、5000 cm^{-2}、10 000 cm^{-2} 和 20 000 cm^{-2} 的细胞密度进行接种培养,发现低密度组增殖率高于高密度组,但在统计学上无显著性差异,他们认为最理想的细胞接种密度是 10 000 cm^{-2},可以在第三代获得(1.0~2.5)千万个细胞,且细胞能维持 CEC 特有的形态。Patel 等[36]认为培养的 CEC 细胞密度低于 2000 cm^{-2} 时细胞增殖明显。Singh 等[37]将牛 CEC 以 < 1000 cm^{-2}、≥1000~2000 cm^{-2} 和 > 2000 cm^{-2} 密度培养,发现细胞密度低时会出现膜屏障功能降低,对小分子物质通透性增加。

6 促进 CEC 增殖的方法

6.1 带温度反应性的培养表面 带温度反应性的

培养表面 PIPAAm 能促进 CEC 的黏附和生长,可获得较高的细胞密度和细胞活性,估计与 CEC 存在温度敏感性瞬时受体电位通道有关[21]。

6.2 半胱氨酸 Shin 等[38]发现高浓度的半胱氨酸可以抑制 ROS 生成,减少氧化损伤介导的 CEC 细胞死亡,然而半胱氨酸对于无氧化应激的 CEC 有毒性作用。

6.3 Rho 激酶抑制剂 Rho 激酶抑制剂 Y-27632 通过抑制 Rho/ROCK 信号,可改变 CEC 的静息电位和回路,促进食蟹猴 CEC 的黏附,抑制细胞凋亡,增加细胞数量[39-41]。

6.4 基因转染技术的应用 近年来,利用基因转染的方式,将一些特定的基因转染到 CEC 上,建立细胞系进行培养,可以抑制细胞凋亡和促进增殖,显著延长了传代代数和增殖能力。转染的基因包括逆转录病毒 pLXSN 16 E6-E7、Cdk4R24、端粒酶逆转录酶基因、HIV-1、鼠白血病毒、疱疹性口腔炎病毒糖蛋白基因、改良的泡沫病毒膜蛋白基因和原型泡沫病毒假型等[42-45],可建立"永生化"CEC 细胞系。

7 问题与展望

CEC 在很多实验室体外培养均获得成功,基因转染技术可以建立"永生化"细胞系。Fan 等[46]应用非基因转染技术也可以将人 CEC 培养传代长达 224 代。尽管如此,CEC 体外长期培养并应用于临床仍面临许多挑战。(1)大部分长期培养的培养基配方中都需要加入牛血清,存在病原体污染的风险。(2)CEC 增殖能力有限,长期培养后易出现细胞形态改变,趋向于成纤维细胞形态、功能蛋白表达减少等问题。(3)大部分培养的人 CEC 来源于年老供体,传代时间短和增殖能力差,更容易出现不规则形细胞。(4)基因转染技术建立的细胞系存在成瘤的风险和病毒的细胞毒性问题。

如何更优化长期培养,使 CEC 传代后仍能良好地保持细胞形态、功能表型和增殖能力,避免潜在的感染、成瘤和细胞毒性成为未来的研究热点。关于 CEC 细胞周期、信号通路和离子通道的研究将进一步促进 CEC 体外培养的研究进展,使体外培养长期传代的 CEC 在临床上的应用变为现实。

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(上接第 **484** 页)

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