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原代小鼠肝脏细胞的高效分离纯化与培养*

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摘要 目的:建立一种能稳定获得高活性和高纯度原代小鼠肝脏细胞的分离、纯化及培养方法。**方法:**应用改良的 Seglen 二步法原位灌注和机械离心分离肝脏细胞,并用改良的高糖 DMEM 培养基进行培养。台盼蓝拒染法检测接种时肝脏细胞的存活率,倒置显微镜动态观察肝脏细胞形态变化,应用免疫荧光技术对肝脏细胞进行 Albumin 染色。**结果:**每只小鼠可获取肝脏细胞的总产量平均为 1.35×10^6 /g 体重,肝脏细胞存活率 $> 90\%$ 。倒置显微镜下观察贴壁前肝细胞直径为 $35.14 \mu\text{m} \pm 4.35 \mu\text{m}$,肝脏细胞在接种后 3 h 基本完成贴壁;肝脏细胞接种后 24h,所有肝脏细胞均强阳性表达成熟肝脏细胞标志物 Albumin,肝细胞纯度 $> 95\%$ 。**结论:**改良的分离纯化及培养方法能稳定获得高产量、高活率及高纯度的小鼠肝脏细胞。

关键词:肝脏细胞;原代细胞培养;分离;纯化;小鼠

中图分类号:Q95-3,Q813.11,R322.47 **文献标识码:**A **文章编号:**1673-6273(2014)06-1005-04

Isolation, Purification and Primary Culture of Mouse Hepatocytes with High Viability*

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ABSTRACT Objective: A stable method was established for isolation and primary culture of mouse hepatocytes with high purity and viability. **Methods:** The C57BL/6 mouse hepatocytes were isolated by a modified two-step collagenase perfusion and low-speed mechanical centrifugation. The hepatocytes were cultured in modified DMEM with high level of glucose. The viabilities of cells were evaluated by Trypan blue exclusion before cells were seeded. Cellular morphological changes were observed using a phase contrast microscopy. The Albumin and CK19 staining were performed on hepatocytes by immunofluorescence. **Results:** The total cell yield of hepatocytes was 1.35×10^6 cells/g wet weight. Total cell viability was more than 90%. The diameter of hepatocytes was $35.14 \mu\text{m} \pm 4.35 \mu\text{m}$ before adherence. The hepatocytes with enlarged and flattened round shape were attached to the surface of collagen-coated dishes 3 hours after seeding. Albumin was positively expressed in all hepatocytes at 24 h after attachment, and the purity of hepatocytes was more than 95%. **Conclusion:** Mouse hepatocytes with high quantity, purity and survival rate were obtained by the improved method for isolation, purification and culture.

Key words: Hepatocytes; Primary cell culture; Isolation; Purification; Mouse

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前言

肝脏是机体最重要的器官之一^[1],肝细胞具有丰富的酶系及多种特异性功能^[2]。体外分离和培养的肝细胞被广泛用于病理学、药代动力学、毒理学和致癌作用等基础实验研究^[3-5],并且肝细胞移植对于急性肝功能衰竭、肝硬化等是一种具有巨大潜力的治疗方式^[6-8]。与其他动物相比,小鼠的基因分析系统更加完善,对小鼠基因的研究和了解也更为广泛和深入,并且在已建立的人类疾病模型中,小鼠模型最多^[9,10]。因此,迫切需要建立一种高效的小鼠肝细胞体外培养模型。目前大鼠肝细胞的分离方法相对成熟,然而与小鼠相比较,小鼠形体较小,肝细胞分离操作困难,细胞模型创建过程中的技术细节也与小鼠差异较

大,将研究大鼠肝脏细胞所采用的技术应用到小鼠研究中并不能达到预期的效果^[11,12]。因此我们在 Seglen 二步法原位灌注的基础上进行一系列的改进和优化,并且采用改良的高糖 DMEM 培养基进行培养,成功的建立了高效的小鼠肝脏细胞体外培养模型。

1 材料与方法

1.1 材料及主要试剂

6~8 周 C57BL/6 雄性小鼠(SPF 级),体重(21 ± 1)g,由复旦大学附属中山医院实验动物中心提供。NB4 胶原酶(德国 Serva 公司);胎牛血清(Sigma 公司,美国);高糖 DMEM 培养基等其它试剂均购于美国 Sigma 公司。Axiovert 200 倒置生物显

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显微镜(Carl Zeiss, 德国); Steril-GARD III Advance II 级生物安全柜(Baker, 美国); 二氧化碳孵育箱(Heraeus, 德国); BD Intima-II 静脉留置针(BD Falcon, 美国); 70 μm 细胞筛网(CORNING, 美国); 35 mm 细胞培养皿(CORNING, 美国)。

1.2 肝脏细胞的分离和培养

应用改良 Seglen 二步法原位肝脏灌注和机械离心分离肝脏细胞。具体方法为: 小鼠麻醉后打开腹腔, 行门静脉插管, 灌注 D-Hank's 缓冲液(添加 0.5 mmol/L EGTA、100 $\mu\text{g/ml}$ Streptomycin 和 100 IU/ml Penicillin, 使用前 37 $^{\circ}\text{C}$ 预热), 待肝脏鼓起后剪破下腔静脉引流, 持续灌注 8~10 min 后换用胶原酶灌注液(D-Hank's 液中含 2 mmol/L, 0.12 PZ-u/ml 的 Collagenase NB4, 使用前 37 $^{\circ}\text{C}$ 预热)持续灌注约 10 min。摘除肝脏, 置于平皿中, 加入高糖 DMEM 培养基(添加 0.2% BSA、20 mmol/L Hepes、100 $\mu\text{g/ml}$ Streptomycin 和 100 IU/ml Penicillin, 使用前 4 $^{\circ}\text{C}$ 预热), 撕破肝脏被膜收集肝脏细胞悬液。用 70 μm 细胞筛网过滤后, 50 g 离心 1 min 取沉淀物, 沉淀物稀释后再以同样参数离心 1 次, 取沉淀物稀释后再 50 g 离心 2 min, 沉淀物即富含肝脏细胞的部分。用改良的高糖 DMEM 培养基(添加 10% FBS、10 mmol/L Nicotinamide、10 $\mu\text{g/ml}$ Insulin、 10^{-7} mol/L Dexamethasone、100 $\mu\text{g/ml}$ Streptomycin 和 100 IU/ml Penicillin), 将细胞稀释成 $1.8 \times 10^5/\text{ml}$, 并接种于包被 I 型鼠尾胶原的 35 mm 培养皿中, 每皿中接种 2 mL 细胞液, 接种第 12 h 换液, 后每周 3 次更换培养基。

1.3 细胞计数和测定细胞活力(台盼蓝拒染法)

取 0.4 mL 细胞液添加 0.6 mL 台盼蓝, 轻轻混匀, 3 min 后

进行细胞计数, 形态学选取肝脏实质细胞进行单独计数, 测算细胞活力。

1.4 抗体和免疫荧光染色

抗 -Albumin (1:100) (Abcam, 美国); 驴抗羊 IgG-cy3 (1:1000) (Jackson ImmunoResearch Laboratories, 美国)。免疫荧光染色方法: DMEM 培养基冲洗细胞两次, 用 1% 多聚甲醛固定, 加一抗 4 $^{\circ}\text{C}$ 过夜, PBS 冲洗, 加入二抗 37 $^{\circ}\text{C}$ 孵育 2 h。用 DAPI 染核。

2 结果

2.1 产量及存活率

肝脏细胞的总产量平均为 $1.35 \times 10^6/\text{g}$ 体重。用台盼蓝拒染法检测分离的新鲜肝细胞存活率, 结果显示 90% 以上肝细胞均排斥染色, 整个细胞透明无色。细胞接种后, 肝细胞约占贴壁细胞总数的 95% 以上, 仅存在少量的上皮细胞和星状细胞等肝脏非实质细胞。肝脏细胞在改良的 DMEM 培养基进行培养, 细胞可持续培养超过 2 周。

2.2 形态学观察

肝细胞贴壁前呈圆形或椭圆形, 单核或双核, 测得贴壁前直径为 $(35.14 \pm 4.35) \mu\text{m}$ (AxioVision 4.0 软件)。肝脏细胞接种于 I 型胶原包被的 35 mm 培养皿中, 接种后 30 min~1 h 细胞开始贴壁, 接种 3 h 后可达到较好的贴壁效果。在未进行包被的培养皿中, 肝细胞贴壁效果相对差, 细胞聚集成团。肝细胞贴壁后胞体增大、平展并有向外延伸的趋势, 肝细胞呈不规则多角形, 相邻的细胞开始建立连接, 形成岛状或条索状结构见图 1。

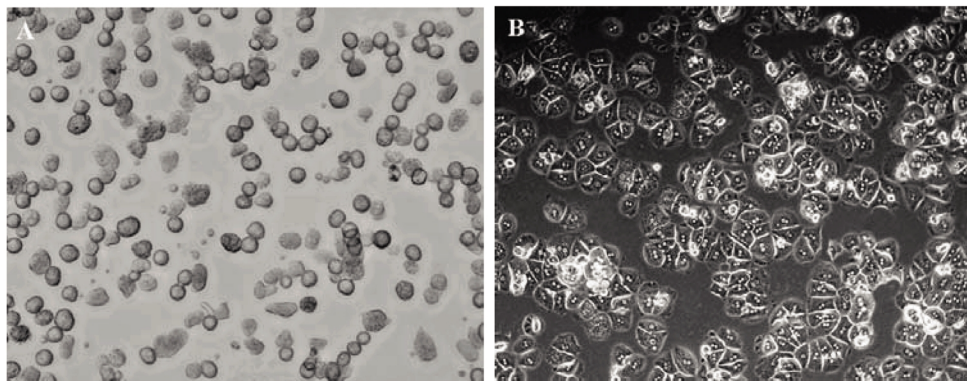


图 1 肝细胞形态学观察

A 未贴壁肝脏细胞的相差图像(100 $\times$); B 贴壁 24h 肝细胞的相差图像(100 $\times$)

Fig.1 Morphology of cultured hepatic cells

A. Phase contrast images of hepatocytes before attachment(100 $\times$); B. Phase contrast images of hepatocytes at 24 h(100 $\times$)

2.3 免疫荧光染色

肝脏细胞接种后 24 h, 所有肝细胞均强阳性表达成熟肝脏细胞标志物 Albumin, 表明获取的实质细胞为成熟的肝脏细胞见图 2。

3 讨论

原代肝细胞分离的目的是获得生物活性高并且形态完整的肝细胞。Seglen 原位二步灌注法是目前最常用的用于分离肝脏细胞的理想灌注方法, 该方法分离过程短、肝细胞的产量和

活性较高^[3]。目前, 肝脏的原位灌注主要分为下腔静脉逆行灌注和门静脉插管顺行灌注两种。本实验中对上述两种方法分离得到的肝脏细胞含量及活性, 发现门静脉顺行灌注为更好的灌注方法。虽然下腔静脉逆行插管不需要借助特殊的显微器械, 肉眼操作即可保证较高的成功率, 但是逆行灌注不符合肝内门静脉正常的血流方向, 且灌注时需夹闭上腔静脉, 极易造成肝脏的灌注不均, 因此, 逆行灌注的效率相对较低, 分离得到的肝细胞含量及纯度也较低。门静脉插管顺行灌注对细节技术要求较高, 但是反复练习后亦可以保证较高的成功率, 并且

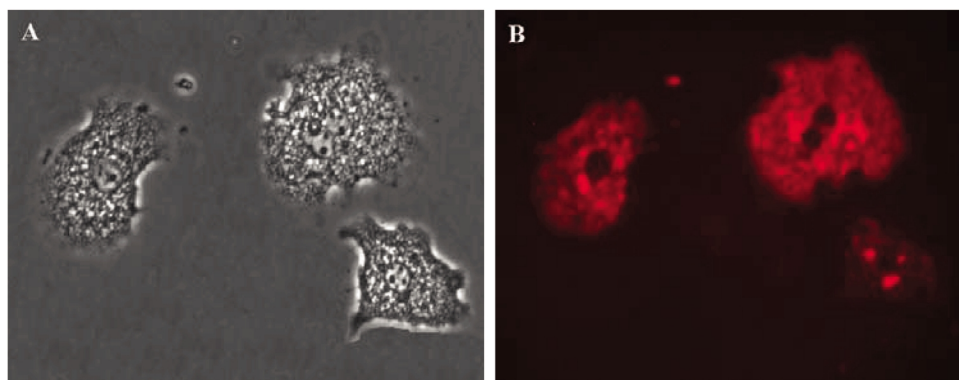


图2 体外培养肝细胞的免疫荧光鉴定

A 肝脏细胞的相差图像(400×); B 肝脏细胞的 ALB 表达阳性(400×)

Fig.2 Expression of albumin in the hepatic cells by immunofluorescence staining

A. Phase contrast image of hepatocytes(400×); B. The hepatic cells stain positively for the albumin(400×)

顺行灌注符合肝脏内门静脉的血流特点,使灌注更加均匀,减少血细胞及杂质的污染,获得的肝脏细胞产量及活性均较高。

我们在实验中发现胶原酶浓度、灌注温度、速度等条件均影响细胞的产量及活性。本实验采用不间断的单向二步灌注法,第一步灌注时采用无钙的 D-Hank's 灌流液,其中添加 0.5 mmol/L 的 EGTA,移去了肝脏中的血细胞及肝细胞间质中的 Ca^{2+} ,从而减少细胞之间的黏附。第二步灌注中,预实验的结果显示德国 Serva 公司生产的 NB4 及 NB4G 型胶原酶分离肝细胞的效果明显优于其他公司所售 IV 型胶原酶。 Ca^{2+} 是维持胶原酶活性所必需,胶原酶灌流液中需添加钙。我们通过多次试验最终确定了灌流液中胶原酶和 Ca^{2+} 的浓度,以达到最佳分离效果并提高了肝细胞存活率。根据不同细胞之间沉降率的差别,经过 3 次低温(4°C)及低速(50 g)离心肝细胞悬液,可有效的将肝细胞与肝脏非实质细胞分离开来,减少肝细胞损伤,且提高肝细胞纯度。

成熟肝细胞是高分化的终末细胞,对培养条件要求较高,在体外不易培养。本实验中肝细胞在包被于 I 型鼠尾胶原的培养皿中贴壁较好,细胞平铺于皿底,而接种于未包被于鼠尾胶原的肝细胞贴壁效果较差,细胞极易聚集成团。肝细胞培养在改良的高糖 DMEM 培养基中进行,在培养基中添加了 10% FBS,满足肝细胞存活的需要。在肝细胞培养过程中,胰岛素具有重要作用,可以增加细胞的贴壁率,促进核蛋白磷酸化及肝细胞铁的摄入^[14-16]。培养基中添加的尼克酰胺在细胞内可转化为 NAD 和 NADP,二者参与机体的多种生化反应,可以抑制肝细胞的炎症反应及氧化应激^[17],原代肝细胞培养时,细胞内 NAD 大量丢失,通过添加尼克酰胺,弥补细胞内 NAD 的减少,维持肝脏细胞的正常功能^[18]。地塞米松促进肝细胞的线粒体再生,提高肝细胞稳定性,可以减轻炎症反应中脂多糖诱导的肝损伤^[19,20]。在培养基中添加合适的细胞因子和激素,对维持肝细胞的生物学功能具有重要意义。

综上所述,本研究在传统的原位胶原酶灌注法的基础上,通过一系列改良和优化,明显提高了体外分离纯化获得小鼠肝脏细胞的存活率和纯度,并且在改良的 DMEM 培养基中可稳定生长并维持正常功能,为后续与肝细胞相关的多种实验研究奠定了细胞学基础。

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