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World Chin J Digestol 2003 December;11(12):1915-1918
世界华人消化杂志 ISSN 1009-3079 CN 14-1260/R

同种肝细胞脾内移植治疗大鼠肝衰竭的实验研究

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收稿日期: 2003-06-27 接受日期: 2003-08-16

Hepatocyte transplantation intrasplenically for rats with acute hepatic failure

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Received: 2003-06-27 Accepted: 2003-08-16

Abstract

AIM: To study the effects of intrasplenic transplantation of primary cultured hepatocytes and isolated hepatocytes on rats with acute hepatic failure (AHF) induced by D-galactosamine (D-gal), and to observe the biological characteristics of transplanted hepatocytes in the spleen.

METHODS: AHF rats were induced by D-gal. Hepatocyte transplantation was carried out 24 hours after intoxication, and the rats were divided into three groups randomly. In Group I, rats received intrasplenic injection of 2×10^7 hepatocytes cultured for 3 days, whereas in Group II, rats received 2×10^7 hepatocytes incubated for 8 hours intrasplenically. In Group III, rats received 1mL normal saline intrasplenically as control. Then the survival rates, liver function, liver histology and the biological characteristics of transplanted hepatocytes in all rats were observed.

RESULTS: Compared with Group III rats, a significant improvement in survival rates was observed in Group I and II (78 % *vs* 23 %, $P<0.01$ and 71 % *vs* 23 %, $P<0.05$ respectively).Three days after hepatocyte transplantation, the liver functions in Groups I and II rats were restored significantly compared with those of Group III, and the liver pathologic changes were mild . But no difference was found in the survival rates and liver functions between Groups I and II. By the HE and PAS staining, our data indicated that the structure and function of hepatocytes transplanted kept basically well.

CONCLUSION: Intrasplenic transplantation of primary cultured hepatocytes and isolated hepatocytes can prolong the survival of AHF rats and improve the liver function and histology, and no statistically significant differences are observed between Groups I and II. Our data suggest that hepatocytes cultured for 3 days may be an alterative for hepatocyte transplantation.

Teng GJ, Bai XF, Huang CX, Sun YT, Xu Z, Nie QH, Liu J, Gu BQ. Hepatocyte transplantation intrasplenically for rats with acute hepatic failure. Shijie Huaren Xiaohua Zazhi 2003;11(12):1915-1918

摘要

目的: 探讨经脾内同种异体移植培养的原代肝细胞与肝细胞悬液治疗大鼠药物性肝衰竭的疗效,并观察脾内移植肝细胞的生物学特性.

方法: 采用D-氨基半乳糖(D-gal)建立大鼠急性肝衰竭模型, 24 h后随机分为3组进行治疗, Ⅰ组: 经脾内移植体外培养3d的肝细胞2×10^7; Ⅱ组: 经脾内移植预孵育8 h的肝细胞悬液2×10^7; Ⅲ组: 经脾内注射生理盐水1 mL. 观察受体大鼠的存活率、肝脏功能和病理变化及移植肝细胞的生物学特性.

结果: Ⅰ组、Ⅱ组大鼠存活率(78 %, 71 %)与Ⅲ组大鼠存活率(23 %)相比具有显著性差异($P<0.01$, $P<0.05$), 肝功能各项指标均有明显改善, 肝组织病理改变较轻. 而Ⅰ, Ⅱ组大鼠的存活率、肝功能改变方面, 没有统计学差异. 经HE和PAS染色证实,移植的肝细胞在受体脾内结构和功能保持较好.

结论: 经脾内移植的培养肝细胞和肝细胞悬液均能提高大鼠药物性肝衰竭的存活率、改善肝功能及肝脏组织病理变化.而培养肝细胞与细胞悬液相比,统计学上没有明显差异, 但结果显示培养肝细胞仍具有一定的优越性.

滕光菊, 白雪帆, 黄长形, 孙永涛, 徐哲, 聂青和, 刘节, 顾炳权. 同种肝细胞脾内移植治疗大鼠肝衰竭的实验研究. 世界华人消化杂志 2003;11(12):1915-1918
http://www.wjgnet.com/1009-3079/11/1915.asp

0 引言

近年来, 随着肝细胞分离技术和培养方法的快速发展, 肝细胞移植已开始用于临床并取得了一定的疗效[1-9]. 但用于移植肝细胞的最佳状态和适宜时机, 目前还不太

清楚.为此我们用D-gal制备大鼠急性肝衰竭模型，经脾内同种异体移植培养肝细胞治疗大鼠急性肝衰竭，并设脾内移植肝细胞悬液和生理盐水为实验对照和空白对照，对移植后大鼠存活率、肝脏生化指标、肝脏组织学及移植后脾内肝细胞形态和功能进行了观察，以比较体外短期培养的肝细胞和新分离的肝细胞悬液经脾内移植治疗急性肝衰竭大鼠的疗效，为探索移植肝细胞的最佳状态和适宜时间提供实验依据.

1 材料和方法

1.1 材料 供受体均为♂SD大鼠，2-3月龄，体质量200-300 g，由第四军医大学实验动物中心提供，实验室温度21-25 ℃，实验前12 h禁食. 胶原酶Ⅳ、胰岛素、胰高血糖素和表皮生长因子等购于美国Sigma生物公司，Williams'E培养基、胎牛血清为Gibco公司产品.主要仪器设备包括BT01-100蠕动泵(Langepump保定格兰蠕动泵有限公司)、Sorvall低温离心机(美国Heraeus公司)，CL-800全自动生化分化仪(日本岛津)，倒置显微镜(上海海鸥)等.

1.2 方法 经大鼠腹腔内注射不同剂量的D-gal (1.8，2.0，2.2 g/kg)，根据动物死亡率、肝脏功能及病理改变选择2.0 g/kg D-gal为大鼠急性肝衰竭的最适诱导剂量. 肝细胞分离及培养参照Seglen报道的方法略加修改. SD♂大鼠肌注0.3 mg/kg^{-1}速眠新进行麻醉，胸腹部消毒，开腹，门静脉插管固定，剪断下腔静脉，用37 ℃水浴预热的无钙、镁Perfusion buffer循环灌流，10 min后分离肝脏，将其置于12 cm培养皿内，改用0.5 g/L的胶原酶25 mL循环灌注，至肝包膜下组织呈龟背状裂隙或门静脉破裂时终止.取下消化成熟的肝脏，轻轻撕去肝包膜，将肝细胞置于预冷的Suspension Buffer中重悬，然后置37 ℃恒温水浴箱内预孵育30 min，分别经100目和200目滤网过滤，300 r/min离心1 min × 4次，每次用Wash buffer洗涤. 向沉淀的肝细胞中加入Williams'E培养液制成肝细胞悬液.用台盼蓝排斥法测肝细胞活力，并计算产率.肝细胞产量为(0.5-1.21)$\times 10^8$/鼠，细胞活率为93.6 %，肝实质细胞纯度大于96 %. 将活率90 %以上的肝细胞密度调至(2-5)$\times 10^8$/L接种于铺有鼠尾胶的50 mL培养瓶或6孔板的Williams'E混合培养基中，置37 ℃，50 mL/L孵箱培养. 100只大鼠，在D-gal诱导肝衰竭后24 h，药物性中毒死亡14只. 存活86只，随机分为3组: Ⅰ组，29只，移植体外培养3 d的肝细胞: 2×10^7个肝细胞作为实验组; Ⅱ组，29只，移植预孵育8 h的肝细胞悬液1 mL含2×10^7个肝细胞作为实验对照; Ⅲ组，28只，同上经脾内注射生理盐水1 mL，作为空白对照. 移植方法: 均经脾尾段向脾实质内注射，注射同时阻断脾蒂血流，注射后用纱布轻压注射点以防渗漏和出血，然后逐层缝合. 同时每只受体大鼠肌注青霉素20万U. 随后每组分别取10只大鼠用于观察大鼠肝功能改变; 其余各组大鼠进行存活率和组织形态学的研究.分别于肝细胞移植前及移植后1 d，3 d，7 d，经腹主动脉穿刺取血，在全自动生化分析仪上检测丙氨酸氨基转移酶(ALT)、门冬氨酸氨基转移酶(AST)和总胆红素(TBil). 另取6只正常大鼠作对照.移植后1 d，3 d，7 d，14 d，取各组存活大鼠脾脏、肝脏标本，用40 g/L甲醛固定，按常规方法行HE，PAS染色，观察受体鼠肝脏恢复、移植肝细胞在脾内的生物学特性.

统计学处理 所有数据均以$\bar{x}\pm s$表示，死亡率比较采用*Fisher*确切概率计算，各项肝功能指标比较采用*t*检验，用SPSS软件作统计学分析.

2 结果

2.1 大鼠存活率 药物性肝衰大鼠3组，肝细胞移植后2 h各组均有1只死亡，未列入统计. 受体大鼠2 wk存活率Ⅰ组为78 %(11/14)，Ⅱ组为71 %(10/14)，Ⅲ组为23 % (3/13). 统计学处理，Ⅰ，Ⅱ组与Ⅲ组相比有显著性差异($P=0.007$，$P<0.01$; $P=0.021$; $P<0.05$)，Ⅰ组与Ⅱ组相比无显著性差异($P>0.05$，表1).

表1 肝细胞移植后2 wk内大鼠存活情况(n)

分组	n	t(移植后)d			
		1	3	7	14
Ⅰ组	14	13	12	11	11[b]
Ⅱ组	14	12	10	10	10[a]
Ⅲ组	13	8	6	4	3

[a]$P<0.05$; [b]$P<0.01$ *vs* Ⅲ组.

表2 肝细胞移植大鼠肝脏生化指标变化($\bar{x}\pm s$)

分组		ALT(μkat/L)	AST(μkat/L)	TBil(μmol/L)
0 d		0.9 ± 0.2	1.5 ± 0.2	2.8 ± 0.6
Model		16.9 ± 5.8	28.0 ± 8.7	17.9 ± 11.5
1st d	Ⅰ	14.9 ± 7.5	26.8 ± 10.5	15.1 ± 11.2
	Ⅱ	17.0 ± 5.2	25.5 ± 6.6	18.2 ± 9.9
	Ⅲ	18.91 ± 5.2	25.7 ± 7.8	20.0 ± 9.1
3rd d	Ⅰ	6.0 ± 2.4[a]	5.2 ± 3.1[b]	7.3 ± 3.5[a]
	Ⅱ	7.8 ± 3.7	6.5 ± 2.9[b]	5.9 ± 2.9[a]
	Ⅲ	11.4 ± 3.6	18.3 ± 4.3	12.6 ± 3.3
7th d	Ⅰ	1.6 ± 0.5	1.9 ± 0.9	3.4 ± 0.7[a]
	Ⅱ	1.7 ± 0.6	2.2 ± 1.1	3.7 ± 0.8
	Ⅲ	2.1 ± 0.8	2.7 ± 1.2	5.3 ± 1.6

[a]$P<0.05$，[b]$P<0.01$ *vs* Ⅲ组.

2.2 肝脏功能变化 实验前3组大鼠24 h ALT，AST和TBil差异无显著性($P>0.05$)，表明实验前三组大鼠肝功能状态基本一致. 在细胞移植后1 d各组肝功能指标间无显著性差异($P>0.05$). 移植后3 d，除Ⅱ组与Ⅲ组的ALT之间没有显著性差异外，Ⅰ，Ⅱ组其余各

项指标与 Ⅲ 组相比有显著性差异(P <0.05); Ⅰ组 与Ⅱ组各项指标相比无显著性差异.移植后7 d，各组大鼠肝功均明显恢复，除Ⅰ组与 Ⅲ 组的 TBiL 之间具有显著性差异(P <0.05)外，各组之间无显著差异(表 2).

2.3 组织学改变 肝细胞移植后 3 d，Ⅲ 组大鼠肝细胞呈片状坏死，细胞核皱缩碎裂，肝小叶结构模糊，肝窦扩张、出血，汇管区有炎性细胞浸润(图 1). Ⅰ组和Ⅱ 组肝脏组织病变均轻于Ⅲ 组，表现为肝细胞变性，点状坏死及嗜酸性变，炎性细胞浸润减少，出血减轻(图 2).但Ⅰ 组和Ⅱ 组肝脏组织学病理改变无明显差异. 肝细胞移植 7 d 以后，各组大鼠肝脏组织学结构大致恢复正常.Ⅰ 组和Ⅱ 组移植后 1 d，3 d，7 d 脾脏红髓内均可见弥散分布小灶状的肝细胞，圆形或椭圆形，胞核清楚，胞质蓝染，至 14 d 脾内仍可见到少许肝细胞聚集成团或 2-3 个呈索状排列，还可见双核细胞(图3). 同样PAS染色显示肝细胞胞质内丰富的红棕色糖原颗粒，随时间延长，染色更深(图 4).

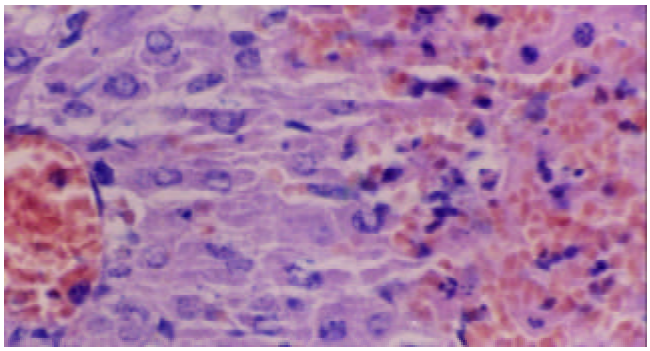

图 1 Ⅲ 组大鼠生理盐水注射后 3 d HE × 400.

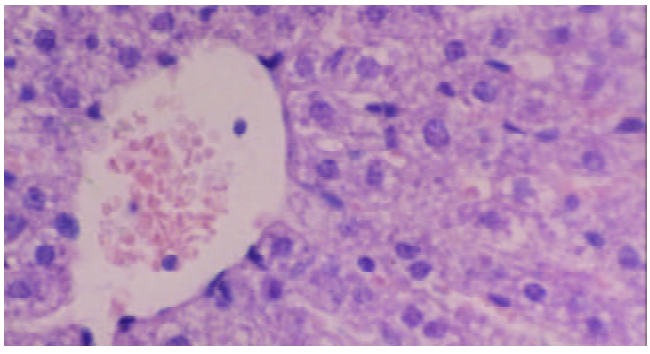

图 2 Ⅰ组 肝细胞移植后 3 d HE × 400.

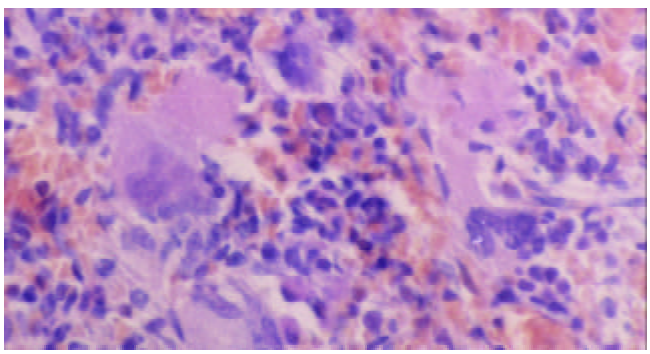

图 3 肝细胞移植后 3 d, 脾内肝细胞 HE × 200.

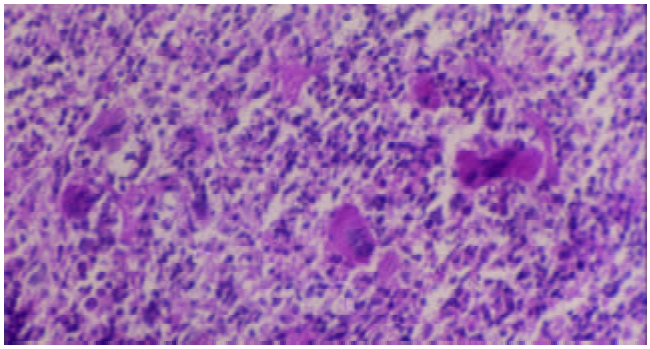

图 4 肝细胞移植后 3 d, 脾内见丰富糖原颗粒 PAS × 200.

3 讨论

多年来，肝功能衰竭的治疗仅限于传统的保肝解毒、支持治疗，但疗效不佳，未能改善患者的预后. 原位肝移植(OLT)被认为是最有效的治疗手段[10-12]，但在临床推广应用仍受到诸多条件限制[13-15]. 而肝细胞移植可以将一次获得的大量肝细胞分别移入多个受体，使多个患者同时受益，同时操作较 OLT 相对简单，移植失败或产生排斥反应对受体影响较小，故越来越受到人们的重视[16-20]. 肝细胞移植途径主要有门静脉、脾及腹腔等部位. 目前多数学者认为脾脏是肝细胞移植的最佳场所[21, 22]，其原因在于脾红髓的网状结构有利于细胞间的相互作用及细胞着床，同时脾内丰富的血供也为移植肝细胞的长期存活、增生及保持良好的生理功能提供有利条件. 本实验采用同种肝细胞异体脾内移植治疗 D-gal 诱导的急性肝衰竭大鼠，结果表明能明显改善受体鼠肝功能、肝脏病理变化和提高存活率. 与相关文献报道一致[23].

不论是对于肝细胞移植还是生物人工肝，都需要具有良好生物活性的肝细胞，国内外学者在此方面进行了广泛的研究[24-36]. 我们在第一次试验中发现大鼠肝细胞原代培养的 3 d，肝细胞处于功能活性的最佳状态，在此基础上我们选取培养第 3 d 的大鼠原代肝细胞和预孵育 8 h肝细胞进行脾内移植治疗急性肝衰竭大鼠，结果显示经脾内移植的培养肝细胞和肝细胞悬液均能提高大鼠药物性肝衰竭的存活率、改善肝功能及肝脏组织学病理变化，培养第 3 d 肝细胞疗效较好，但与细胞悬液相比无显著性差异，考虑可能与培养肝细胞具有很强的贴壁性在消化时受到损伤以及此次实验的样本量较小有关.

肝细胞移植后1-14 d在受体鼠脾红髓内均可见弥散分布大而圆的肝细胞，部分肝细胞聚集成团，有的排列呈条索状，还可见双核细胞，说明植入的肝细胞在受体内可以存活并有分裂现象.PAS 染色可见大量棕红色的糖原颗粒，进一步说明植入脾脏的肝细胞仍具有正常肝细胞的特点，参与了受体糖代谢活动.

总之，我们的研究结果显示脾内肝细胞移植治疗药物诱导急性肝衰竭大鼠，可以提高大鼠的存活率、改善肝功能和肝脏组织病理学变化，植入脾内的肝细

胞在没有免疫抑制剂的情况下可以在受体内短期存活，并具有糖代谢功能. 虽然，统计学上没有明显差异，但从大鼠的死亡率、肝功能的改善和病理改变来看，培养3 d的肝细胞与肝细胞悬液相比，显示出了一定的优势，可能更适合于肝细胞移植. 究竟肝细胞需培养多长时间更适合用于临床肝细胞移植或生物人工肝还有待进一步的研究.

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