

生物人工肝用肝细胞低温保存的现状与进展

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■背景资料

目前肝功能衰竭病死率高达80%左右, 现有病因治疗及支持治疗疗效甚微。肝移植被认为是最有效的治疗手段, 但存在供体严重不足、终生免疫治疗及费用昂贵等缺点。生物人工肝的发展为肝衰竭的治疗提供了新的途径。

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Hypothermic storage of hepatocytes used for bioartificial liver support system: current status and recent advances

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Abstract

The problem that high-quality hepatocytes are difficult to obtain restricts the use of bioartificial liver support system (BLASS) in clinical practice. Finding an effective way to preserve hepatocytes and constructing a “ready-to-use” hepatocyte bank would efficiently promote the development of the BLASS. Nowadays, the methods for hypothermic storage of hepatocytes could be classified

into two types: conventional hypothermic storage at 4 °C or subzero nonfreezing storage, and cryopreservation at -80 °C or -196 °C. Each type of hypothermic storage method has its advantages and disadvantages. Many factors may affect the effect of hypothermic storage (cryopreservation), such as storage solution and cryoprotective agent. Although the precise mechanism underlying the death of hepatocytes during hypothermic storage is not well understood, numerous studies have indicated that apoptosis plays an important role in hypothermic storage injury.

Key Words: Bioartificial liver support system; Hypothermic storage; Cell apoptosis

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摘要

大量功能好的肝细胞是生物人工肝支持系统(bio-artificial liver support system, BLASS)的核心, 是制约BLASS临床广泛应用的瓶颈。探索出一种实用的肝细胞低温保存方法, 建立一个随时可用(ready to use)的肝细胞库是BLASS普遍推广的基础。目前肝细胞低温保存分为4 °C或零下非结冰保存和-80 °C或-196 °C深低温冻存两大类, 两大类保存方法各有优缺点。影响肝细胞低温保存的因素很多, 如保存(冻存)液、(冻存)保护剂等。有关肝细胞在低温保存过程中死亡的机制尚未完全阐明, 但大量研究发现细胞凋亡是除了坏死之外低温保存肝细胞死亡的另一个重要的途径。

关键词: 生物人工肝; 低温保存; 细胞凋亡

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0 引言

肝功能衰竭(liver failure)是多种因素引起的严

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重肝功能损害^[1], 现有病因治疗及一般支持治疗疗效甚微, 病死率高达80%左右^[2]. 除了肝移植^[3,4], 生物人工肝支持系统(Bio-artificial liver support system, BLASS)的发展为肝功能衰竭的治疗开辟了新的途径^[5]. 一方面通过BLASS短期有效的支持, 部分患者肝脏可望通过残存的正常肝细胞代偿增生而恢复功能^[6], 另一方面也可以通过BLASS的体外支持, 为不可逆急慢性肝功能衰竭患者最终的肝移植赢得宝贵的待肝时间, BLASS起到了“肝功能衰竭-肝移植”的桥梁作用^[7]. 肝细胞是BLASS的核心, 是制约BLASS临床广泛应用的瓶颈^[8]. 所以探索出一种可靠的低温保存体系, 建立一个随时可用(ready to use)的肝细胞库^[9-11], 是BLASS技术普遍推广的基础. 目前肝细胞低温保存主要分为两大类: 4℃低温保存(hypothermic preservation)^[12]或者零下非结冰保存(subzero nonfreezing storage)^[13]; -80℃或者-196℃深低温冻存(cryopreservation)^[14]. 有关肝细胞在低温保存过程中死亡的机制尚未完全阐明, 但研究发现细胞凋亡在细胞低温保存损伤中发挥着重要的作用^[15,16]. 本文就肝细胞低温保存的现状与进展及其同细胞凋亡的关系作一综述.

1 4℃常规低温保存

影响4℃低温保存的主要因素有: (1)低温保存液: UW液(University of Wisconsin solution)多用于供肝的短期保存和运输, 被认为是器官保存的标准液^[17]. 随着肝细胞移植与BLASS的发展, 对肝细胞的需求明显增加, UW液广泛被用于肝细胞的短期低温保存且效果明显. Calligaris等^[18]用UW液4℃保存分离的鼠肝细胞72 h, 发现低温保存的肝细胞氨基清除能力及尿素合成能力同新鲜细胞无明显差别. Abrahamse等^[19]分别用UW液, Celsior液及HTK液4℃保存原代猪肝细胞48 h, 发现UW液组肝细胞存活率同Celsior液无差别但优于HTK液, UW液组DNA片段释放及LDH释放明显低于Celsior液及HTK液. Kao等^[12]用UW液4℃保存鼠肝细胞48 h, 细胞存活率及LDH释放同新鲜细胞无差异, 但明显优于林格式乳酸液(Ringer's lactate solution, RL)及磷酸缓冲液(phosphate-buffer saline, PBS)4℃保存鼠肝细胞的效果. 因此, UW液在肝细胞短期4℃保存方面优势明显, 但是UW液比较昂贵, 许多学者开始探索优化其他保存液, 以期获得同UW液同样甚至更佳的保存效果: Mamprin等^[17]发现由

氨基磺酸类缓冲剂BES、葡萄糖酸锑钠和蔗糖构成的BGS液低温保存肝细胞的效果同UW液无差异. 也有关于使用Williams E、L-15和RPMI 1640等培养基4℃保存肝细胞的研究^[20,21], 但是保存时间超过48h后细胞存活率往往降至50%以下, 不及UW液的保存效果. (2)低温保护剂: 聚乙二醇(polyethylene glycol, PGE)的相对分子质量大约8 000 Da, 是一种非渗透性保护剂, 其机制可能是同低温保存时维持肝细胞内外渗透压, 减轻细胞水肿和保护细胞膜和细胞器有关^[22]. 刘鸿凌等^[23]用含5% PGE的RPMI 1640培养基和UW液4℃低温保存原代乳猪肝细胞, 48 h内均可获得较高的细胞存活率及贴壁率, 保存72 h后PGE组细胞存活率有所下降不及UW液组, 但仍然可达60%. 各时间点PGE组肝细胞药物代谢功能及合成功能同UW液组无明显差异. 乙酰半胱氨酸(*N*-acetyl-cystein, NAC)及葡萄糖(glucose, Glu). NAC是一种活性氧自由基清除剂, 具有抗氧化的作用. 葡萄糖是细胞能量代谢最主要的物质. Gómez-Lechón等^[24]发现在磷酸缓冲液(phosphate-buffer saline, PBS)的基础上增加2 mmol NAC及15 mmol Glu 4℃低温保存原代人肝细胞, 肝细胞存活率、生长贴壁率、糖原合成、谷胱甘肽水平及细胞色素酶P450药物代谢水平单纯PBS液(对照组)明显增加. (3)降温速率: Kebis等^[25]0℃-4℃ HTK液低温保存鼠肝组织24 h, 一组采用计算机程控降温(45 min内, HTK液由初始的13℃缓慢降至3℃), 另一组将肝组织直接置于0℃-4℃ HTK液(对照组). 发现程控降温组天冬氨酸转氨酶(AST)及丙氨酸转氨酶(ALT)的释放量是对照组的1/2-1/3. 因此, Kebis认为直接用低温HTK液保存肝组织是不恰当的, 低温保存前控制降温速率可以减轻低温保存肝细胞损伤.

2 零下非结冰低温保存

温度是影响细胞代谢活性的重要因素, 一般而言温度越低细胞代谢活性就越低, 保存时间越长^[26]. 零下非结冰温度(subzero nonfreezing temperature, SNFT)是指0℃到某溶液冰点(freezing point)之间的温度范围. 超冷温度是指溶液冰点之下温度至溶液开始形成冰晶之间的温度范围^[27]. 比如: UW液的冰点是-1℃, 但是只有外界温度降至-4℃(超冷温度阈值)时才开始结冰, 因此, UW液的零下非结冰温度是0℃至-4℃, 超冷温度是-1℃至-4℃. 零下非结冰温度及超冷

■研发前沿

获得大量的生物转化和分泌功能好且能快速临床应用的肝细胞是生物人工肝的核心. 而探索出一种有效的肝细胞低温保存体系, 建立一个随时可用“ready to use”的肝细胞库, 临床随时可以得到大量高活率有功能的肝细胞, 可有效的解决制约人工肝发展的瓶颈, 是人工肝普遍推广的基础.

■相关报道

目前肝细胞低温保存分为两大类: (1)-196 °C深低温冻存; (2)4 °C常规低温保存。为提高低温保存复温后肝细胞的存活率, 学者们对多种冻存保护剂进行了研究, 同时发现细胞凋亡是除了坏死之外, 低温保存肝细胞死亡的一个重要途径。

温度不仅最大限度的降低了细胞的代谢活性, 而且又避免了冰晶形成引起的冻存损伤, 理论上是一种理想的肝细胞低温保存方式。目前, 有关零下非结冰低温保存肝细胞的文献报道较少: Yoshida等^[28]用UW液保存分离的鼠肝组织, 试验分为两组: 4 °C组(对照组), -0.8 °C组(零下非结冰组)。低温保存24、48、72及96 h后60 min内测定肝组织的ATP(三磷酸腺苷)含量、TAN(总腺苷)含量、LDH释放(乳酸脱氢酶)及DNA片段释放。结果显示各时间点-0.8 °C组ATP含量和TAN含量明显高于零下非结冰组; -0.8 °C组各时间点LDH释放(乳酸脱氢酶)明显低于零下非结冰组; 但是各时间点两组DNA片段释放无明显差异。Matsuda等^[29]用UW液保存通过酶消化法分离的鼠肝细胞, 实验分为3组: -4 °C组(subzero non-freezing group), 0 °C组(zero nonfreezing group), 4 °C组(conventional temperature group)。低温保存24和48 h后测定细胞存活率、ATP含量、LDH释放、乳酸释放和尿素合成能力。结果显示: 各时间点-4 °C组肝细胞存活率及细胞内ATP含量明显优于4 °C组(低温保存48 h: 细胞存活率分别为60.3%±5.4%, 46.0%±6.0%, $P<0.01$; 细胞内ATP含量: $4.47\pm2.11\ \mu\text{g}/10^6$, $1.82\pm1.09\ \mu\text{g}/10^6$, $P<0.05$); -4 °C组肝细胞LDH释放及乳酸释放较0 °C组和4 °C组明显降低; 各组间尿素合成能力无明显差异。Rodriguez等^[30]用UW液保存鼠肝细胞, 试验分为3组: 0 °C组(zero nonfreezing group), -4 °C组(subzero nonfreezing group), -4 °C加非渗透性保护剂丁二醇组。低温保存120 h后, -4 °C组肝细胞存活率及细胞内ATP含量较0 °C组明显增加, LDH释放及乳酸产物明显降低。-4 °C UW液保存肝细胞的基础上添加非渗透性保护剂丁二醇组, 可进一步提高细胞的存活率及细胞内ATP含量。

3 -80 °C或者-196 °C(液氮)深低温冻存

目前-80 °C或者-196 °C(液氮)深低温冻存复苏后细胞存活率在80%左右, 如何优化冻存方法, 提高复苏后细胞存活率及保持细胞功能, 减轻细胞冻存损伤, 满足BLASS的需要是目前研究的重点^[31]。影响深低温冻存肝细胞活性及存活率的因素很多, 包括冻存液, 冻存保护剂, 降温速率, 复苏方式, 保存形式及细胞密度, 冻存前孵育, 复温后培养等^[32]。

3.1 冻存液 冻存液(cryopreservation solution)对肝细胞的冻存效果影响很大^[33], 合适的冻存液有

助于帮助细胞抵抗渗透压的变化和减少冻存过程中细胞内冰晶的形成, 从而提高冻存后肝细胞的存活率。目前比较常用的深低温冻存液有DMEM, William E, RPMI 1640, F12, L-15等培养基^[34-36]。不同保存液的成分及浓度各不相同, 冻存肝细胞的效果也不尽相同: Baldini等^[37]用含50 mL/L FBS, 100 mL/L DMSO, 125 U/L胰岛素和10 ng/L胰高血糖素的DMEM液, 液氮保存猪肝细胞长达数月, 细胞功能无明显影响。Kunieda等^[38]分别用UW液+DMSO及DMEM液+DMSO于-80 °C冻存原代猪肝细胞3 d、5 d和5 mo, 结果UW液+DMSO组的肝细胞存活率、贴壁率、尿素合成能力及LDH释放量均优于DMEM液+DMSO组。Illouz等^[39]用ETK液±DMSO及UW液±DMSO液氮冻存原代人肝细胞, 四组间细胞存活率无统计学差异。但是, UW液+DMSO组的肝细胞存活率低于UW液+其他保护剂, 而ETK液+DMSO组的肝细胞存活率同ETK液+其他保护剂无差异。提示ETK液是一种有效的细胞冻存液, 其保存效果同UW液无差异, 加入冻存保护剂可进一步减轻肝细胞冻存损伤。

3.2 冻存保护剂 目前冻存保护剂(cryoprotective agent, CPA)主要分为两大类: 渗透性保护剂, 如二甲基亚砜(dimethyl sulfoxide, DMSO); 非渗透性保护剂, 如羟乙基淀粉(hydroxyethyl starch, HES), 低分子右旋糖酐(low molecular weight dextran, D-40), 蔗糖(sucrose), 聚乙烯吡咯烷酮(polyvinylpyrrolidone, PVP)等。DMSO是一种渗透性保护剂, 可以自由通过细胞膜, 一方面可以降低细胞内冰点, 减少细胞内冰晶形成所致的“冰晶性损伤”, 另一方面可以防止冻存过程中细胞外渗透压升高引起的细胞过度脱水所致的“渗透性休克性损伤”^[40]。DMSO是肝细胞深低温冻存最常用的渗透性保护剂, 常用浓度为5%-20%, DMSO浓度过低不能起保护作用, 浓度过高存在严重的毒性作用(DMSO可与蛋白质疏水基团发生作用, 导致蛋白质变性)^[41]。羟乙基淀粉和低分子右旋糖酐是比较常用的非渗透性保护剂, 具体保护机制目前仍然不明确, 可能是与冻存过程中减少细胞内冰晶形成及复苏时减轻由于渗透压改变引起的细胞肿胀有关。非渗透性保护剂HES同渗透性保护剂DMSO在肝细胞低温冻存中联合应用优于单用DMSO且可以降低DMSO浓度, 减轻DMSO的肝细胞毒性作用^[42]。另外, 试验发现在DMSO的基础上添加膜稳定剂^[43,44](如海藻糖, 牛磺酸等)、生物抗氧

化剂^[45,46](如过氧化氢酶, 氨乙酰半胱氨酸, 还原性谷胱甘肽, 胎牛血清等), 凋亡抑制剂^[47,48](如Z-DEVD-FMK, IDN-1965等)可以提高冻存肝细胞存活率, 减轻生物性细胞损伤。

3.3 降温速率 降温速率(freezing rate)是影响细胞深低温冻存复苏后存活率的一项关键因素。如果降温过快, 降温过程中随着细胞外冰晶的形成, 细胞内水分渗出, 可导致致死性的细胞脱水性“溶质性损伤”。如果降温过慢, 降温过程中细胞内不及外渗便在细胞内形成小冰晶, 可引起致死性的“冰晶性损伤”。不同类型的细胞最佳冻存速率不同^[49]。大量研究发现, 肝细胞降温速率曲线呈倒置的“U”型, 最佳降温速率区间-1℃至-5℃/min可以获得较高的细胞存活率及功能恢复^[50]。Hengstler等^[51]通过电脑程控降温: 以-1.8℃/min(降10 min)降至0℃(保持8 min), 继以-2℃/min(降4 min)降至-8℃, -8℃在0.1 min内迅速降至-28℃, 后以-2.5℃/min(降2 min), 又以+2.5℃/min(升2 min), 再以-2℃/min(降16 min)及-10℃/min(降4 min)降至-100℃, 最后至液氮-196℃保存, 肝细胞复苏后存活率可达89%。Neyzen等^[49]研究发现, 在冻存的初级阶段(4℃至-10℃)降温速率非常重要, 此阶段偏离-0.8℃至-1.1℃/min, 将严重影响肝细胞冻存后存活率和细胞功能。周霖等^[52]比较了电脑程控降温(Hengstler法)和梯度降温(4℃/-85℃/-196℃)大规模深低温冻存猪肝细胞1 mo后的差别, 结果两组细胞存活率分别为76.3%±1.9%, 72.4%±1.5%, $P>0.05$; 尿素合成、白蛋白分泌、葡萄糖合成及利多卡因转化功能无统计学差异。

3.4 复苏方式 复苏是指将低温冻存的肝细胞恢复至常温的过程。目前常用的是快速复温法, 目的是使细胞迅速通过变相易损期(0℃至-5℃), 以防止细胞内重结晶形成及渗透性肿胀, 提高肝细胞的存活率和细胞功能^[53]。一般用37℃水浴快速复温, 有人认为40℃复温速度更快。Baldini等^[37]在冷冻的肝细胞放入40℃水浴中快速复温(10 s内), 然后移去上清。Wu等^[54]用电脑程控降温法于-196℃保存肝细胞一段时间, 置于37℃水浴震荡1-2 min, 复苏后电镜观察细胞活力和细胞器形态结构, 经比较冻存细胞同未冻存细胞无明显差别, 把上述两种肝细胞放入生物人工肝反应器, 治疗急性肝衰竭模型猪取得良好的效果。Loven等^[55]用梯度降温冻存肝细胞1-2 d后, 取出肝细胞于37℃水浴震荡1-2 min并用William E液洗涤, 复

苏后冻存肝细胞的存活率及I相、II相代谢活性同未冻存细胞无差异。

3.5 保存形式及细胞密度 目前大多数是将肝细胞悬液直接冻存, 其他肝细胞冻存的形式有单层胶原、三明治夹心、微囊化和微载体冻存等。Khalil等^[56]将肝细胞埋入含胶原基质, -196℃保存肝细胞2 mo, 复温后细胞保持良好的生物学功能。Sugimachi^[34]通过胶原酶消化猪肝脏获得肝细胞进行三明治夹心培养, 3 d后采用程控法降温至-70℃后于冰箱保存3 h或者14 d, 复苏后细胞形态及功能均保持良好。微囊化及微载体是目前应用较多的肝细胞大规模培养方法, 应用于冻存效果较好。Canaple等^[57]于-196℃保存微囊化的肝细胞发现, 冻存30 d和70 d的微囊化肝细胞的白蛋白分泌量相当于包裹的新鲜肝细胞, 冻存4 mo的微囊化肝细胞仍然保持较好的白蛋白合成及转铁蛋白分泌能力。最成功的是Watanabe等^[58]将微载体培养的猪肝细胞电脑程控降温至-70℃保存, 复温后置于中空纤维管中行人工肝支持治疗, 疗效确切。细胞密度对细胞冻存效果也有一定的影响, Madan等^[59]总结既往大量研究发现, 肝细胞以 10^6 - 10^7 /mL冻存比较合适。

3.6 冻存前孵育和复苏后培养 细胞膜的完整性对冻存后细胞质量有重要的影响, 而冻存前孵育可以有效地修复损伤的细胞膜, 使肝细胞对冻存和复温的抵抗力明显增强^[60]。Terry等^[61]进一步研究发现: 含100-300 mmol/L葡萄糖的培养基预孵育可以明显细胞冻存复苏后鼠肝细胞的存活率、贴壁能力及减少LDH的释放; 100-300 mmol/L果糖的培养基预孵育可以明显细胞冻存复苏后鼠肝细胞的存活率、贴壁能力; 含0.5-5 mmol/L α -脂酸的培养基预孵育可以明显细胞冻存复苏后鼠肝细胞的存活率、贴壁能力和人白蛋白分泌能力; 预孵育温度在4℃的效果优于37℃。复苏后培养同样可以减轻细胞冻存损伤, 促进细胞功能恢复。

4 细胞凋亡与肝细胞低温保存损伤

有关肝细胞在低温保存过程中死亡的机制尚未完全阐明, 除了冰晶形成、渗透压改变如pH值外, Fu等^[62]发现肝细胞冻存复苏后, 采用膜联蛋白相关的TUNEL染色、流式细胞仪和DNA梯度分析, 冻存复苏后细胞凋亡指数较新鲜细胞明显增加。Kao等^[12]用UW液4℃低温保存鼠肝细胞24 h后, 发现凋亡细胞明显增加, 同时伴有凋

■创新盘点

本文详细论述了目前肝细胞低温保存的方法及其优缺点, 同时对低温保存肝细胞的效果以及同细胞凋亡的关系也做了一些探讨。

■应用要点

零下非结冰不同于深低温冻存和常规低温保存,是一种比较新的有效的肝细胞低温保存方法。

亡相关蛋白热休克蛋白的改变。因此,细胞凋亡是除了坏死之外冻存肝细胞死亡的另一个重要的途径。

细胞凋亡有两个独立的途径:死亡受体途径^[63,64](外源性途径即由Fas/CD95和TNFR介导的通路);线粒体途径^[65,66](内源性途径即Cyt.C, caspase-2, 3, 9等介导的通路)。关于冻存引起肝细胞凋亡的具体机制目前仍然不明确,但也取得了一定的进展: Fu等^[67]将肝细胞培养温度由37℃降至32℃后培养12 h,使用流式细胞仪及DNA梯度分析测定Fas受体介导的细胞凋亡作用,结果显示:低温保存抑制了Fas受体介导的细胞凋亡及细胞色素C的释放。Matsushita等^[68]在培养液基础上增加25 mmol/L的广谱细胞凋亡蛋白酶抑制剂IDN-1965液氮冻存肝细胞2 wk, TUNEL染色测定细胞凋亡指数,同时测定caspase-3活性及细胞色素C的释放。结果显示凋亡蛋白酶抑制剂IDN-1965明显提高了冻存复苏后肝细胞存活率,抑制了细胞凋亡、caspase-3活性及细胞色素C的释放。Fujita等^[69]进一步研究发现,冻存肝细胞复苏过程中伴有氧化应激活性氧产生和凋亡酶活性增加,冻存前添加抗氧化剂及凋亡酶活性抑制剂可以提高冻存复苏后细胞存活率,抑制细胞凋亡。相反Vanhulle^[48]发现冻存前添加caspase-3抑制剂Z-DEVD-FMK(2.5 μg)未发现肝细胞存活率增加,但是添加caspase-3上游靶点(GSK-3)抑制剂氯化锂,则明显提高了冻存复苏后肝细胞的存活率,抑制了细胞凋亡。因此线粒体途径相关的细胞凋亡在细胞冻存损伤中发挥着重要的作用^[70]。

5 结论

肝细胞低温保存目前分为4℃低温保存或零下非结冰保存和-80℃或-196℃深低温冻存两大类。4℃低温保存肝细胞具有操作简单,随取随用,便于运输和推广等优点,但是保存时间较短(不易超过48 h)。-80℃或-196℃深低温冻存是比较成熟的肝细胞长期低温保存方法,但也在操作比较复杂,程控降温成本高且不便运输等缺点。零下非结冰一方面可以使代谢降至最低,不同于常规低温保存(4℃),另一方面可以避免“细胞内冰晶”、“渗透性休克”等深低温冻存损伤,可能是比较理想的肝细胞低温保存方法。有关肝细胞在低温保存过程中死亡的机制尚未完全阐明,但大量研究发现细胞凋亡可能是除了坏死之外冻存肝细胞死亡的另一个重

要的途径,而线粒体途径相关的细胞凋亡可能在细胞冻存损伤中发挥着重要的作用。总之,探索出一种实用的肝细胞低温保存方法,建立一个随时可用(ready to use)的“血库样”肝细胞库可有效的促进BLASS的发展。

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同行评价

本文综述内容系统全面, 条理清楚, 语言流畅, 引文详实, 文章内容对于相关领域研究人员有较高的参考价值, 是一篇较好的综述。

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