最小体积玻璃化冻存技术在生物医学领域的进展和应用

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摘要 细胞和组织的生物保存在辅助生殖医学、再生医学和器官移植等领域均具有至关重要的作用, 对公共健康具有广泛影响. 生物保存方法主要包括传统的程序化慢冻法(slow freezing)和玻璃化冻存技术(vitrification). 玻璃化冻存技术可通过降低或避免结晶的产生而显著提高细胞解冻后的活性和功能. 但玻璃化冻存技术通常采用较高浓度的冷冻保护剂(cryoprotectant agent, CPA), 会对细胞和组织产生一定的毒性和渗透压力. 研究显示, 通过减小玻璃化冻存中样品的体积($<1 \mu\text{L}$)可显著提高冷却速率, 从而降低玻璃化冻存所需的CPA浓度. 而微纳技术的迅速发展使得皮升至纳升量级样本的操作成为可能. 因此, 微纳技术与低温学的有机结合将为未来生物保存技术的发展提供巨大的潜能.

关键词 冷冻保存, 冷冻保护剂, 微液滴玻璃化冻存, 最小体积玻璃化冻存

生物冷冻保存是指在超低温条件下对细胞和组织实现长期保存. 冷冻保存在现代医学中已被广泛使用, 如对人体生殖能力的保存^[1~4]、移植细胞或组织的保存^[5~7]以及对组织再生和细胞治疗中的细胞或组织储存^[8~11]等. 迄今为止, 研究人员已在冷冻保存技术的改进以及多种细胞/组织(如胚胎^[12,13]、肝细胞^[14,15]、干细胞^[16,17]和血管^[18~20])冻存方法的优化等方面做出了巨大努力.

在冷冻保存过程中, 细胞和组织的温度降至零度以下, 其生物活性急剧减慢甚至完全停止. 理想情况下, 冻存之后复苏的细胞可恢复其冻存前的生物活性.

为此, 冻存过程中使用的冷冻保护剂(cryoprotectant agent, CPA)起着至关重要的作用. CPA不仅可避免或减少降温过程中冰晶形成, 且作为一种渗透压缓冲剂^[21~24]可阻止冻存过程中较大电解质浓度梯度的产生, 最终对细胞活性起到保护作用^[25~27]. 此外, CPA还可以稳定细胞膜, 保持生物大分子的天然形态^[27~29]. 目前使用较广泛的CPA主要包括二甲基亚砜(dimethyl sulfoxide, DMSO)、1,2-丙二醇(1, 2-propanediol, PROH)、乙二醇(ethylene glycol, EG)、蔗糖、海藻糖和甘露糖醇^[30,31].

目前, 程序化慢冻和玻璃化冻存两种方法在实验

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室和临床均有广泛使用^[32,33], 两种方法的基本冷冻过程示意如图1所示. 程序化慢冻建立于20世纪70年代初, 主要是通过控制生物样品的冷冻速率来减少细胞内冰晶的形成, 从而降低对细胞膜^[34]和细胞骨架^[35]的损伤. 慢冻法使用的CPA浓度通常相对较低(如1.5 mol/L)^[30,36], 已成为许多细胞组织冻存^[37-41]的标准方法. 程序化慢冻法中, 首先需要将细胞缓慢降温, 即控制细胞的降温速率保持在 $-3^{\circ}\text{C}/\text{min}$ 以内将细胞降温至结晶温度以下(一般选择 -80°C 或液氮温度 -196°C), 之后将冷冻的细胞转移至液氮中长期储存. 复温时, 需要将细胞以尽可能快的速度升温, 目前以水浴中对流复温为主要实现方法^[42]. 但是细胞在缓慢冷冻过程中很难完全避免因冰晶形成、极高渗透压和脱水所造成的损伤^[43].

玻璃化冻存技术作为另一种冷冻保存方法, 因可有效地阻止冰晶形成和增加解冻液离子强度而显示出更好的冻存效率^[32,44-50]. 玻璃化是指液相不发生结晶而直接转化成玻璃态固体的现象. 相比于程序化慢冻, 玻璃化冻存需要更高浓度的CPA(6~8 mol/L)和超高冷却速率($>2000^{\circ}\text{C}/\text{min}$). 超高冷却速率可通过将细胞或组织直接浸入液氮(-196°C)或液氮蒸气(-160°C)来实现^[50]. 具体来说, 首先将细胞或组织进行CPA的梯度加载, 一般分为三到五步, 最终达到需要的CPA浓度. 之后, 将充分加载CPA的细胞或组织通过载体或直接浸入液氮中, 以极高的降温速率使得细胞或组织快速通过相变温度区, 最大程度地减小结晶. 接下来, 将冻存的样品转入液氮罐长期储存. 复温时, 大部分玻璃化冻存与程序慢冻法使用相同的水浴对流传热复温^[42]. 在玻璃化冻存中, 使用高浓度CPA主要通过降低凝固点和提高介质黏度来促进玻璃化的实现. 然而, 高浓度CPA的使用可能对细胞产生渗透性休克效应和化学毒性, 导致细胞骨架变形^[51-53]、纺锤体解体 and 染色体扩散^[54].

目前, 很多研究专注于开发最小样品体积(minimum volume, $<1\ \mu\text{L}$)玻璃化冻存的技术方法, 期待通过进一步有效地提高冷却速率, 最大程度地降低冻存中所需的CPA浓度, 从而减小对细胞造成的毒性^[55,56]. 当前已开发的相关方法包括开口式拉长麦管(open pulled straw, OPS)^[57,58]、玻璃毛细管^[58,59]、电子显微镜铜网(electron microscopic grid, EMG)^[58,60,61]、冷冻环TM(cryoloop)^[58,62,63]、冷冻帽(cryotop)^[64,65]、凝胶上

图1 两种主要冷冻方法的降温复温过程示意图. 玻璃化冷冻(红色), 程序化慢速冷冻(黑色)

Figure 1 Schematic diagram of the cooling and rewarming process of two main cryopreservation methods: vitrification (red) and programmed slow freezing (black)

样管(gel-loading tip, GLT)^[7,66,67]和基于液滴的玻璃化冻存方法^[14,68-73]. 虽然研究已证实最小样本体积的玻璃化冻存方法对冷冻生物学的有效性, 但目前这些方法对操作技术要求苛刻, 阻碍了其在临床的广泛应用.

近年来, 微纳技术的迅速发展实现了对纳升体积样品准确操控的可能, 有助于对冻存方法的改进. 例如, 将微流体技术用于CPA加载和去除以降低对细胞的渗透性休克^[42,74], 将石英毛细管微通道作为载体用于玻璃化冻存提高冷却速率^[16,75], 以及将高通量的液滴喷射系统用于细胞玻璃化冻存^[14,68,70,76,77]. 液滴生成的方法还有很多, 如声学方法^[70]、喷墨打印方法^[78]和微流控方法^[79]. 目前这些方法都已经被成功应用于细胞的冷冻保存, 在不影响细胞活性的前提下, 通过先进的机械化装置展现出巨大的高通量冻存优势. 微纳技术的巨大进步使其已显现出改变现代医学的巨大潜力, 为诊断、手术和治疗提供了全新的思路^[80-83]. 将这些技术运用到生物保存, 可以使最小体积玻璃化冻存方法被广泛用于生物保存领域. 本文综述了现有的玻璃化冻存方法, 并对这些技术在细胞、组织以及工程化组织保存中的潜在应用与局限性进行了着重剖析.

1 玻璃化冻存

19世纪80年代, 研究者就初次描述并研究了玻璃化现象^[84], 但直到1937年玻璃化才被Luyet^[85,86]应用于冷冻保存. 在玻璃化过程中, 当温度迅速通过相变温度时, 液体转变成黏稠的玻璃状固体, 而液体分子天然无

序的状态被保留. 在这个过程中, 所有基于分子扩散的化学反应都被终止, 随后生物的新陈代谢达到静止^[87,88]. 与此同时, 细胞和组织避免了因冰晶形成而可能造成的重大损伤.

玻璃化冻存通常需要使用高浓度CPA(渗透性和/或非渗透性, 6~8 mol/L). 非渗透性CPA, 如糖类(如蔗糖、海藻糖)和聚合物(如聚乙烯吡咯烷酮和聚乙烯醇), 不仅可以降低所需渗透性CPA的总浓度, 而且可以促进玻璃化转变过程^[89~91]. 另外, 也可通过分步加入CPA(即增加一个或多个低浓度CPA预处理步骤)的方法减小CPA对细胞或组织造成的渗透性休克和毒性效应^[32].

提高冷却速率可使细胞加速通过相变温度, 从而减轻或避免了降温过程对细胞膜造成的冷冻伤害^[92]. 此外, 在冷冻和复苏过程中, 提高降温 and 升温速率均可降低玻璃化所需的CPA浓度, 从而减轻毒性和渗透压对细胞产生的影响^[93]. 为解决这些问题, 研究人员开发了最小化样本体积的玻璃化冻存载体系统以期最大程度地提高降温 and 升温的速率. 然而, 大多数最小体积玻璃化系统的通量较低, 虽然已应用于冷冻保存临床收集量小的生殖细胞(如卵母细胞), 但是对容量较大的细胞或组织(如全血、血液细胞和干细胞)进行玻璃化冻存仍存在很大的局限性.

2 基于载体的玻璃化冻存系统

基于最小样品体积的冻存方法的需求^[89,94,95], 研究人员迄今已开发出多种用于玻璃化冻存的载体系统(表1)^[96~105]. 传统的塑料吸管是最早用于胚胎玻璃化冻存的载体^[12], 可实现2500°C/min的降温速率和1300°C/min的升温速率. 但该载体样本体积较大(45 µL)^[106], 不仅难以实现超高冷却速率, 而且需要使用高浓度的CPA方可实现玻璃化. 为进一步减小样品体积, 研究人员研发了一系列不同的玻璃化冻存载体, 如开口式拉长麦管(~1 µL)^[57]和半吸管系统(~0.3 µL)(图2A)^[96]. 这些载体因提高了玻璃化冻存的降温 and 升温速率(OPS: 16700和13900°C/min; 半吸管系统: >20000°C/min), 显著降低了对细胞或组织的冷冻损伤.

另有研究者提出将玻璃毛细管用作玻璃化载体, 通过改变玻璃毛细管的直径控制降温 and 升温的速率. 例如, 直径2 mm与440 µm的毛细管分别可以实现2000和12000°C/min的降温速率, 以及5000和62000°C/min的升温速率^[59]. 这种方法已被用于牛卵母细胞^[59]和胚胎的玻璃化冻存^[107]. 其孵化率与OPS技术相当(玻璃毛细管: 19%; OPS: 27%; 对照组: 80%), 降温 and 升温速率无显著差异. 为避免因直接接触液氮可能导致的

表 1 总结玻璃化载体系统

Table 1 Summary of vitrification carrier systems

玻璃化载体	样本体积	降温速率 (°C/min)	升温速率 (°C/min)	临床应用	优点/缺陷	参考文献
传统吸管	0.25 mL	2500	1300	是	样本制备简单/降温速率不足	[12]
开口式拉长麦管(OPS)	~1 µL	16700	13900	否	易加载, 高降温速率/潜在污染	[57]
半吸管	~0.3 µL	>20000	N/A	否	降温速率高/污染的风险	[96,105]
电子显微镜铜网(EMG)	<1 µL	150000	150000		极高降温速率/操作难度高, 设备需求	[60,61]
冷冻环(cryoloop)	0.7~1.0 mm(内直径)	700000	N/A	是	极高降温速率/操作难度较高	[62]
冷冻帽(cryotop)	<0.1 µL	23000	42000	否	极高降温速率/操作复杂耗时	[64]
冷冻头(cryotip)	N/A	12000	24000	否	操作相对简便/降温速率不足	[98]
凝胶上样管(GLT)	0.6~0.7 µL	—	—	否	操作简便/样本制备复杂	[66,67]
石英微毛细管	~3 mm(内直径)	250000	N/A	否	高降温速率/技术要求较高, 材料易碎	[16,75,97]
最小液滴尺寸	0.1~0.5 µL	130000	N/A	否	高降温速率, 操作简便/不适用于大体积样本	[76,99~101]
液滴喷射	~1 nL	N/A	N/A	否	高降温速率, 高通量/不适用于大体积样本	[14,68,70,102~104]

图2 最小体积玻璃化载体(网络版彩图)。A: 半吸管, 插入0.5 mL的吸管来储存; B: cryoloop^[67]; C: cryotop^[67]; D: 凝胶上样管^[67]

Figure 2 Minimum volume vitrification carriers (color online). A: Hemi-straw, which was inserted into a 0.5 mL straw for storage; B: Cryoloop^[67]; C: Cryotop^[67]; D: gel loading tip^[67]

交叉污染, 进一步开发了闭口式拉长麦管. 将其用于小鼠(*Mus musculus*)卵母细胞的玻璃化冻存, 细胞成活率(79%)和纺锤体形态^[108]均优于OPS方法(63%).

由于石英微毛细管具有更高的热传递速率(冷却速率达250000°C/min), 近年来被用于玻璃化冻存, 获得了优于玻璃毛细管和塑料毛细管的冻存效果^[16,75,97]. 薄壁石英微毛细管(外径0.2 mm, 壁厚0.01 mm)可以实现高达250000°C/min的超快冷却速率^[16], 进而降低玻璃化所需的CPA浓度水平. 最近也有实验证明, 使用石英微毛细管进行玻璃化冻存后的细胞存活率(小鼠胚胎干细胞^[16]和小鼠卵母细胞^[109])高于传统方法, 相比于传统玻璃化6 mol/L以上的CPA浓度, 在大大降低浓度之后(分别为2.5与2 mol/L)仍然保持了70%以上的干细胞存活以及90%以上的小鼠卵母细胞的存活率.

此外, 电子显微镜铜网也被用于玻璃化冻存体积小小于1 μL的样本, 其理论降温 and 升温速率接近150000°C/min^[60,61]. 将其用于牛囊胚的玻璃化冻存^[110], 得到的胚胎成活率与未冻存的囊胚无显著差异, 孵化率(68%)高于用塑料吸管载体玻璃化后的孵化率(53%). 将简化的EMG方法用于玻璃化冻存小鼠卵母细胞, 获得的成活率和受精率分别为90.0%和56.7%^[111].

冷冻环是近年来临床用于玻璃化冻存胚胎及卵母

细胞最常采用的方法之一. 这一方法使用的载体是在不锈钢细管上安装一个小的尼龙环(直径0.7~1.0 mm), 另一端插入冻存管的盖子(图2B), 将样本放置在冻存环上的CPA薄膜上, 再封存进冻存管, 随后注入液氮. 由于样本体积小(<1 μL), 无固体支撑, 可实现的冷却速率高达700000°C/min^[64,112]. 实验证实, 采用这种方法冻存后的细胞存活率高于管状载体系统^[62].

cryotop是最近研发的另一种玻璃化冻存方法, 即将聚丙烯条带固定于一个带有保护帽的支撑物上(图2C). 具体使用方法如下: 先使用玻璃毛细管将样本(<0.1 μL)置于条带上, 然后移除多余的CPA溶液, 仅留下足够覆盖细胞的一个薄层用于冷冻. 这种最小样本体积玻璃化冻存的方法可将降温 and 升温速率分别提高到23000和42100°C/min^[64]. 因此, 利用该体系对牛卵母细胞进行玻璃化冻存复苏后获得的受精率和囊胚发育相比于塑料吸管和OPS法更高, 典型纺锤体及染色体形态保持更好^[98,113].

相比之下, cryotip方法是将细胞加载到一根微毛细管中后, 将两端热封闭来形成一个闭合的系统. 虽然这种载体系统的降温 and 升温速率(分别为12000及24000°C/min)低于cryotop方法, 但是使用两种方法玻璃化冻存后的人囊胚成活率、妊娠率及出生率均无显著差异^[114].

在GLT玻璃化冻存方法中, 样本体积由移液枪枪头的尺寸(如0.6~0.7 μL)决定(图2D)^[66,67]. 实验结果显示, 将其用于牛胚胎及兔受精卵的玻璃化冻存, 得到的分裂率低于cryotop技术^[67], 这可能是由于实验中使用的CPA浓度较高以及GLT方法实现的冷冻速率不足导致的.

2.1 基于载体玻璃化冻存系统的应用

在现代临床医学中, 冷冻保存为保存和延长人类的生育能力提供了一种可靠的选择^[115,116]. 目前, 胚胎冷冻保存已成为临床试管婴儿(*in vitro fertilization*, IVF)的常规程序^[117]. 但由于部分患者对胚胎冷冻存有道德或宗教因素的顾虑, 冷冻保存卵母细胞成为他们更好的选择方案. 另外, 卵细胞冻存技术对于女性癌症患者具有更特别的意义, 为其在化疗或放射治疗前提供了保留生育能力的机会. 然而, 经缓慢冷冻或传统玻璃化冷冻方法冻存的卵母细胞, 植入体内后得到的出生率(<5%)较低, 不足以在临床上广泛应用^[118,119].

其主要原因是由于冻存过程中卵母细胞受到了不可逆损害,如透明带硬化和染色体位移^[115]。玻璃化冻存的突出优势吸引了大量生育保存领域的研究者在该领域开展广泛研究,尤其是研究其在提高卵母细胞存活、妊娠和出生率方面的潜力。目前,基于载体的玻璃化冻存由于其已经被发展成为较成熟的方法体系,并实现了高速的玻璃化冷冻,因而其在生殖保存中被广泛应用。

(1) 卵母细胞的玻璃化冻存. 牛、小鼠和人的卵母细胞被用于各种玻璃化载体系统的研究^[106,108,120]。Vajta等人^[57]使用OPS方法冷冻保存牛卵母细胞,复苏后IVF达到50%分裂率,其中25%在体外受精后发育成囊胚,在卵母细胞和囊胚阶段植入均成功实现了妊娠。使用EMG和cryoloop方法可以得到与新鲜卵母细胞相似的存活率,但IVF后分裂率(EMG: 30%, cryoloop: 26%, 新鲜的卵母细胞: 50~70%)和胚泡发育率(EMG: 15%, 新鲜的卵母细胞: 40%)显著降低^[61,121]。Morató等人^[113]比较了cryotop和OPS玻璃化冻存方法对牛卵母细胞的冻存效果,结果表明使用Cryotop比OPS方法获得了更高的分裂率(46%比32%)和囊胚率(5%比无胚泡),以及更高的纺锤体和染色体结构的保持率(60%比38%)。使用封闭式拉长麦管玻璃化冻存小鼠卵母细胞获得的存活率和纺锤体形态保持率(79%和93%),高于传统吸管(77%和79%)、显微镜网格(39%和95%)和OPS(63%和89%)等玻璃化冷冻载体^[108]。当使用EMG法玻璃化冷冻小鼠卵母细胞后,能明显观察到孵化率在降低(39%, 新鲜的卵母细胞: 85%)^[111]。实验证明Cryoloop方法对于小鼠卵母细胞是一种成功的玻璃化冻存方法,其存活率、受精率、囊胚发育率以及纺锤体和染色体形态保持都与新鲜的卵母细胞相当^[122,123]。用石英微毛细管作为载体玻璃化小鼠卵母细胞时,可得到相似的存活率、受精率和囊胚发育率。但是,在该方法中,将玻璃化冻存后的卵母细胞培育出囊胚转移到代孕母亲体内时,比新鲜的卵母细胞得到的出生率低(19%)^[109]。

对于人卵母细胞的情况,OPS玻璃化冻存方法实现了高存活率和受精率(68.6%和71.7%),然而妊娠率(15.3%)和出生率(4.6%)仍然相对较低^[124]。cryoloop和半吸管系统也对人卵母细胞进行了玻璃化测试^[125],观察到了相似的存活率(分别为81%和85%)。另一项使用cryoloop玻璃化冻存人卵母细胞的研究^[126]报告了相似

的结果,即可观的受精率(72%)和较低的妊娠率(5%~6%),以及较低的纺锤体和染色体正常减数分裂率^[127]。另一方面,有研究指出,使用cryotop方法对人卵母玻璃化冻存,得到了令人鼓舞的结果,即具有更高的存活率(90%~99%)、受精率(81%~93%)和妊娠率(32%~41%)^[64,115,128,129]。然而,仍然需要进一步改进,以获得更高的成功率。

(2) 胚胎的玻璃化冻存. 早期胚胎. 研究证实,使用0.25 mL常规吸管对牛早期胚胎进行玻璃化冷冻,解冻后胚胎的分裂率低于5%^[130]。但使用OPS和封闭式拉长麦管时进行玻璃化冻存可实现更高的存活率(OPS: 88%; 封闭式拉长麦管: 85%)和保持更好的正常细胞形态^[131]。使用半吸管玻璃化冷冻的人类早期胚胎表现出比cryoloop方法更高的发育潜力(38%比29%)^[125]。然而,在另一项研究中,用cryoloop方法玻璃化冷冻人胚胎,并最后培育至胎儿出生,得到了较高的存活率(85%)和妊娠率(44%)^[132]。与上述方法相比,用cryotop方法玻璃化冷冻人早期胚胎也得到了成功的结果,以及更高的存活率(100%)、分裂率(93%)和囊胚率(52%)^[64]。

囊胚. OPS和EMG玻璃化方法都已用于牛囊胚的冷冻保存。采用OPS法时,虽然复苏后观察到的成活率较低,但是将囊胚植入母体后,成功实现了妊娠^[57]。当EMG方法被用于牛囊胚玻璃化,得到囊胚的存活率和未经玻璃化冻存的无明显差异。然而玻璃化冷冻48 h后,存活率仅为73%,低于对照组的84%^[110]。

采用OPS法冷冻小鼠囊胚可以获得比传统塑料吸管法(69%)更高的存活率(82%)^[133],这可归因于OPS方法降低了玻璃化冻存样品体积。另一方面,将采用封闭式拉伸细管方法玻璃化冷冻扩张期囊胚移植到假孕小鼠子宫,实现了较高的妊娠率和出生率(70%和45%),高于OPS(20%和8%)和传统吸管(10%和5%)^[134]。将cryoloop方法冻存后的小鼠囊胚移植到假孕受体中,也得到了高着床率(80%)和胎儿发育率(55%),与未经玻璃化的细胞相当^[62]。

许多玻璃化冻存方法已经用于人囊胚的冷冻保存,包括EMG,半吸管, cryoloop和cryotop等。EMG法得到的高存活率(83%~90%)、孵化率(49%)、着床率(29%)和妊娠率(34%~48%)^[135,136],高于使用cryoloop^[62,137~140]和cryotop获得的结果^[64,141](存活率和妊娠率分别为90%和53%)。相比之下,半吸管方法得到的结

果并不理想, 存活率、孵化率(19%)、着床率(13%)和妊娠率(19%)均比较低^[96]。

(3) 干细胞的玻璃化冻存. 在修复或替换基因疾病、损伤或先天性缺陷丧失功能的组织/器官方面, 再生医学表现出极大的潜力^[142]。目前正在开发的技术包括干细胞移植、患者自身的干细胞的调控以及使用支架材料提供生化信号以刺激干细胞开始分化等。因此, 实现干细胞的高效保存是未来的再生疗法中至关重要的部分。

对于干细胞的生物保存目前仍存在许多挑战, 这主要是因为细胞分裂过程中保存细胞未分化表型具有一定难度。因此, 为临床使用提供持久的、高质量的细胞来源成为干细胞临床应用的关键^[143]。冷冻保存技术为科研和临床使用干细胞的储存提供了经济、快捷、高效的解决方案。玻璃化冻存技术已被成功用于多种干细胞的保存, 如胚胎干细胞^[144]、间充质干细胞^[145]和造血干细胞^[146]。当前的干细胞玻璃化冻存方法主要将冻存液阶梯式的加入方式与最小体积化冻存结合, 以最大化降低对细胞的渗透性损伤。解冻后, 通过逐步稀释将冻存液彻底去除。利用这种方法冻存复苏后的干细胞可保持80%以上的细胞活性^[147]。

cryotip和石英微毛细管方法都已用于小鼠胚胎干细胞的玻璃化冻存研究。采用cryotip方法, 解冻后有的细胞保持了高于99%的细胞存活率^[148]。使用石英微毛细管方法时, 小鼠胚胎干细胞被成功冻存, 且复苏后细胞未分化表型未受到影响^[16]。相比之下, 将OPS方法用于人囊胚内细胞团分离人胚胎干细胞的玻璃化冻存^[149,150], 复苏后的细胞有一小部分(24%)发生非特异性分化, 但细胞贴附特性与未冻存干细胞相当, 总的来说干细胞的多功能性在解冻后得到保存^[150]。其他玻璃化冻存方法用于人类胚胎干细胞保存的效率还需要进一步研究。

当前的干细胞玻璃化冻存方法虽然取得了一定进展, 但这些方法对需要冻存的大量干细胞来说相当耗时, 而且以防细胞损失需要高水平的操作技能。此外, 由于需要保存的干细胞数量较多, 急需高通量的冻存方法。因此, 基于喷射器的无载体液滴玻璃化冻存技术不仅具有高通量的处理能力, 且可通过减小冻存液滴尺寸进一步降低所需CPA浓度, 目前已经成功地应用于小鼠卵母细胞的冻存, 可作为一种适用于干细胞玻璃化冻存的可靠技术^[73]。

(4) 工程化组织的玻璃化冻存. 组织工程技术利用再生手段在治疗一些难治愈疾病方面显示出极大的潜力, 如糖尿病、心脏疾病、肾功能衰竭、骨质疏松症和脊髓损伤^[151~156]。工程化组织(tissue engineered cartilage, TEC)的低温长期保存, 对实现替代组织从实验室到临床的成功转化十分重要。提高替代组织的保存限期, 在按需的基础上向患者分配基于细胞的产品。

近年来低温科学的发展为冷冻保存组织和TEC打下了坚实的技术基础。研究发现, 不适宜的冷冻方法会导致组织细胞内皮损坏, 以致组织高度血管化。至今, 冷冻保存的动物肠道和卵巢在复苏和移植后保持了较好的功能性。在对组织和TEC进行冷冻和复苏时, 首要任务是防止冰晶的形成, 以及保持细胞和细胞外基质(extracellular matrix, ECM)的完整性和机械性能。此外, 还要防止冷冻过程中发生脱水, 避免胶原纤维之间的交联增加, 导致组织硬化^[143]。由于生物材料和再生组织在液-固相变过程中很难得到完整的保存, 因此使用程序化慢冻法进行保存并不理想。玻璃化冻存技术由于避免或降低了冰晶的形成, 因而可维持组织的完整性, 相比慢冻法更具有潜力^[143]。

利用玻璃化冻存技术对工程化组织, 如TE骨骼(TEB)^[157]、TE替代胰腺(TEPS)^[158,159]和TE血管(TEBVs)^[160]均取得令人鼓舞的结果。Song等人^[159]的研究证实, 使用DMSO和1,2-丙二醇作为CPA对TEPS进行玻璃化冻存后的结果与未冷冻对照组相当。同样兔软骨在玻璃化解冻后也保持了较高的存活率(>80%)^[161]。此外, 将玻璃化冻存技术和程序化慢冻法分别用于天然血管的冷冻保存, 玻璃化方法展现出了更好的效率^[162,163], 复苏后的血管能够保持超过80%的最大收缩, 且对药物刺激显示出对照组类似的反应。相比之下, 冻存过程中产生结晶的血管仅保留了低于30%的最大收缩^[162]。在另一项研究中, 将DMSO, 丙二醇和甲酰胺的混合液用于基于胶原的TEBV的冷冻保存, 几乎见不到结晶的产生。与此相反, 使用程序化慢冻法冷冻的样品中能观察到大量的冰晶形成。因此, 玻璃化冻存方法更适于TEBV的冷冻保存。然而无论采用哪种冷冻方法, 复苏后细胞发育能力(38% vs. 39%)相较于新鲜样品(63%)都有所降低^[143]。

虽然与慢冻法相比, 玻璃化冻存技术用于TEC冷冻保存取得的效果更好一些。但是现有的冻存方法仍存在许多挑战。例如, TEC尺寸较大, 利用基于载体的

最小体积方法对其实现超快速冷冻存在技术局限性, 因此必须要使用高浓度的CPA才能实现玻璃化冻存, 且需要确保CPA遍及整个组织结构及每个细胞. 此外, 使用高浓度CPA产生的细胞毒性和渗透性休克是TEC玻璃化冻存应用的另一挑战. 因此, 尽量降低CPA浓度来减少对细胞的伤害是十分必要的. 此外, 优化CPA去除的方法也很关键, 这可避免或降低解冻过程中因去玻璃化导致的细胞活力下降^[19].

2.2 基于载体玻璃化冻存方法的局限性

各种最小体积玻璃化载体对卵母细胞和胚胎的冻存结果都较传统吸管有所改善. 但是由于目前基于载体的玻璃化冻存方法基本的操作流程都是基于单个或多个胚胎或卵母细胞的单独操作, 并且在冻存过程中对于操作时间有严格限制. 例如, 在最小体积玻璃化载体中常见的石英毛细管方法中, 冻存卵母细胞的步骤必须严格控制在每个步骤5 min内完成, 连续完成5个冻存步骤^[109], 这需要操作人员对于精准的微量操作具有极高的操作水平, 这些方法实现的结果高度依赖于胚胎学家的操作技能, 因此未来在比较生物保存技术时需要考虑这个因素. 由于手工操作具有不确定性, 如果可以采用自动化系统, 未来手工流程有可能被淘汰. 另一点需要注意的是, 这些方法的有效性也取决于保存的生物样本自身的特性和需求.

基于微纳尺寸液滴的玻璃化冻存方法作为一种无载体的冻存方法, 通过将包裹细胞的CPA液滴直接射入液氮实现冷冻保存^[14]. 由于不需要玻璃化载体或容器, 极大地提高了降温 and 升温的速率. 此外, 近年来微纳液滴发生及喷射技术的迅速发展为实现连续液滴玻璃化冻存提供了可靠的技术平台, 在高通量生物保存方面显现出巨大的潜力, 如血液的冷冻保存^[164].

3 基于液滴的无载体玻璃化冻存方法及其应用

基于液滴的玻璃化冻存方法主要是将细胞或组织先载入冷冻保存液, 然后将包裹细胞的液滴直接转入液氮^[14,68,74]. 另外, 也可将包裹细胞的液滴置于高导热率基质表面(如铝、铜), 随后快速浸入液氮. 这种技术的优势在于不使用体积较大的载体, 微液滴的热容量较小, 进而可以达到更高的降温 and 升温速率. 无载体

技术降低了冰晶形成的可能性以及所需的CPA浓度^[68]. 基于液滴的玻璃化技术已在多个植物种类的冻存中得到了理想的结果^[165,166]. 这些结果为冷冻保存哺乳动物细胞(如卵母细胞和胚胎)拓展了新思路^[64,69,167,168].

Landa等人^[167]已将基于液滴的玻璃化冻存方法用于小鼠胚胎的保存^[64]. 在该研究中, 胚胎首先被转入10%甘油的培养基中预平衡, 之后包裹胚胎的液滴(体积为5~20 μL)被直接滴入液氮实现冻存. 8个胚胎在冷冻复苏后均表现出较高的存活率(>90%)和发育潜力, 其中83%以上的胚胎发育成了囊胚, 超过73%的胚胎在体外培养48和96 h后实现着床^[167]. 然而, 冻存过程中, 将液滴(5~20 μL)直接放入液氮通常导致液滴被液氮蒸气膜包裹并在液氮表面漂浮(图3), 因此蒸气膜的存在阻碍了热传递的有效性, 这种现象被称为Leidenfrost现象^[68]. 因此对Leidenfrost现象的进一步研究以及去除方法的探索, 对基于液滴玻璃化冻存方法的使用至关重要. Song等人^[68]建立了液滴玻璃化冷冻过程中的冷冻结晶模型, 从物理层面阐述了Leidenfrost现象产生的负面影响, 并提出了利用纳升体积液滴进行冷冻来尽可能减小因Leidenfrost现象导致液滴结晶的方法.

尽管基于液滴的玻璃化冻存方法仍存在一定局限性, 但如果采用更小的液滴, 无载体玻璃化冻存系统仍然可以得到比载体系统更高的热传导率. Papis等人^[69]

图3 液滴产生与表面漂浮过程(网络版彩图). A: 实验装置及液滴状态; B: 液滴浸入液氮. 液滴被液氮蒸气包裹; C: 液滴沉入液氮中, 液滴表示产生结晶

Figure 3 Generation of droplets and levitation of droplets (color online). A: Experimental setup and behavior of droplets; B: Droplets immersed in the liquid nitrogen are wrapped by liquid nitrogen vapor; C: The droplets sink into the liquid nitrogen. The white color indicates the crystals

和Dhali等人^[168]采用了类似的方法, 直接将更小体积(即6 μL)的液滴浸入液氮, 用于玻璃化冻存牛卵母细胞和小鼠受精卵。相比于大液滴, 利用小液滴冻存后的卵细胞具有更高的分裂率和囊胚率。其冻存效率的提高可以归因于更小液滴体积实现了更高的降温速率。

固体表面玻璃化(solid-surface vitrification, SSV)的冻存方法是使液滴不与液氮直接接触而实现玻璃化冻存^[169]。在这种方法中, 将包含细胞/组织的CPA液滴用移液器或玻璃毛细管置于金属表面上(如铝箔), 用液氮或液氮蒸气进行冷却(图4A)。SSV方法即1~2 μL 玻璃化冻存液液滴中包裹5~10个卵母细胞, 已用于冷冻保存来源于牛^[170]、山羊^[171]和猪^[172,173]的体外成熟卵母细胞。然而, 这些研究显示玻璃化卵母细胞经激活^[169]或体外受精^[172,174]后发育能力较低。而且玻璃化的卵母细胞的分裂率(14%)远低于未经过冷冻保存的卵母细胞(46%), 这表明即使该卵母细胞在玻璃化冻存后细胞膜被完整保存, 冷冻损伤仍然存在。发育能力的降低可能是由于玻璃化冻存导致纺锤体和/或染色体的结构损害^[174,175]。这种冷冻伤害可能是由于单液滴中包裹5~10个卵母细胞且体积较大而致使传热效率较低。由此推测, 传热效率可通过一个液滴仅包裹单个卵母细胞以减小冻存的液滴体积来改善。

减小包裹细胞/组织的玻璃化冻存液体积不仅可以提高降温和升温速率, 也同时降低了冰晶成核/形成的可能性^[176]。虽然对于玻璃化冻存中“最小”体积没有

明确的定义, 但通常是指体积在1 μL 左右或小于1 μL ^[64]。基于这一原则, Arav等人^[99-101]研发了最小液滴尺寸(drop size technique, MDS)技术, 仅使用纳升(0.1~0.5 μL) CPA溶液用于细胞玻璃化冻存。MDS技术已成功用于猪^[99,100]、牛^[101]的卵母细胞, 以及牛、羊胚胎的玻璃化冻存^[176]。最近MDS方法被进一步改进, 即将0.1~0.5 μL 的液滴置于玻璃盖玻片上, 随后浸入液氮(图4B)。这种技术将冷冻速率最大化至130000 $^{\circ}\text{C}/\text{min}$, 与传统玻璃化冻存方法相比, 最多可将CPA浓度降低50%。与传统的程序化慢冻法和基于传统吸管的玻璃化冻存方法相比, 基于液滴的无载体玻璃化冻存方法在卵母细胞和胚胎的冻存方面展现出明显优势, 细胞的存活率及功能均有所提高。然而这些方法通常属于时间和人力密集型操作, 极大地限制了细胞玻璃化冻存的通量。另外, 由于液滴尺寸难以准确控制, 生成的液滴尺寸非均匀分布, 这会导致玻璃化冻存结果的不一致性。因此, 上面提到的技术对于需要大通量玻璃化冻存的细胞, 如红细胞(red blood cell, RBC)、干细胞和肝细胞, 缺乏实用性。

Demirci等人^[14,70]报道了基于喷射装置的液滴玻璃化方法, 减小液滴体积(1.5~500 nL)使单个液滴携带一个或少数几个细胞, 同时不降低细胞活性。实验证明, 这种液滴发生和喷射技术具有高通量的特点, 每秒可产生1000个液滴^[14,68,70,102-104]。采用这种方法对红细胞进行玻璃化冻存, 优化参数后可以实现低于20%的溶血率^[77]。将喷射液滴玻璃化方法用于冻存卵细胞,

图4 液滴玻璃化冻存方法(网络版彩图)。A: 固体表面玻璃化冻存装置。一个铝箔包裹的金属柱体被部分浸入液氮中。将包裹卵母细胞的冻存溶液的微液滴滴在金属柱体的上表面, 瞬间即被玻璃化; B: 优化后的最小液滴尺寸技术。各种尺寸液滴被毛细管放置在玻璃盖玻片上

Figure 4 Droplet vitrification (color online). A: The solid surface vitrification device. A metal cube covered with aluminum foil was partially submerged into liquid nitrogen. Microdroplets of vitrification solution containing the oocytes were dropped onto the cold metal cube and are instantaneously vitrified. B: The modified minimum drop size technique. Droplets of various sizes were placed on glass cover slips using capillaries

可以得到高细胞活性(玻璃化: 83.2%, 对照组: 94.9%)、高分裂率(玻璃化: 96%, 对照组: 88.6%)以及较高的囊胚率(玻璃化: 26%, 对照组: 49.4%)^[73]. 此外还有基于声学喷射的液滴发生技术能够实现最小液滴体积(<1 nL)^[70,177~180], 可产生比喷射装置更小体积的液滴.

生物打印方法具有液滴位置可控、体积小及高通量的特点, 打印频率可达到20 kHz, 单个液滴的体积可小至380 pL^[180], 因而也被尝试用于生物保存. 研究者将生物打印与SSV方法相结合, 避免了液滴与液氮直接接触, 将其应用于干细胞冷冻保存获得了高存活率及功能性^[71].

由于液滴体积减小可显著提高降温及升温速率, 因此可以使用更低浓度的CPA(~1.5 mol/L)实现玻璃化冻存, 从而使产生的渗透压和机械应力最小化. 实验证明, 液滴尺寸和玻璃化冻存所需CPA浓度存在如下关系: 液滴越小, 实现玻璃化冻存所需的CPA浓度越低^[14]. MDS方法中, 液滴被喷出并滴入液氮时保持球形形状, 可以实现各向均匀冷却. 各类型的哺乳动物细胞, 如AML-12肝细胞、NIH-3T3成纤维细胞、HL-1心肌细胞、小鼠胚胎干细胞和RAJI细胞, 在冻存液滴中包裹喷射后均保持高存活率(>89%)^[14,68].

虽然最小体积的玻璃化冻存方法在设计之初大多

用于生殖细胞(如胚胎和卵母细胞)的保存, 但随着生物医学和再生医学的发展而得到了更广泛的应用, 如保存用于移植和细胞治疗的高品质的细胞与组织, 以及延长组织再生应用中组织结构的贮藏寿命等. 在未来的临床应用中, 可将喷射系统灭菌后在无菌罩中操作^[120,181]. 此外, 采用无菌的液氮可避免潜在的污染^[182]. 液氮灭菌可通过无菌的聚四氟乙烯(poly tetra fluoroethylene, PTFE)管壳式过滤器过滤^[183]或紫外(ultraviolet, UV)辐射^[184]的方法实现.

4 总结与展望

细胞/组织的冷冻保存效果取决于多个因素, 包括细胞大小、细胞质体积和细胞的类型. 大多数哺乳动物细胞使用冷却速度约为-1 °C/min的缓慢冷冻法进行冻存后可维持90%以上的细胞活性. 然而, 在保存细胞质体积较大的细胞(如卵母细胞)时, 由于细胞含水量高胞内易结晶而很难避免细胞损伤. 对于不同大小的细胞群混合组成的组织, 由于每种细胞最佳冻存条件的差异而产生更多挑战. 如上所述, 相对于传统方法, 使用玻璃化冻存技术冻存的细胞复苏后具有更高的存活率, 展现出应对上述挑战的巨大潜力(表2)^[185~193].

本文着重介绍了最小体积玻璃化冻存方法及其

表 2 用于细胞及组织冷冻保存的玻璃化冻存方法^{a)}

Table 2 Vitrification methods for cell and tissue cryopreservation

细胞/组织	冷冻保存的挑战	通常应用的玻璃化冻存方法	存活率/活性	限制	参考文献
角膜移植	内皮细胞的损伤 (高冷冻速率下的内细胞冻结, 较低冷却速率下产生溶解引起的损伤)	非渗透性CPA, 将镀聚四氟乙烯的袋子作为玻璃化载体	<10%	CPA毒性, 升温过程中去玻璃化	[129,185~187]
人胚胎干细胞	对CPA敏感, 需严格控制时间及高水平技巧	OPS (1~20 µL), 玻璃吸管 (20~130 µL)	>75%	耗费时间, 劳动密集型	[18,147]
成体干细胞	未分化阶段损失及活性损失	OPS	>80%	耗费时间, 劳动密集型	[18,143,147,188]
工程化组织	CPA需要渗透整个TEC来避免结晶	玻璃小瓶	95%	组织太大难以玻璃化	[18,19,189,190]
软骨细胞/软骨	需要一定的CPA浓度避免结晶	玻璃小瓶	软骨细胞: 93% 软骨组织: 85%	复苏升温过程的需要足够快	[18,190]
组织工程血管	生物材料在冷冻过程中硬化	冻存管/玻璃小瓶	50%	复苏过程中活性损失	[18,191]
精子	对渗透性休克效应敏感, 体积大	多重热梯度	50%运动性减弱	样本体积大, 需要优化降温速率	[76,192]
卵巢组织	样本尺寸大	吸管及网格	固缩, 血管化, 检测到细胞肿大	难以保存大样本, 必须切成片, 冷冻过程结晶	[89,193]

a) CPA: 冷冻保护剂; EM: 电子显微镜; OPS: 开口式拉长麦管; TEC: 工程化组织

在临床和实验室中的应用。最小体积玻璃化冷冻方法可提高降温与升温速率以降低玻璃化所需的CPA的浓度,进而降低冷冻保存过程中CPA对细胞和组织产生的毒性和渗透性休克效应。该方法为液滴玻璃化的进一步发展提供了方向,即减小液滴尺寸来获得更高的冷却速率。然而,这一策略仍存在许多挑战,如通量的局限性和对操作人员熟练度的苛刻需求。因此,今后的研究工作需要把重点放在新型玻璃化系统的开发以及改进现有玻璃化冻存方法上,最

大限度地减少对操作人员熟练度的依赖和人工操作的不稳定性。

近年来,微纳技术的发展已经使小体积样本的操控得以实现^[42,73,194~197]。同时,传热学领域对传热效率的机理研究^[198~200]以及对冻存过程中Leidenfrost现象^[201~204]的研究也取得了较大进展。将这些技术理论与目前冻存技术结合,具有实现自动化和高通量生物保存的巨大可能,并期望借此获得临床认可的高效率生物保存。

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Progress and applications of minimum volume vitrification in biomedicine

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Cell and tissue cryopreservation has played important roles in multiple fields such as assisted reproductive medicine, regenerative medicine and organ transplantation, and has created broader impacts on public health. Slow freezing and vitrification are the two main methods for biopreservation. Vitrification has demonstrated significant improvement in cell viability and function post thawing by reducing or even avoiding crystallization during freezing process. However, high concentrations of cryoprotectants often required for vitrification may induce toxicity and osmotic stress to cells and tissues. It has been indicated that vitrification using reduced sample volumes (i.e., <1 μ l) can significantly elevate the cooling rate, thus reducing the CPA concentrations needed. The rapid development of nano- and micro-scale technologies in recent years has made it possible to manipulate samples in picoliter to nanoliter. Therefore, the integration of nanotechnologies with cryogenics would create great potential for the progress in biopreservation technology.

cryopreservation, cryoprotectant agents, droplet vitrification, minimum volume vitrification

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