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评 述

哺乳动物卵母细胞和胚胎冷冻保存研究进展: 现存问题与未来展望

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摘要 在过去的几十年中, 哺乳动物卵母细胞和胚胎冷冻保存技术因在辅助生殖技术及实际生产中应用广泛, 其研究发展尤为迅速. 多个物种(包括人)的卵母细胞和胚胎冷冻保存研究获得了受孕和活产的成功佐证, 为冷冻保存技术的临床应用提供了理论依据, 并成为体外受精不可缺少的环节. 尽管哺乳动物卵母细胞和胚胎的冷冻保存技术的研发和应用取得了巨大进展, 例如为可能不育的患者贮藏生殖潜能、卵子冻存库的建立以及卵母细胞和胚胎的跨国运输, 但成功率仍达不到使用新鲜卵母细胞和胚胎的水平, 还存在许多难题有待解决. 本文针对低温保存技术中制约冷冻效率和成功率的主要问题进行了综述, 旨在解决技术研发中遇到的瓶颈, 为改进技术提供借鉴和参考, 鼓励低温生物学研究者继续开展深入研究, 加快推进冷冻保存技术在人类和动物辅助生殖技术中的实际应用.

关键词
冷冻保存
哺乳动物
卵母细胞
胚胎
问题

在过去的几十年中, 辅助生殖技术(assisted reproductive technology, ART)已被用于帮助不孕不育患者和珍贵的动物妊娠. 鉴于此, 随着技术的不断改进, 在过去几年中, 可利用的卵母细胞和可移植的胚胎比例显著增加. 但产生富余的卵母细胞和胚胎则需要通过冷冻保存技术的完善来实现.

雌配子和胚胎冷冻保存概念的出现是从Chang[1,2]报道低温贮藏对兔(*Oryctolagus cuniculus*)卵母细胞、受精卵及胚胎的影响结果开始的. 随后, 配子、胚胎及卵巢组织的冷冻保存方法相继得到了长足的发展. 1977 年, 首次报道了冻存小鼠(*Mus musculus*)卵母细胞成功妊娠并产仔[3], 之后在这个领域开展一系列物种包括人类(*Homo sapiens*)[4]、兔[5]、牛(*Bos tauru*)[6]和马(*Equus ferus caballus*)[7]的研究.

目前认为, 慢速冷冻和玻璃化这 2 种方法的出现可满足哺乳动物卵母细胞和胚胎的成功冷冻保存的

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要求. 研究表明, 缓慢冷冻的成活率和抑制率低, 并可能导致纺锤体异常[8,9], 这些不良影响限制了慢速冷冻技术的应用. Rall 和 Fahy[10]发明了玻璃化法, 已被广泛应用于人类[11]、各种家畜和实验动物卵母细胞[12~21]以及哺乳动物胚胎的冷冻保存[21~30]. 研究认为, 玻璃化法是比慢速冷冻更好的冷冻保存方法[31]. 无论使用何种冷冻保存方法, 经冻融的配子或胚胎受孕率仍不及新鲜的配子或胚胎[32]. 这意味着为找到提高卵母细胞和胚胎冷冻保存成功率的办法, 需不断优化冻存技术. 本文阐述了制约低温保存技术朝着高效成功方向发展的主要问题, 旨在为今后改善冷冻保存技术和方法提供参考, 并期待随着本领域更深入地探索, 将会取得更显著的研究成果.

1 冷冻损伤的类型

哺乳动物的卵母细胞和胚胎在冷冻保存过程中很容易受到不同类型冷冻损伤的危害[33]. 为此, 要达到成功冷冻保存的效果, 就要建立一种损伤最小化同时能保持高存活率的方法. 这些损伤的发生是与卵母细胞或者胚胎的低温生物学特性密切相关的, 如对低温的敏感性、质膜对水和冷冻保护剂(cryoprotectant agents, CPAs)的渗透性、对冷冻保护剂化学毒性的灵敏度以及对渗透性膨胀和收缩的耐受性等. 温度降至零度以下, 细胞可能会受到冷冻损伤, 其发生机制总结如下.

1.1 低温损伤

研究发现, 低温损伤通常发生在–5~15℃, 低温会导致脂肪滴、富脂膜以及有丝分裂或减数分裂纺锤体的微管发生局部不可逆的变化[34,35]. 这在慢速冷冻技术应用中是一种常见的冷冻损伤, 而玻璃化冷冻保存法则避免了经过危险温区时所造成的低温损伤[36], 因而成为目前唯一能够成功冷冻保存完整的猪(*Sus scrofa*)胚胎(冷敏感脂肪滴含量极高)[37]与猪、牛、绵羊(*Ovis aries*)、马等动物的低温敏感性卵母细胞[38]的冻存技术.

1.2 冰晶体的形成

细胞外的培养液与细胞内胞质和胞核内液体是造成损伤的主要来源[39], 可能发生在–5~–80℃. 与慢速冷冻相比, 玻璃化冷冻过程中, 卵母细胞和胚胎与高浓度的冷冻保护剂接触, 以高达 2000~20000℃/min 的降温速度冷冻后(图 1), 再投入液氮(liquid nitrogen, LN2)中, 因此可避免冰晶体的形成[39].

1.3 断裂损伤

温度在–50~–150°C, 由于固凝溶液的机械作用, 尤其是相对较大的生物体如卵母细胞和胚胎, 易发生断裂损伤[40].

1.4 多重星状体的形成

有研究提出了一种新型冷冻损伤[41]. 在玻璃化冷冻中, 卵母细胞置于高浓度的冷冻保护剂并以超速冷冻降温时会导致雄原核附近形成多重星状体, 从而使纺锤体迁移和原核的发育受阻, 会延迟第一次卵裂, 损害囊胚的形成[41]. 作者推测其中一个原因可能是卵母细胞中谷胱甘肽的浓度过低亦或质量差的卵母细胞不能维持精子星状体的唯一性, 致使玻璃化的卵母细胞中星状体的形成增多. 最近另一项研究发现, 玻璃化复温的卵母细胞体外受精(*in vitro* fertilization, IVF)后, 在成熟的卵母细胞中高浓度谷胱甘肽并没有减少多重星状体形成的发生率[42].

1.5 渗透力

在冷冻保存过程中, 当细胞与高渗透性的保护剂接触时, 会因细胞内外的渗透压差而立即脱水皱缩. 据报道, 细胞脱水的速度比失去细胞质中的大分子和其他溶质快了大约 5000 倍[43]. 解冻是冷冻过程的逆过程, 也是同样重要的. 因为卵母细胞和胚胎比冷冻保护剂更渗水, 当不加冷冻保护剂直接将它们置于溶液中, 冷冻的细胞解冻后会膨胀或爆裂. 因此, 通常使用高浓度的非渗透性保护剂(如蔗糖)来缓冲渗透压, 以抵消细胞内高浓度的冷冻保护剂. 细胞形态的这种变化可导致细胞骨架损伤以及透明带的断裂[44,45].

2 低温冷冻对卵母细胞和2-细胞胚胎的影响

2.1 骨架

卵母细胞的细胞骨架由3个主要部分组成: 微管(聚合的微管蛋白)、微丝(聚合的肌动蛋白)和中间丝. 冷冻保存时, 为了达到平衡, 渗透压发生急剧的变化, 可能会导致卵母细胞萎缩变形, 推测原因可能是破

坏了细胞骨架. 研究已证实卵母细胞在降温冷冻[35]、冷冻保护剂[46]或冷冻/解冻过程中[45], 会发生微管解聚、DNA 碎片化[47]、纺锤体轴构型异常[48,49]、染色体异常[49]、皮质颗粒分布或胞外分泌改变[48]以及细胞质膜断裂[50]. 同样, 卵母细胞冷冻保存后, 对微丝骨架的功能产生负面影响[46]. 这些发育干扰导致卵膜线粒体的异常分布[51,52], 从而导致减数分裂能力和卵母细胞受精率降低, 而且会引起植入前胚胎发育失败.

卵母细胞的减数分裂纺锤体由微管构成, 微管的结构是由α-和β-微管蛋白聚合而成. 纺锤体对减数分裂过程和染色体重组与分离起到至关重要的作用[53]. 通过许多技术可以使纺锤体结构可视化, 共聚焦显微镜和偏振光显微镜是其中的 2 种方法. 共聚焦显微镜法因为需要对卵母细胞染色、固定而使其丧失活性, 具有一定的局限性[54]. 而偏振光显微镜法的优点是可以观察活细胞的动态, 即在冷冻保存前后均能观察到纺锤体[55,56]. 但由于它无法区分正常的双极纺锤体构象与重度混乱的构象, 无法预测解冻的卵母细胞 MⅡ期纺锤体微管聚合度, 观测 MⅡ期纺锤体(特别是冷冻保存后)效率较低[57]. Gomes 等人[58]利用一种无创性成像的极化视野显微镜(polarized field microscopy, PMF)方法, 采用免疫组化(immunocytochemistry, ICC)法比较小鼠卵母细胞减数分裂中期Ⅰ(metaphaseⅠ, MⅠ)、分裂末期Ⅰ(telophaseⅠ, TⅠ)和分裂中期Ⅱ(metaphaseⅡ, MⅡ)不同阶段纺锤体在不同温度(37℃、室温、4℃及玻璃化)下培养 0, 10, 30 和 60 min 的聚合动态. 他们观察到, 减数分裂不同时期下的卵母细胞纺锤体的解聚/再聚合平衡状态与温度和时间呈相关性, 研究表明, 在室温、4℃以及玻璃化冷冻后复温培养下的卵母细胞, TⅠ期比 MⅠ, MⅡ期纺锤体的解聚程度低.

据报道, 卵母细胞经过缓慢冷冻和玻璃化冷冻法, 解冻后纺锤体都会立即呈现严重错乱无序或者消失的状态[55,59,60], 比慢速冷冻法[61]的危害程度更大. 但玻璃化和缓慢冷冻后 MⅡ期纺锤体消失与重现的结果也被报道过[35,61~63], 这取决于解冻后的时间间隔、冻融方法以及物种的不同[55,59,60,64]. 有研究还提出, 温度导致卵母细胞的微管解聚可能与卵母细胞的核成熟状态有关[58,65]. 寻找能避免冻存卵母细胞在生发泡期(germinal vesicle, GV)纺锤体解聚的方法是非常必要的. 然而, 未成熟的卵母细胞因其对水和 CPAs 的渗透性较小[66], 与成熟卵母细胞比, 冷冻对保存率[67]和活产率的影响就小多了[68].

细胞角蛋白是一种中间丝, 在卵母细胞成熟和胚胎发育中起着重要的作用[69]. 研究发现, 成熟[70]和未成熟的卵母细胞[71]进行玻璃化冷冻都会使细胞角蛋白(cytokeratin, CK)的结构受到影响, 其中最有可能导致卵母细胞的死亡[70]. 玻璃化过程中使用细胞松弛素 B 和紫杉醇的细胞骨架稳定剂后, 得到的结果仍然是有争议的. 卵母细胞使用细胞骨架稳定剂升温后, 发育能力得到改善, 在小鼠[72]、牛[73]、猪[74,75]和羊[76]的卵母细胞中均有报道, 相反, 牛[77]、猪[78]和兔卵母细胞[79]却报道没有得到改善. 所以需要优化冷冻保存的程序, 做进一步验证和评价, 以解决细胞骨架损伤的问题.

2.2　透明带

透明带是包裹在卵母细胞和植入前胚胎外的质膜糖蛋白膜. 已知透明带在整个受精过程中起关键

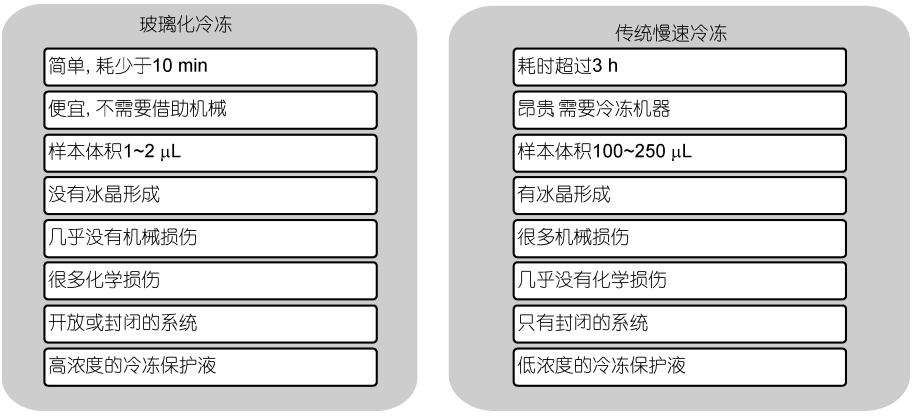

图 1　玻璃化冷冻与慢速冷冻的对比

作用, 可引发皮质颗粒胞吐作用, 在第一个精子进入后阻止其余精子穿越阻止多精受精. 皮质反应通过修饰透明带(透明带反应)、卵膜或两者都作用来阻止多精受精. 在卵母细胞的冷冻保存中, 发现冷冻保护剂会引起卵母细胞短暂的钙离子增加[80], 因此诱发皮质颗粒胞吐作用提前发生[81], 足以导致透明带变硬, 给精子穿透和受精带来损害[82]. 细胞结构的快速变化是低温冷冻对卵母细胞的另一个负面影响. 观察到这种细胞变形因其自身折叠形成新月或凹形, 从而导致透明带破损[44], 是卵母细胞冷冻保存后发生多精受精可能性最大的原因.

2.3 线粒体

线粒体是哺乳动物卵母细胞中最为丰富的细胞器, 其功能障碍或异常与否是决定卵母细胞和胚胎发育能力的关键. 线粒体是卵母细胞中产生能量的唯一来源, 为受精和植入前胚胎发育提供三磷酸腺苷(ATP), 线粒体生成的 ATP 减少与植入前胚胎发育失败率呈正相关[83]. 此外, 植入前胚胎发育障碍可能导致卵膜线粒体的异常分布[52]. 在人[84]和牛[85]的研究中都报道过玻璃化冷冻保存后会危及线粒体功能[85]. 卵母细胞 ATP 含量降低, 可能会导致卵母细胞发育不良. 线粒体在细胞内的分布取决于微管[86], 其在细胞内的分布对 ATP 的再分配起着重要作用, 当高能量需求时, 可以让细胞内的不同区域产生 ATP 的水平增加[86,87]. 研究表明, 冷冻保存会损害微管功能[88], 导致线粒体的异常分布[51], 从而改变了细胞内 ATP 的分布. 有人提出, 在受精和发育过程中线粒体无法恢复到正常的分布模式致使 ATP 的分布发生改变, 可能会减弱卵母细胞的功能[51]. 另外, 冷冻能导致线粒体发生膨胀[70,89], 线粒体畸形以及内外膜的破裂[44,90]. 为减少玻璃化对线粒体功能的负面影响, 有实验在玻璃化溶液中添加 1 mmol/L 甘氨酸, 可维持卵母细胞的线粒体功能并改善后续囊胚的发育率[51].

3 冷冻保存对分子的影响

冷冻保存对基因表达的负面影响已经在氧化应激、细胞凋亡、细胞周期和精卵相互作用的重要基因等方面进行了相关的研究报道[90~97]. 基因表达发生改变可能是冷冻保存后的卵母细胞受精能力下降的重要原因. 临床研究表明, 2 种冷冻保存技术对生物学功能的影响不同, 慢速冷冻过程更为有害. 慢速冷冻导致参与保持染色体结构和细胞周期调控的基因表达下调[98], 人的卵母细胞 MⅡ期 mRNA 保存率(39.4%)比玻璃化冷冻(63.3%)低[99]. 与玻璃化相比, mRNA 的低表达对蛋白表达和卵母细胞生理机能有不良影响[100]. 细胞凋亡是卵母细胞变性和胚胎分裂的基本过程[101]. *BCL2*(B-cell leukemia/lymphoma 2)基因家族对细胞凋亡的调控起着重要作用, 并被视为抗凋亡和促细胞存活因子, 而 BAX(Bcl2-associated X protein)是促凋亡和促细胞死亡因子[102]. 研究表明, 犬(*Canis lupus familiaris*)卵母细胞和小鼠胚胎的玻璃化并没有改变 *BAX* 基因的表达模式[90,103], 而玻璃化复温组中 *Bcl2* 发生了高表达[90]. 但也有与此相反的报道, 例如, 在牛卵母细胞玻璃化后凋亡基因(*Fas* 和 *FasL*, *Bax* 和 *Bcl-2*)表达上调[93]; 与对照组相比, 小鼠胚胎玻璃化后 *Bcl2* 基因表达下调[104].

CD9 属于四次跨膜蛋白超家族, 位于小鼠卵母细胞质膜上[105], 在配子融合中[106]发挥至关重要的作用. 与非玻璃化对照组比较, 发现玻璃化冷冻的牛[91]和绵羊的[94]卵母细胞在解冻后 *CD9* mRNA 表达量降低. 研究人员对小鼠胚胎玻璃化后的影响展开了全面广泛的研究, 结果表明, 对新陈代谢和细胞生理活动的调控影响最大, 如影响细胞增殖、细胞周期、发育、生物合成、呼吸以及应激相关的基因的表达[107,108]. 有趣的是, 玻璃化后牛胚胎IVF的基因表达发生了很大变化, 而玻璃化后体内发育胚胎的基因表达没有太大的变化[109]. 有 268 个基因在 IVF 和体外扩张囊胚调控中差异表达, 表明 IVF 胚胎比体内繁殖(*in vivo* derived, IVV)胚胎对玻璃化更敏感[109].

研究人员对玻璃化冷冻的表观遗传效应也进行了研究[93,110~120]. 有研究表明, 卵母细胞[110,115,119]和囊胚[112]玻璃化并没有显著改变基因的甲基化模式. 与此相反, 对小鼠卵母细胞[113]、胚胎[112]和胎儿[117]的研究报道了玻璃化后基因的甲基化减少. 还有其他的研究表明, 玻璃化不但能显著增加小鼠卵母细胞的基因甲基化[116], 而且能最终产生牛的 2-细胞胚胎和体外扩张囊胚[111]. 至于玻璃化冷冻对乙酰化的影响, 几项研究表明, 玻璃化显著改变了卵母细胞的乙酰化模式[92,114,116,118,120]. 研究结果并不一致, 这表明玻璃化对基因组甲基化模式的影响与物种、发育阶段、基因的不同均有关系, 并且可能取决于所分析的基因组片段的大小[110]. 在以上这些研究中也报道了表

观遗传修饰的异常可能是导致玻璃化的卵母细胞发育能力降低的原因.

4 卵母细胞与胚胎冻存的比较

不管用何种冷冻保存方法, 卵母细胞比卵裂期的胚胎冻存更为困难[121]. 卵母细胞特别容易受到冷冻损伤, 因此要特别注意卵母细胞的冷冻保存程序, 原因如下:

(1) 大小差异. 众所周知, 卵母细胞是哺乳动物体内最大的细胞. 从低温生物学的角度考虑, 样品体积越小冷冻保存效果越好. 在胚胎发育的第一周, 细胞团总量减小; 而到了扩张囊胚期, 总量比原卵母细胞降低了十倍甚至百倍. 因此, 由于卵母细胞表面或体积比较大而导致渗水性低, 也就是说, 冻结时可能由于尚存水分而产生细胞内冰晶体, 对细胞造成极大损害[39]. 有研究指出, 血浆膜的渗透性对卵母细胞和胚胎在成熟与发育阶段的变化起着重要的作用. 例如, 牛的卵母细胞比其桑椹胚和囊胚对水和冷冻保护剂的渗透性都低[66]. 在哺乳动物卵母细胞或早期胚胎中, 水和抗冻保护剂通过简单的扩散跨质膜缓慢迁移. 因此, 通过长时间与冷冻保护剂溶液的接触和两步处理法对细胞脱水更为必要, 还可以让冷冻保护剂得到充足的渗透. 相反, 在桑椹胚和囊胚中, 因为水分子的运动和冷冻保护剂是迅速通过膜通道渗透的, 所以单步处理和短时间的接触即可达到良好的效果. 另外, 在降温和复温前, 卵母细胞和冷冻保护剂作用时的温度是至关重要的, 而在桑椹胚和囊胚中, 由于膜通道扩散的速度受温度影响较小, 渗透方面对温度要求不是太高.

(2) 含水量差异. 卵母细胞比胚胎中的水分含量高. 但是囊胚腔的液体可能会形成冰晶体造成冷冻损伤[122,123]. 若这种情况存在于卵母细胞间隙, 将会产生更为严重的不利影响.

(3) 细胞数量的差异. 活体组织切片检测、胚胎分割结果证明, 多细胞胚胎可以弥补自身细胞 50% 的损失, 但是卵母细胞只有一次机会且没有备份, 经过重创后不能再生.

5 冷冻保存的成功实验

为了降低冷冻损伤并能维持高的存活率, 研究人员做了大量实验, 获得了较为成功的冷冻保存方法, 这些研究的主要成果总结如下:

(1) 为避免冰晶体的形成, 降温前的平衡备受研究人员的关注. 已有 2 种方法被应用: (ⅰ) 无论冷冻稀释液还是冷冻浓缩液都进行超短平衡法[124]; (ⅱ) 延长第 1 次冷冻保护液的平衡时间, 随即进行短时间的第 2 次平衡, 但需相对延长培养时间[125]. 有研究指出, 平衡时间越短, 卵母细胞和胚胎的玻璃化效果越好[126]. 有人提出时间应小于 10 s 的结论[127], 而另一种结论却指出缩短平衡时间会导致玻璃化保存后卵母细胞存活率和囊胚形成率降低[128,129], 表明卵母细胞与玻璃化溶液接触时间过短, 卵母细胞间的水分可能无法完全被冷冻保护剂取代, 这可能会造成卵母细胞内的某些细胞器受损. 相反, 延长接触时间可确保冷冻保护剂良好的渗透效应, 达到对整个卵母细胞最佳的保护效果.

(2) 为了达到良好的冷冻保存效果, 要求冷冻保护剂具有即高渗又低毒的特性. 为此, 乙二醇(ethylene glycol, EG)、甘油(glycerol, GLY)、二甲基亚砜(dimethylsulphoxide, DMSO)、丙二醇(propylene glycol, PROH)和乙酰胺等物质被作为冷冻保护剂使用[130]. 乙二醇因其高渗低毒, 被认为是一种良好的冷冻保护剂[131]. 已有研究显示, DMSO 可提高 EG 的渗透性, 对纺锤体聚合有良好的效果, 因此在卵母细胞玻璃化时起到保护作用[132]. 在猪卵母细胞冻存研究中, EG 通常与 DMSO 结合使用[39,133], 并进行了最佳浓度的相关研究[134]. 而在牛[135]和人[136]的研究中, 卵母细胞的玻璃化液体使用 EG 和 PROH 组合比在 EG 中加 DMSO 的效果更好, 这可能是由于 PROH 比 DMSO 毒性低. 目前认为理想的玻璃化溶液不但具有非常低的毒性, 而且在生殖低温生物学特性方面具有一定的优势. 例如, 小鼠卵子玻璃化溶液有 90% VM3, 无需使用卵胞浆内单精子显微注射技术(intracytoplasmic sperm injection, ICSI)就能够受精并发育成囊胚, 与未处理的卵子对照组相比, 囊胚率达到 80%[137]. 在低温保存介质中加入低毒、非渗透性的冷冻保护剂, 以促进脱水, 从而最大限度地减少了玻璃化溶液的毒性影响. 在这些物质中, 海藻糖或蔗糖可能更有效[138], 也可抵消因冷冻保护剂渗透作用对卵母细胞存活率的影响[21]. 引入和移除保护剂的方法是减少毒性对卵母细胞损坏的另一途径. 冷冻中, 逐步加入冷冻保护剂或逐渐增加其浓度, 以及在升温/解冻过程中逐步去除这些化合物, 使渗透压影

响最小化.

(3) ICSI 法的发明及广泛应用[139], 从冷冻液中移除钙离子法[80], 以及利用牛胎儿血清[140]的方法可避免卵母细胞冷冻保存后[80]透明带变硬继而产生受精水平下降的问题.

(4) 卵母细胞胞质内含有大量的脂滴, 所以对冷冻损伤具有高度敏感性[74], 机械去除脂滴[74,141]、用化学试剂还原脂质[142]或在培养液中添加左旋肉碱这一在脂肪代谢中起着关键作用的物质[143~145]可以克服这一问题.

(5) 极快的冷冻速度是提高玻璃化效果的最重要因素之一. 借助细微的载体工具, 如电镜铜网(electron microscope grids)、开口式拉长麦管(open pulled straw, OPS)、尼龙环(cryoloops)、冷冻帽(cryotop)、半麦管(hemi-straw)、冷冻管尖(cryotip)或铝制薄片(aluminum sheets)等, 使玻璃化溶液体积最小化, 从而加快了降温和复温的速度. 用不同系统装载的玻璃化冷冻的哺乳动物卵母细胞均获得了囊胚(表 1), 有的也已获得怀孕和存活的后代(表 2), 经过玻璃化冷冻的胚胎也获得了同样的成功(表 3).

表 1 哺乳动物玻璃化的卵母细胞与新鲜卵母细胞对照组的囊胚率(GⅤ/MⅡ期)

物种	卵母细胞发育阶段	使用的载体	囊胚率(%) *vs.*对照组(%)	参考文献
人	成熟卵母细胞	冷冻帽	48.7 *vs.* 47.5	[146]
牛	未成熟卵母细胞	冷冻帽	1.6 *vs.* 34.4	[147]
牛	成熟卵母细胞	电镜铜网	15 *vs.* 42	[124]
小鼠	成熟卵母细胞	尼龙环	83.9 *vs.* 84.1	[51]
猪	未成熟(GV)和成熟卵母细胞	固体表面玻璃化冷冻	3(GV) *vs.* 60 9(MⅡ) *vs.* 20	[18]
绵羊	未成熟卵母细胞	冷冻环	29.4 *vs.* 45.1	[12]
猫(*Felis catus*)	成熟卵母细胞	冷冻环	36.7 *vs.* 55.2	[148]
猫	体外成熟卵母细胞	冷冻帽	10 *vs.* 25	[15]
水牛(*Bubalus bubalis*)	剥离的成熟卵母细胞	固体表面玻璃化冷冻	(7.0±7.1) *vs.* (5.8±4.6)	[19]
水牛	剥离的成熟卵母细胞	冷冻环	(2.8±7.1) *vs.* (5.8±4.6)	[19]

表 2 哺乳动物卵母细胞成功玻璃化使用的不同载体工具

物种	卵母细胞发育阶段	使用的载体	临床结果	参考文献
牛	未成熟卵母细胞	铝片与尼龙网架	通过铝片玻璃化产生一头小牛, 活产率为 1.8%	[14]
人	体内, 成熟卵母细胞	冷冻片	有一位妇女生产了一个健康成活的婴儿, 活产率为 5.8%	[149]
小鼠	体外, 成熟卵母细胞	冷冻帽	玻璃化冷冻, 后代率为 56.7%; 鲜卵母细胞, 后代率为 57.8%	[13]
猫	体外, 成熟卵母细胞	冷冻帽	出生 4 只小猫成活, 活产率为 10%	[15]

表 3 哺乳动物胚胎成功玻璃化使用的不同载体工具

物种	卵母细胞发育阶段	使用的载体	临床结果	参考文献
牛	体细胞核移植产生的囊胚期	0.25 mL 塑料麦管	鲜囊胚, 获得 2 头健康小牛(25%); 玻璃化/解冻囊胚, 获得 1 头健康小牛(11%)	[27]
人	囊胚期	电镜铜网	临床妊娠率 34.1%, 活产 11 个婴儿	[30]
人	囊胚期	冷冻环	18 位供体, 有 23 名健康婴儿出生, 37 人在怀孕中	[150]
人	囊胚期	半麦管	获得 27%持续妊娠率	[151]
猪	囊胚期	开口式拉长麦管	9 只受体猪妊娠, 受孕率为 42.9%, 平均产仔数为(5.4±0.8)个(3~9 个)	[152]
兔	桑椹胚期和囊胚期	改进的封闭式开口式拉长麦管	玻璃化胚胎活产率为 51.7%, 新鲜胚胎活产率为 58.5%	[29]
山羊(*Capra aegagrus hircus*)	桑椹胚期和囊胚期	开口式拉长麦管	当玻璃化溶液为 40%(体积比)的 EG 或配比为 15%(体积比)的 EG 加 15%(体积比)的 DMSO, 产羔率分别为 46.2%和 51.4%. 与鲜胚 57.1%比没有显著差异	[25]
绵羊	体内剥离的胚胎	开口式拉长麦管	产羔率为 56%	[28]
欧洲臭鼬(*Mustela putorius*)	桑椹胚期和囊胚期	开口式拉长麦管	2 个受体共传承 8 个后代, 存活率 16%	[26]
梅花鹿(*Cervus nippon*)	八细胞期、桑椹胚期和囊胚期	0.25 mL 塑料麦管	鲜胚胎出生率, 64.3%; 玻璃化冷冻胚胎出生率, 53.9%	[23]

6 结论与展望

与玻璃化冷冻技术开始使用的最初几年相比, 如今的技术应用成果取得了显著进展. 哺乳动物卵母细胞或胚胎的冷冻保存方法可分为慢速冷冻和玻璃化冷冻法, 根据本文提到的大量文献资料明显可以看出, 玻璃化法是更为行之有效的冷存方法, 并为其在人工辅助生殖技术众多领域的应用提供了广阔的前景. 人类胚胎冷冻保存技术的完善将允许移植尽可能少的胚胎, 从而降低多胎妊娠治疗中最严重的并发症的发病率. 随着玻璃化冷冻的物理和生物学原理更深层次地探索, 未来冷冻保存技术将会取得更高效更成功的应用成果. 对卵母细胞/胚胎冷冻保存技术的展望如下:

(ⅰ) 卵母细胞或胚胎细胞质膜的渗透性, 即便是同一物种也会随着其成熟、发育阶段的变化而变化. 因此, 即使采用相同的冷冻方案, 卵母细胞或胚胎在冷冻保存后的存活情况也是有差异的.

(ⅱ) 合理选择毒性低的冷冻保护剂, 密切监测其温度、时间和溶液稀释浓度, 以及从细胞中逐步添加和脱除的情况. 玻璃化保护剂的最大优势是对细胞内的水分扰动极小, 为哺乳动物卵母细胞和胚胎玻璃化成功冻存的实现带来了新的希望.

(iii) 由于卵母细胞或胚胎会与液氮的开放式容器直接接触, 导致污染的高风险性[153], 因此需要不断完善能够降低污染风险的安全操作方法. 最近, 在使用不同类型的“封闭式”系统后取得了理想的结果[22,154], 为过渡到更密封更安全的玻璃化冷冻系统奠定了基础. 结果强调必须更加努力优化封闭式玻璃化系统方案.

为了实现卵母细胞或胚胎在冷冻保存后“零损害”的目标, 并且确保卵母细胞或胚胎冷冻保存技术最安全、最迅速的发展, 今后该领域的研究热点需要探索如何提升和完善玻璃化冷冻工艺, 以减小玻璃化冷冻损伤并提高冷冻后发育效果. 通过对各种冷冻保护剂的分子和生物化学特性地不断研究和探索, 合理选择最有利、最高效的单一或者多种组合的存储方式, 这无疑会对加推进快冷冻保存技术的研究进展, 使之作为人类和动物辅助生殖技术领域的核心技术更为广泛地应用产生深远的影响.

参考文献

1 Chang M. The effects of low temperature on fertilized rabbit ova *in vitro*, and the normal development of ova kept at low temperature for several days. J Gen Physiol, 1948, 31: 385–410
2 Chang M. Fertilizability of rabbit ova and the effects of temperature *in vitro* on their subsequent fertilization and activation *in vivo*. J Exp Zool, 1952, 121: 351–381
3 Whittingham D. Fertilization *in vitro* and development to term of unfertilized mouse oocytes previously stored at −196°C. J Reprod Fertil, 1977, 49: 89–94
4 Chen C. Pregnancy after human oocyte cryopreservation. Lancet, 1986, 327: 884–886
5 AI-Hasani S, Kirsch J, Diedrich K, et al. Successcul embryo transfer of cryopreserved and *in-vitro* fertilized rabbit oocytes. Hum Reprod, 1989, 4: 77–79
6 Fuku E, Kojima T, Shioya Y, et al. *In vitro* fertilization and development of frozen-thawed bovine oocytes. Cryobiology, 1992, 29: 485–492
7 Maclellan L, Carnevale E, Coutinho da Silva M, et al. Pregnancies from vitrified equine oocytes collected from super-stimulated and non-stimulated mares. Theriogenology, 2002, 58: 911–919
8 Oktay K, Cil A P, Bang H, et al. Efficiency of oocyte cryopreservation: a meta-analysis. Fertil Steril, 2006, 86: 70
9 Bromfield J, Coticchio G, Hutt K, et al. Meiotic spindle dynamics in human oocytes following slow-cooling cryopreservation. Hum Reprod, 2009, 24: 2114–2123
10 Rall W, Fahy G. Ice-free cryopreservation of mouse embryos at −196°C by vitrification. Nature, 1985, 313: 573–575
11 Smith G D, Serafini P C, Fioravanti J, et al. Prospective randomized comparison of human oocyte cryopreservation with slow-rate freezing or vitrification. Fertil Steril, 2010, 94: 2088–2095
12 Moawad A R, Zhu J, Choi I, et al. Production of good-quality blastocyst embryos following IVF of ovine oocytes vitrified at the germinal vesicle stage using a cryoloop. Reprod Fert Develop, 2013, 25: 1204–1215
13 Kohaya N, Fujiwara K, Ito J, et al. Generation of live offspring from vitrified mouse oocytes of C57BL/6J strain. PLoS One, 2013, 8: e58063

14 Aono A, Nagatomo H, Takuma T, et al. Dynamics of intracellular phospholipid membrane organization during oocyte maturation and successful vitrification of immature oocytes retrieved by ovum pick-up in cattle. Theriogenology, 2013, 79: 1146–1152
15 Pope C E, Gómez M C, Kagawa N, et al. *In vivo* survival of domestic cat oocytes after vitrification, intracytoplasmic sperm injection and embryo transfer. Theriogenology, 2012,77: 531–538
16 de Leon P M M, Campos V F, Corcini C D, et al. Cryopreservation of immature equine oocytes, comparing a solid surface vitrification process with open pulled straws and the use of a synthetic ice blocker. Theriogenology, 2012, 77: 21–27
17 Vieira A, Forell F, Feltrin C, et al. Calves Born after direct transfer of vitrified bovine *in vitro*-produced blastocysts derived from vitrified immature oocytes. Reprod Domest Anim, 2008, 43: 314–318
18 Gupta M K, Uhm S J, Lee H T, et al. Cryopreservation of immature and *in vitro* matured porcine oocytes by solid surface vitrification. Theriogenology, 2007, 67: 238–248
19 Gasparrini B, Attanasio L, De Rosa A, et al. Cryopreservation of *in vitro* matured buffalo (*Bubalus bubalis*) oocytes by minimum volumes vitrification methods. Anim Reprod Sci, 2007, 98: 335–342
20 Begin I, Bhatia B, Baldassarre H, et al. Cryopreservation of goat oocytes and *in vivo* derived 2- to 4-cell embryos using the cryoloop (CLV) and solid-surface vitrification (SSV) methods. Theriogenology, 2003, 59: 1839–1850
21 Vajta G, Holm P, Kuwayama M, et al. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev, 1998, 51: 53–58
22 Panagiotidis Y, Vanderzwalmen P, Prapas Y, et al. Open versus closed vitrification of blastocysts from an oocyte-donation programme: a prospective randomized study. Reprod Biomed Online, 2013, 26: 470–476
23 Wang L, Zhou G, Shi W, et al. First live offspring born in superovulated sika deer (*Cervus nippon*) after embryo vitrification. Theriogenology, 2012, 78: 1627–1632
24 Nagashima H, Hiruma K, Saito H, et al. Production of live piglets following cryopreservation of embryos derived from *in vitro*-matured oocytes. Biol Reprod, 2007, 76: 900–905
25 Hong Q H, Tian S J, Zhu S E, et al. Vitrification of boer goat morulae and early blastocysts by straw and open-pulled straw method. Reprod Domest Anim, 2007, 42: 34–38
26 Piltti K, Lindeberg H, Aalto J, et al. Live cubs born after transfer of OPS vitrified-warmed embryos in the farmed European polecat (*Mustela putorius*). Theriogenology, 2004, 61: 811–820
27 Gong G, Dai Y, Fan B, et al. Birth of calves expressing the enhanced green fluorescent protein after transfer of fresh or vitrified/thawed blastocysts produced by somatic cell nuclear transfer. Mol Reprod Dev, 2004, 69: 278–288
28 Isachenko V, Alabart J L, Dattena M, et al. New technology for vitrification and field (microscope-free) warming and transfer of small ruminant embryos. Theriogenology, 2003, 59: 1209–1218
29 López-Béjar M, López-Gatius F. Nonequilibrium cryopreservation of rabbit embryos using a modified (sealed) open pulled straw procedure. Theriogenology, 2002, 58: 1541–52
30 Cho H J, Son W Y, Yoon S H, et al. An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. Hum Reprod, 2002, 17: 2419–22
31 Kuleshova L L, Lopata A. Vitrification can be more favorable than slow cooling. Fertil Steril, 2002, 78: 449–454
32 Forman E J, Li X, Ferry K M, et al. Oocyte vitrification does not increase the risk of embryonic aneuploidy or diminish the implantation potential of blastocysts created after intracytoplasmic sperm injection: a novel, paired randomized controlled trial using DNA fingerprinting. Fertil Steril, 2012, 98: 644–649
33 Kasai M. Advances in the cryopreservation of mammalian oocytes and embryos: development of ultrarapid vitrification. Reprod Med Biol, 2002, 1: 1–9
34 Martino A, Pollard J W, Leibo S, et al. Effect of chilling bovine oocytes on their developmental competence. Mol Reprod Dev, 1996, 45: 503–512
35 Aman R R, Parks J E. Effects of cooling and rewarming on the meiotic spindle and chromosomes of *in vitro*-matured bovine oocytes. Biol Reprod, 1994, 50: 103–110
36 Rall W. Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology, 1987, 24: 387–402
37 Berthelot F, Martinat-Botté F, Perreau C, et al. Birth of piglets after OPS vitrification and transfer of compacted morula stage embryos with intact zona pellucida. Reprod Nutr Dev, 2001, 41: 267
38 Ledda S, Bogliolo L, Succu S, et al. Oocyte cryopreservation: oocyte assessment and strategies for improving survival. Reprod Fertil Dev, 2006, 19: 13–23
39 Paynter S. A rational approach to oocyte cryopreservation. Reprod Biomed Online, 2005, 10: 578–586
40 Rall W, Meyer T. Zona fracture damage and its avoidance during the cryopreservation of mammalian embryos. Theriogenology, 1989, 31:

683–692
41 Hara H, Hwang I S, Kagawa N, et al. High incidence of multiple aster formation in vitrified-warmed bovine oocytes after *in vitro* fertilization. Theriogenology, 2012, 77: 908–915
42 Hara H, Yamane I, Noto I, et al. Microtubule assembly and *in vitro* development of bovine oocytes with increased intracellular glutathione level prior to vitrification and *in vitro* fertilization. Zygote, 2013, 26: 1–7
43 Jackowski S, Leibo S, Mazur P, et al. Glycerol permeabilities of fertilized and unfertilized mouse ova. J Exp Zool, 1980, 212: 329–341
44 Wu C, Rui R, Dai J, et al. Effects of cryopreservation on the developmental competence, ultrastructure and cytoskeletal structure of porcine oocytes. Mol Reprod Dev, 2006, 73: 1454–1462
45 Aigner S, Van der Elst J, Siebzehnriibl E, et al. The influence of slow and ultra-rapid freezing on the organization of the meiotic spindle of the mouse oocyte. Hum Reprod, 1992, 7: 857–864
46 Vincent C, Garnier V, Heyman Y, et al. Solvent effects on cytoskeletal organization and *in-vivo* survival after freezing of rabbit oocytes. J Reprod Fertil, 1989, 87: 809–820
47 Sharma G T, Dubey P K, Chandra V, et al. Morphological changes, DNA damage and developmental competence of *in vitro* matured, vitrified-thawed buffalo (*Bubalus bubalis*) oocytes: a comparative study of two cryoprotectants and two cryodevices. Cryobiology, 2010, 60: 315–321
48 Morató R, Mogas T, Maddox-Hyttel P, et al. Ultrastructure of bovine oocytes exposed to Taxol prior to OPS vitrification. Mol Reprod Dev, 2008, 75: 1318–1326
49 Boiso I, Marti M, Santaló J, et al. A confocal microscopy analysis of the spindle and chromosome configurations of human oocytes cryopreserved at the germinal vesicle and metaphase II stage. Hum Reprod, 2002, 17: 1885–1891
50 Zhou G B, Li N. Cryopreservation of porcine oocytes: recent advances. Mol Hum Reprod, 2009, 15: 279–285
51 Zander-Fox D, Cashman K S, Lane M, et al. The presence of 1 mM glycine in vitrification solutions protects oocyte mitochondrial homeostasis and improves blastocyst development. J Assist Reprod Genet, 2013, 30: 107–116
52 Nagai S, Mabuchi T, Hirata S, et al. Correlation of abnormal mitochondrial distribution in mouse oocytes with reduced developmental competence. Tohoku J Exp Med, 2006, 210: 137–144
53 Schatten G, Simerly C, Schatten H, et al. Microtubule configurations during fertilization, mitosis, and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. Proc Natl Acad Sci USA, 1985, 82: 4152–4156
54 Rienzi L, Ubaldi F, Iacobelli M, et al. Meiotic spindle visualization in living human oocytes. Reprod Biomed Online, 2005,10: 192–198
55 Rienzi L, Martinez F, Ubaldi F, et al. Polscope analysis of meiotic spindle changes in living metaphase II human oocytes during the freezing and thawing procedures. Hum Reprod, 2004, 19: 655–659
56 Bianchi V, Coticchio G, Fava L, et al. Meiotic spindle imaging in human oocytes frozen with a slow freezing procedure involving high sucrose concentration. Hum Reprod, 2005, 20: 1078–1083
57 Coticchio G, Sciajno R, Hutt K, et al. Comparative analysis of the metaphase II spindle of human oocytes through polarized light and high-performance confocal microscopy. Fertil Steril, 2010, 93: 2056–2064
58 Gomes C, Merlini M, Konheim J, et al. Oocyte meiotic-stage-specific differences in spindle depolymerization in response to temperature changes monitored with polarized field microscopy and immunocytochemistry. Fertil Steril, 2012, 97: 714–719
59 Eroglu A, Toth T L, Toner M, et al. Alterations of the cytoskeleton and polyploidy induced by cryopreservation of metaphase II mouse oocytes. Fertil Steril, 1998, 69: 944–957
60 Chen S U, Lien Y R, Chen H F, et al. Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws. Hum Reprod, 2000, 15: 2598–2603
61 Martínez-Burgos M, Herrero L, Megías D, et al. Vitrification versus slow freezing of oocytes: effects on morphologic appearance, meiotic spindle configuration, and DNA damage. Fertil Steril, 2011, 95: 374–377
62 Ciotti P M, Porcu E, Notarangelo L, et al. Meiotic spindle recovery is faster in vitrification of human oocytes compared to slow freezing. Fertil Steril, 2009, 91: 2399–2407
63 Chang C C, Lin C J, Sung L Y, et al. Impact of phase transition on the mouse oocyte spindle during vitrification. Reprod Biomed Online, 2011, 22: 184–191
64 Gook D A, Osborn S M, Johnston W, et al. Cryopreservation of mouse and human oocytes using 1,2-propanediol and the configuration of the meiotic spindle. Hum Reprod, 1993, 8: 1101–1109
65 Goud A, Goud P, Qian C, et al. Cryopreservation of human germinal vesicle stage and *in vitro* matured M II oocytes: influence of cryopreservation media on the survival, fertilization, and early cleavage divisions. Fertil Steril, 2000, 74: 487–494
66 Agca Y, Liu J, Peter A, et al. Effect of developmental stage on bovine oocyte plasma membrane water and cryoprotectant permeability

characteristics. Mol Reprod Dev, 1998, 49: 408–415
67 Ghetler Y, Yavin S, Shalgi R, et al. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. Hum Reprod, 2005, 20: 3385–3389
68 Tucker M J, Wright G, Morton P C, et al. Birth after cryopreservation of immature oocytes with subsequent *in vitro* maturation. Fertil Steril, 1998, 70: 578–579
69 Ian Gallicano G, McGaughey R W, Capco D G, et al. Cytoskeleton of the mouse egg and embryo: reorganization of planar elements. Cell Motil Cytoskel, 1991, 18: 143–154
70 Valojerdi M R, Salehnia M. Developmental potential and ultrastructural injuries of metaphase II (MII) mouse oocytes after slow freezing or vitrification. J Assist Reprod Gen, 2005, 22: 119–127
71 Xia W, Fu X W, Zhou G B, et al. Cytokeratin distribution and expression during the maturation of mouse germinal vesicle oocytes after vitrification. Cryobiology, 2013, 66: 261–266
72 Park S E, Chung H M, Cha K Y, et al. Cryopreservation of ICR mouse oocytes: improved post-thawed preimplantation development after vitrification using Taxol, a cytoskeleton stabilizer. Fertil Steril, 2001, 75: 1177–1184
73 Schmidt D, Nedambale T, Kim C, et al. Effect of cytoskeleton stabilizing agents on bovine matured oocytes following vitrification. Fertil Steril, 2004, 82: S26
74 Ogawa B, Ueno S, Nakayama N, et al. Developmental ability of porcine *in vitro* matured oocytes at the meiosis II stage after vitrification. J Reprod Dev, 2010, 56: 356–361
75 Shi W Q, Zhu S E, Zhang D, et al. Improved development by Taxol pretreatment after vitrification of *in vitro* matured porcine oocytes. Reproduction, 2006, 131: 795–804
76 Zhang J, Nedambale T, Yang M, et al. Improved development of ovine matured oocyte following solid surface vitrification (SSV): effect of cumulus cells and cytoskeleton stabilizer. Anim Reprod Sci, 2009, 110: 46–55
77 Mezzalira A, Vieira A, Barbieri D, et al. Abstracts for poster presentation—cryopreservation/cryobiology-vitrification of matured bovine oocytes treated with Cytochalasin B. Theriogenology, 2002, 57: 472
78 Fujihira T, Nagai H, Fukui Y, et al. Relationship between equilibration times and the presence of cumulus cells, and effect of Taxol treatment for vitrification of *in vitro* matured porcine oocytes. Cryobiology, 2005, 51: 339–343
79 Jiménez-Trigos E, Naturil-Alfonso C, Vicente J S, et al. Post-warming competence of *in vivo* matured rabbit oocytes treated with cytoskeletal stabilization (Taxol) and cytoskeletal relaxant (Cytochalasin B) before vitrification. Reprod Domest Anim, 2013, 48: 15–19
80 Larman M G, Sheehan C B, Gardner D K, et al. Calcium-free vitrification reduces cryoprotectant-induced zona pellucida hardening and increases fertilization rates in mouse oocytes. Reproduction, 2006, 131: 53–61
81 Kline D, Kline J T. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. Dev Biol, 1992, 149: 80–89
82 Pickering S J, Braude P R, Johnson M H, et al. Cryoprotection of human oocytes: inappropriate exposure to DMSO reduces fertilization rates. Hum Reprod, 1991, 6: 142–143
83 Brevini T A, Vassena R, Francisci C, et al. Role of adenosine triphosphate, active mitochondria, and microtubules in the acquisition of developmental competence of parthenogenetically activated pig oocytes. Biol Reprod, 2005, 72: 1218–1223
84 Manipalviratn S, Tong Z B, Stegmann B, et al. Effect of vitrification and thawing on human oocyte ATP concentration. Fertil Steril, 2011, 95: 1839–1841
85 Zhao X M, Du W H, Wang D, et al. Effect of cyclosporine pretreatment on mitochondrial function in vitrified bovine mature oocytes. Fertil Steril, 2011, 95: 2786–2788
86 Van Blerkom J. Mitochondria in human oogenesis and preimplantation embryogenesis: engines of metabolism, ionic regulation and developmental competence. Reproduction, 2004, 128: 269–280
87 Cummins J. The role of mitochondria in the establishment of oocyte functional competence. Eur J Obstet Gynecol Reprod Biol, 2004, 115: S23–S29
88 Cobo A, Perez S, De los Santos M, et al. Effect of different cryopreservation protocols on the metaphase II spindle in human oocytes. Reprod Biomed Online, 2008, 17: 350–359
89 Hochi S, Kozawa M, Fujimoto T, et al. *In Vitro* maturation and transmission electron microscopic observation of horse oocytes after vitrification. Cryobiology, 1996, 33: 300–310
90 Turathum B, Saikhun K, Sangsuwan P, et al. Effects of vitrification on nuclear maturation, ultrastructural changes and gene expression of canine oocytes. Reprod Biol Endocrin, 2010, 8: 70
91 Zhou G B, Zeng Y, Meng Q G, et al. Decreased expression of CD9 in bovine oocytes after cryopreservation and the relationship to fertilization capacity. Mol Reprod Dev, 2013, 80: 451–459

92 Li J, Fu X, Mo X, et al. Vitrification alters acH4K12 and acH4K16 levels in sheep oocytes at various developmental stages. Small Ruminant Res, 2013, 112: 108–113
93 Anchamparuthy V, Pearson R, Gwazdauskas F, et al. Expression pattern of apoptotic genes in vitrified-thawed bovine oocytes. Reprod Domest Anim, 2010, 45: 83–90
94 Succu S, Bebbere D, Bogliolo L, et al. Vitrification of *in vitro* matured ovine oocytes affects *in vitro* pre-implantation development and mRNA abundance. Mol Reprod Dev, 2008, 75: 538–546
95 Lee R K K, Li S H, Lu C H, et al. Abnormally low expression of connexin 37 and connexin 43 in subcutaneously transplanted cryopreserved mouse ovarian tissue. J Assist Reprod Genet, 2008, 25: 489–497
96 Wen Y, Quintero R, Chen B, et al. Expression of CD9 in frozen–thawed mouse oocytes: preliminary experience. Fertil Steril, 2007, 88: 526–529
97 Liu H C, He Z, Rosenwaks Z, et al. Mouse ovarian tissue cryopreservation has only a minor effect on *in vitro* follicular maturation and gene expression. J Assist Reprod Genet, 2003, 20: 421–431
98 Monzo C, Haouzi D, Roman K, et al. Slow freezing and vitrification differentially modify the gene expression profile of human metaphase II oocytes. Hum Reprod, 2012, 27: 2160–2168
99 Chamayou S, Bonaventura G, Alecci C, et al. Consequences of metaphase II oocyte cryopreservation on mRNA content. Cryobiology, 2011, 62: 130–134
100 Larman M, Katz-Jaffe M, Sheehan C, et al. 1,2-propanediol and the type of cryopreservation procedure adversely affect mouse oocyte physiology. Hum Reprod, 2007, 22: 250–259
101 Brenner C, Exley G, Alikani M, et al. O-174 apoptosis and human embryo survival. Fertil Steril, 1997, 68: S86
102 Yang M Y, Rajamahendran R. Expression of Bcl-2 and Bax proteins in relation to quality of bovine oocytes and embryos produced *in vitro*. Anim Reprod Sci, 2002, 70: 159–169
103 Dhali A, Anchamparuthy V, Butler S, et al. Effect of droplet vitrification on development competence, actin cytoskeletal integrity and gene expression in *in vitro* cultured mouse embryos. Theriogenology, 2009, 71: 1408–1416
104 Dhali A, Anchamparuthy V, Butler S, et al. Gene expression and development of mouse zygotes following droplet vitrification. Theriogenology, 2007, 68: 1292–1298
105 Barraud-Lange V, Boissonnas C C, Serres C, et al. Membrane transfer from oocyte to sperm occurs in two CD9-independent ways that do not supply the fertilising ability of Cd9-deleted oocytes. Reproduction, 2012, 144: 53–66
106 Miyado K, Yoshida K, Yamagata K, et al. The fusing ability of sperm is bestowed by CD9-containing vesicles released from eggs in mice. Proc Natl Acad Sci USA, 2008, 105: 12921–12926
107 Mamo S, Bodo S, Kobolak J, et al. Gene expression profiles of vitrified *in vivo* derived 8-cell stage mouse embryos detected by high density oligonucleotide microarrays. Mol Reprod Dev, 2006, 73: 1380–1392
108 Boonkusol D, Gal A B, Bodo S, et al. Gene expression profiles and *in vitro* development following vitrification of pronuclear and 8-cell stage mouse embryos. Mol Reprod Dev, 2006, 73: 700–708
109 Aksu D A, Agca C, Aksu S, et al. Gene expression profiles of vitrified *in vitro*- and *in vivo*-derived bovine blastocysts. Mol Reprod Dev, 2012, 79: 613–625
110 Zhao X M, Ren J J, Du W H, et al. Effect of vitrification on promoter CpG island methylation patterns and expression levels of DNA methyltransferase 1o, histone acetyltransferase 1, and deacetylase 1 in metaphase II mouse oocytes. Fertil Steril, 2013, 100: 256–261
111 Zhao X M, Ren J J, Du W H, et al. Effect of 5-aza-2′-deoxycytidine on methylation of the putative imprinted control region of H19 during the *in vitro* development of vitrified bovine two-cell embryos. Fertil Steril, 2012, 98: 222–227
112 Zhao X M, Du W H, Hao H S, et al. Effect of vitrification on promoter methylation and the expression of pluripotency and differentiation genes in mouse blastocysts. Mol Reprod Dev, 2012, 79: 445–450
113 Milroy C, Liu L, Hammoud S, et al. Differential methylation of pluripotency gene promoters in *in vitro* matured and vitrified, *in vivo*-matured mouse oocytes. Fertil Steril, 2011, 95: 2094–2099
114 Li J J, Pei Y, Zhou G B, et al. Histone deacetyltransferase1 expression in mouse oocyte and their *in vitro*-fertilized embryo: effect of oocyte vitrification. Cryoletters, 2011, 32: 13–20
115 Al-Khtib M, Perret A, Khoueiry R, et al. Vitrification at the germinal vesicle stage does not affect the methylation profile of H19 and KCNQ1OT1 imprinting centers in human oocytes subsequently matured *in vitro*. Fertil Steril, 2011, 95: 1955–1960
116 Yan L Y, Yan J, Qiao J, et al. Effects of oocyte vitrification on histone modifications. Reprod Fertil Dev, 2010, 22: 920–925
117 Wang Z, Xu L, He F, et al. Embryo vitrification affects the methylation of the H19/Igf2 differentially methylated domain and the expression of H19 and Igf2. Fertil Steril, 2010, 93: 2729–2733
118 Suo L, Meng Q, Pei Y, et al. Effect of cryopreservation on acetylation patterns of lysine 12 of histone H4 (acH4K12) in mouse oocytes

and zygotes. J Assist Reprod Genet, 2010, 27: 735–741
119 Trapphoff T, El Hajj N, Zechner U, et al. DNA integrity, growth pattern, spindle formation, chromosomal constitution and imprinting patterns of mouse oocytes from vitrified pre-antral follicles. Hum Reprod, 2010, 25: 3025–3042
120 Spinaci M, Vallorani C, Bucci D, et al. Vitrification of pig oocytes induces changes in histone H4 acetylation and histone H3 lysine 9 methylation (H3K9). Vet Res Commun, 2012, 36: 165–171
121 Prentice J R, Anzar M. Cryopreservation of mammalian oocyte for conservation of animal genetics. Vet Med Int, 2011, 2011: 146405
122 Son W Y, Yoon S H, Yoon H J, et al. Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. Hum Reprod, 2003, 18: 137–139
123 Hiraoka K, Hiraoka K, Kinutani M, et al. Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. Hum Reprod, 2004, 19: 2884–2888
124 Martino A, Pollard J W, Leibo S, et al. Effect of chilling bovine oocytes on their developmental competence. Mol Reprod Dev, 1996, 45: 503–512
125 Dinnyés A, Dai Y, Jiang S, et al. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, *in vitro* fertilization, and somatic cell nuclear transfer. Biol Reprod, 2000, 63: 513–518
126 Fahy G M, Lilley T H, Linsdell H, et al. Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. Cryobiology, 1990, 27: 247–268
127 Nakagata N. High survival rate of unfertilized mouse oocytes after vitrification. J Reprod Fertil, 1989, 87: 479–483
128 Wang C T, Liang L, Witz C, et al. Optimized protocol for cryopreservation of human eggs improves developmental competence and implantation of resulting embryos. J Ovarian Res, 2013, 6: 15
129 Wang L, Liu J, Zhou G B, et al. Quantitative investigations on the effects of exposure durations to the combined cryoprotective agents on mouse oocyte vitrification procedures. Biol Reprod, 2011, 85: 884–894
130 Shaw J, Jones G. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. Hum Reprod Update, 2003, 9: 583–605
131 Tan Y J, Xiong Y, Ding G L, et al. Cryoprotectants up-regulate expression of mouse oocyte AQP7, which facilitates water diffusion during cryopreservation. Fertil Steril, 2013, 99: 1428–1435
132 Lucena E, Bernal D P, Lucena C, et al. Successful ongoing pregnancies after vitrification of oocytes. Fertil Steril, 2006, 85: 108–111
133 Bhat M, Yaqoob S, Khan F, et al. Open pulled straw vitrification of *in vitro* matured sheep oocytes using different cryoprotectants. Small Ruminant Res, 2013, 112: 136–140
134 Cuello C, Sanchez-Osorio J, Almiñana C, et al. Effect of the cryoprotectant concentration on the *in vitro* embryo development and cell proliferation of OPS-vitrified porcine blastocysts. Cryobiology, 2008, 56: 189–194
135 Chian R C, Kuwayama M, Tan L, et al. High survival rate of bovine oocytes matured *in vitro* following vitrification. J Reprod Dev, 2004, 50: 685–696
136 Chian R, Son W, Huang J, et al. High survival rates and pregnancies of human oocytes following vitrification: preliminary report. Fertil Steril, 2005, 84: S36
137 Fahy G M, Wowk B, Wu J, et al. Improved vitrification solutions based on the predictability of vitrification solution toxicity. Cryobiology, 2004, 48: 22–35
138 Kasai M, Komi J, Takakamo A, et al. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. J Reprod Fertil, 1990, 89: 91–97
139 Rho G J, Lee S L, Kim Y S, et al. Intracytoplasmic sperm injection of frozen-thawed bovine oocytes and subsequent embryo development. Mol Reprod Dev, 2004, 68: 449–455
140 Carroll J, Wood M, Whittingham D, et al. Normal fertilization and development of frozen-thawed mouse oocytes: protective action of certain macromolecules. Biol Reprod, 1993, 48: 606–612
141 Nagashima H, Nottle M, Kashiwazaki N, et al. Cryopreservation of porcine embryos. Switzerland WIPO Patent, 1995005075, 1995-02-24
142 Men H, Agca Y, Riley L K, et al. Improved survival of vitrified porcine embryos after partial delipation through chemically stimulated lipolysis and inhibition of apoptosis. Theriogenology, 2006, 66: 2008–2016
143 Moawad A R, Tan S L, Xu B, et al. L-carnitine supplementation during vitrification of mouse oocytes at the germinal vesicle stage improves preimplantation development following maturation and fertilization *in vitro*. Biol Reprod, 2013, 88: 1–8
144 Takahashi T, Inaba Y, Somfai T, et al. Supplementation of culture medium with L-carnitine improves development and cryotolerance of bovine embryos produced *in vitro*. Reprod Fertil Dev, 2013, 25: 589–599
145 Chankitisakul V, Somfai T, Inaba Y, et al. Supplementation of maturation medium with L-carnitine improves cryo-tolerance of bovine *in vitro* matured oocytes. Theriogenology, 2013, 79: 590–598

146 Cobo A, Kuwayama M, Pérez S, et al. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. Fertil Steril, 2008, 89: 1657–1664
147 Prentice-Biensch J R, Singh J, Mapletoft R J, et al. Vitrification of immature bovine cumulus-oocyte complexes: effects of cryoprotectants, the vitrification procedure and warming time on cleavage and embryo development. Reprod Biol Endocrinol, 2012, 10: 73
148 Merlo B, Iacono E, Regazzini M, et al. Cat blastocysts produced *in vitro* from oocytes vitrified using the cryoloop technique and cryopreserved electroejaculated semen. Theriogenology, 2008, 70: 126–130
149 Chian R C, Gilbert L, Huang J Y, et al. Live birth after vitrification of *in vitro* matured human oocytes. Fertil Steril, 2009, 91: 372–376
150 Mukaida T, Nakamura S, Tomiyama T, et al. Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles. Hum Reprod, 2003, 18: 384–391
151 Vanderzwalmen P, Bertin G, Debauche C, et al. Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. Hum Reprod, 2003,18: 1504–1511
152 Cuello C, Berthelot F, Martinat-Botte F, et al. Piglets born after non-surgical deep intrauterine transfer of vitrified blastocysts in gilts. Anim Reprod Sci, 2005, 85: 275–286
153 Bielanski A. A review of the risk of contamination of semen and embryos during cryopreservation and measures to limit cross-contamination during banking to prevent disease transmission in ET practices. Theriogenology, 2012, 77: 467–482
154 Desai N N, Goldberg J M, Austin C, et al. The new Rapid-i carrier is an effective system for human embryo vitrification at both the blastocyst and cleavage stage. Reprod Biol Endocrinol, 2013, 11: 41