冷藏在无电解质溶液中人精子的受精能力

人精子冻存作为一种常用的辅助生殖技术广泛应用于不孕的治疗，但是，冷冻可导致精子活动率和受精能力下降[1]。相反，精子冷藏在2~5 ℃的蛋黄丙酮酸缓冲溶液中能保持较冷冻精子更高的活动率，而且人精子冷藏在5 ℃的蛋黄丙酮酸缓冲溶液中24或48 h，具有较新鲜精子更高的仓鼠卵穿透率或更高的体外受精率[2][3][4]，这是由于蛋黄丙酮酸能加速精子膜胆固醇的脱落，显著降低精子膜的胆固醇/磷脂比率，诱发精子获能，促进精子发生顶体反应[3]。精子获能和发生顶体反应后通常伴随精子死亡[5][6]，因此精子冷藏在蛋黄丙酮酸缓冲溶液中的贮期极为有限。Saito等[7][8]发现人精子冷藏在4 ℃无电解质(electrolyte-free，EF)溶液中，可存活较长时间，且证实此方法能延长贮存期并保持人精子的活动率、存活力和功能。但是，冷藏在EF溶液中人精子的穿透和受精能力并未阐明[9]。本研究旨在观察人精子冷藏在EF溶液中精子顶体状态与活动率的关系，并探讨冷藏精子的穿透能力。

1 材料和方法

1.1 精液标本

精液标本由年龄在26~36岁、有生育能力并禁欲3 d的健康男子捐献。通过手淫法将精液收集到无菌广口瓶中，置室温下30 min使其液化，按WHO标准[10]对精液进行分类、分析。精子的浓度和活动率分别为≥20×10^6/ml和38.2%~53.2%。

1.2 精液的处理

每一精液分成3份。第一份新鲜精液作为对照组；第二份精液按Satio等[7]的方法处理，即将精液置于无电解质3层不同浓度Percoll溶液上，具体为：1 ml 66%Percoll(用含0.5% BSA的0.33 mol/L葡萄糖溶液稀释)+4 ml 44% Percoll(用含1.5% BSA的0.33 mol/L葡萄糖溶液稀释)+3 ml 22% Percoll(用含2.5% BSA的0.33 mol/L葡萄糖溶液稀释)+精液，400 ×g离心20 min后，用2 ml含3% BSA的0.33 mol/L葡萄糖溶液(EF溶液)悬浮精子，放入4 ℃冰箱中保存2周；第三份精液加入0.2 ml含3% BSA的缓冲人输卵管液(modified human tubal fluid, mHTF)，4 ℃冰箱中保存2周。

1.3 冷藏精子重激活步骤

用2 ml含0.4% BSA的mHTF液依次洗涤冷藏在EF溶液中第2、7和14天的人精子，300 × g离心5 min后，精子用0.2 ml含0.4% BSA的mHTF液悬浮，并在37 ℃、5% CO_2孵箱中孵育1 h(重激活)。

1.4 精子活动率的测定

依据WHO标准[10]，在显微镜下用血细胞计数器测定。新鲜人精子和重激活后冷藏在EF和mHTF溶液中第2、7和14天的人精子活动率。

1.5 精子的氯四环素(CTC)染色试验

参考DasGupta等[11]的方法，并略加改进。每次试验前准备750 μmol CTC溶液，避光保存在冰箱中。CTC溶液45 μl与等量精子悬液混合后，加入8 μl 12.5%戊二醛溶液固定，取10 μl固定后的精子悬液涂于干

净玻片上，并用8 μl 抗褪色液和无色指甲油封片。当天或次日按Fraser等[12]的标准在荧光显微镜下检测精子顶体状态。每次至少检测和分类100个精子，精子分为3种类型：F型、B型和AR型，其中F型为未获能精子，B型为获能精子，AR型为顶体反应后精子。

1.6　精子穿透试验

雌性仓鼠腹腔注射40 IU孕马血清促性腺激素，72 h后再腹腔注射人绒毛膜促性腺激素(hCG)40 IU，超促排卵。注射hCG 18 h后收集仓鼠卵子，室温下分别用0.1%透明质酸酶和胰蛋白酶去除颗粒细胞和透明带，去除透明带的卵子用含0.4% BSA的mHTF液洗涤2遍。精子悬液浓度调整为3.5×10^6/ml，将50~100 μl精子悬液置于培养皿中，表面覆盖液体石蜡油。每滴精子悬液中加入30个无透明带的仓鼠卵，37 ℃、5%CO_2条件下孵育3 h，使精卵相互作用，再将卵子取出放在载玻片上，表面覆盖盖玻片并适度加压。在400倍相差显微镜下观察、分析，计数每一卵子中肿胀精子头部的百分率，并通过计数每一穿透卵子中肿胀精子头部的平均数确定受精指数。

1.7　统计分析

采用配对t检验和χ^2检验分析数据，$P<0.05$为有统计学意义。

2　结果

2.1　精子冷藏后的活动率

新鲜精子经Percoll 处理前、后的活动率分别为(46.4±6.3)%和(45.8±7.1)%，两者无显著差异($P>0.05$)。冷藏2 d后，EF溶液中精子的活动率与mHTF溶液中精子的活动率无显著差异；但冷藏1周及2周后，EF溶液中精子的活动率显著高于冷藏在mHTF溶液中精子的活动率[1周时为(43.4±7.9)%比(9.5±2.5)%，2周时为(29.4±7.1)%比(0.9±0.8)%，$P<0.01$]。

2.2　人精子冷藏后的顶体状态

精子冷藏在EF或mHTF溶液中2 d，其获能和顶体反应状态在重激活前用CTC染色法检测，并以新鲜精子作为对照。发现冷藏在mHTF溶液中的F型精子百分率较新鲜精子显著下降，B型和AR型精子显著增多；而冷藏在EF溶液的F型、B型和AR型精子百分率与新鲜精子无显著差异(表1)。但是，冷藏在EF溶液中的精子重激活后，F型精子百分率较激活前显著下降，B型和AR型精子百分率则较激活前显著增加(表2)。

表 1　人精子冷藏在 EF 溶液 1 周后的顶体状态($\bar{x}\pm s$)

Tab.1 Acrosome status of human sperm after preservation in EF solution for 1 week (*Mean±SD*)

Group	Spermatozoa patterns (%)		
	F	B	AR
Control	89.4±2.1	7.6±1.8	3.0±1.7
mHTF	58.6±5.8*	23.6±3.4*	17.8±3.6*
EF	91.2±2.6	6.4±1.8	2.4±1.1

*$P<0.01$ *vs* control group. mHTF: Modified human tubal fluid; EF: Electrolyte-free solution; F: Uncapacitated spermatozoa; B: Capacitated spermatozoa (acrosome intact); AR: Acrosome-reacted spermatozoa

表 2 人精子冷藏在 EF 溶液中重激活前、后的顶体状态($\bar{x}\pm s$)

Tab.2 Acrosome status of human sperm preserved in EF solution before and after reinitiation (*Mean±SD*)

Group	Spermatozoa patterns (%)		
	F	B	AR
Control	89.4±2.1	7.6±1.8	3.0±1.7
EF (before reinitiation)	91.2±2.6	6.4±1.8	2.4±1.1
EF (after reinitiation)	74.5±4.0*	16.0±2.3*	9.4±2.1*

*$P<0.01$ *vs* control group and EF (before reinitiation) group

2.3 冷藏精子受精能力

人精子冷藏在EF溶液中1周，具有与新鲜精子相似的去透明带仓鼠卵穿透百分率和受精指数(48.1%比50.9%，1.38±0.16比1.29±0.13，P>0.05)。

3 讨论

人精子冻存必须面临冻融过程及冻结保护剂的损伤，故冻存后精子的穿透率、受精能力均受影响[13][14][15][16][17][18]。虽然人精子冷藏在2~5 ℃蛋黄丙酮酸缓冲液中较冻存的精子具有较高的活动率及受精能力或去透明带仓鼠卵子穿透率[19][20][21]，但不能避免钠离子进入精子细胞内引起肿胀及细胞破裂，这是影响精子较长时间冷藏的主要原因[22][23]。因此，精子冷藏在蛋黄丙酮酸缓冲液的临床应用受到限制。精子冷藏在EF溶液中2周，其活动率、活力、ATP浓度及形态学明显优于冷藏在NaCl和KCl溶液或蛋黄丙酮酸缓冲液[7][8][9]。

精子活动率是判断受精能力的一个重要指标[24]，故我们首先观察人精子冷藏在mHTF和EF溶液中的活动率，结果显示；人精子冷藏在EF溶液中2周，其活动率显著高于mHTF组，与Saito等[7]的报道一致。可以从以下两个方面加以解释：(1)冷藏在EF溶液的精子可以克服由于过多细胞外钠离子进入精子细胞引起的损伤；(2)精子冷藏在EF溶液中较冷藏在含电解质溶液中可维持较高浓度的ATP浓度，ATP浓度与冷冻精子复苏后的活动率呈正相关[9]。我们接着采用CTC荧光染色检测冷藏对保存在EF和mHTF溶液中的精子顶体状态的影响。CTC染色方法不仅可以分辨顶体完整及发生顶体反应的精子，还能将顶体完整的精子分为未获能和获能的[11]。本研究发现冷藏在mHTF中的B型及AR型精子百分率显著高于新鲜精子，F型精子则显著低于新鲜精子，表明部分冷藏在mHTF中的精子已获能并发生顶体反应，其机制是冷藏过程使精子细胞膜稳定性降低，一些结构蛋白和膜内物质发生变化，或共价蛋白丢失导致精子获能，与文献报道冷藏诱导精子发生获能的观点一致[25]。此外，我们还发现冷藏在EF溶液中F型、B型及AR型精子的比率与新鲜精子无显著差别，B型、AR型精子百分率显著低于冷藏在mHTF中的精子，然而冷藏在EF溶液中精子重激活后B型、AR型精子百分率显著增加。这些结果表明，冷藏在EF溶液中的精子能够恢复获能、发生顶体反应并具有受精能力。本实验结果清楚地显示，在EF溶液中，冷藏过程不能诱导精子获能及发生顶体反应，原因是EF溶液中缺少精子获能所需的钙离子和碳酸氢盐[6][26]。我们在动物实验中已经证实精子冷藏在EF溶液中，其体外受精能力与新鲜精子无显著差异[27]，为了进一步阐明冷藏在EF溶液中的人精子受精能力是否正常，而进行精子穿透试验。结果发现，人精子冷藏在EF溶液中1周 ，重激活后精子穿透去透明带的仓鼠百分卵率及受精指数与新鲜精子无显著差异，从而证实人精子冷藏在EF溶液中具有正常穿透能力，其机制为精子冷藏在EF溶液中重激活后具有较高的活动率，并可恢复获能及顶体反应。

本研究的结论为：(1)精子冷藏在EF溶液中其活动率显著高于冷藏在mHTF溶液中；(2)精子冷藏在EF溶液

中，冷藏过程不能诱导精子获能及发生顶体反应；(3)精子冷藏在EF溶液中重激活后可发生获能及顶体反应；(4)精子冷藏在EF溶液中具有和新鲜精子一样的受精能力。

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回结果列表