离心对小鼠卵母细胞纺锤体定位和早期发育潜力的影响

张军超# 叶 荣# 顾培明 鲍柳君 张焕相 朱子玉*

(苏州大学医学部细胞生物学系, 苏州 215123)

摘要 为了评价离心对卵母细胞纺锤体定位和早期发育潜力的影响, 以小鼠MII期卵母细胞经过1 000、3 000和5 000 r/min离心10 min, 通过免疫荧光细胞化学技术检测纺锤体定位的变化, 并通过孤雌激活和体外受精分析离心处理后卵母细胞的发育潜力。结果显示离心后的卵母细胞纺锤体构像正常, 定位于皮层边缘, 且更靠近第一极体, 离心处理的卵孤雌激活后纺锤体动态和减数分裂进程与对照组一致, 并保持了正常的早期胚胎发育潜力。这些结果提示卵母细胞的离心处理可作为小鼠核移植中去核的前处理, 从而改善在没有霍夫曼镜头情况下盲吸去核的效率。

关键词 卵母细胞; 离心; 纺锤体; 早期发育潜力

体细胞核移植(动物克隆)技术已在多种哺乳动物中获得成功, 但克隆效率一般不超过3%~5%。很多因素导致的供核重编程的不完全被认为是克隆低效率的主要原因[1]。而去核的不完全也是一重要因素, 目前的去核大多采用以第一极体(first polar body, PB1)位置作为指示的盲吸法, 但有研究证实哺乳动物卵母细胞中PB1的位置并不能精确地指示纺锤体的定位, 纺锤体相对于PB1的距离与卵龄有关[2]。因此改善去核程序, 提高去核率, 也是提高克隆效率的重要措施之一。

对卵母细胞进行离心处理过去常常是用来分层卵母细胞内的细胞器[3]或去除卵母细胞内的脂滴以提高深低温保存后的存活率[4,5]。有报道称离心后成熟牛卵更支持核移植重构胚发育[6], 且离心处理导致卵皮层的染色体与PB1的相对位置更接近, 从而明显提高了盲吸法去核的效率[7]。离心后的猪卵去核效率也明显改善, 并支持体细胞核发育到囊胚期[8,9]。目前卵母细胞离心处理用于改善去核程序的研究在小鼠中还没有报道。我们以小鼠卵为材料, 检测离心处理后的卵母细胞纺锤体定位的变化, 并通过体外受精(*in vitro* fertilization, IVF)和孤雌活化分析离心对卵发育潜力的影响, 以探讨核移植中应用离心辅助去核程序的可行性。

1 材料与方法

1.1 小鼠超排与卵母细胞收集

6~8周龄的昆明雌性小鼠(购自苏州大学实验动物中心)腹腔注射10 IU PMSG, 间隔48 h注射10 IU hCG。注射hCG后14~16 h, 将小鼠颈椎脱臼处死, 取输卵管, 在HCZB操作液中划开壶腹部, 收集卵丘卵母细胞复合体(cumulus oocyte complexes, COCs)。用于孤雌激活的COCs在含300 IU透明质酸酶(Sigma)的HCZB中处理, 将消化去卵丘细胞的MII卵洗涤后备用。

1.2 卵母细胞的离心处理

无卵丘的卵母细胞或COCs分别移入1.5 ml的离心管中, 分别以1 000、3 000和5 000 r/min离心10 min。前者用于孤雌激活培养和免疫荧光染色分析; 后者进行体外受精。

1.3 孤雌活化

在无Ca^{2+}的CZB中现加$SrCl_2$, 使其终浓度为10 mmol/L, 并加入终浓度为20 μmol/L的细胞松弛素B以抑制极体排放。活化液平衡后加入对照组MII卵或不同强度离心的卵母细胞, 37 ℃、5% CO_2、饱和湿度下培养5 h, 洗涤后继续用CZB培养液培养。

1.4 体外受精

8~10周龄的昆明雄性小鼠(购自苏州大学实验动物中心)颈椎脱臼处死, 取附睾尾和输精管, 剪成小块, 置于获能液(TYH)中在37 ℃、5% CO_2的培养箱中培养0.5 h, 取出组织块后继续获能培养1 h。吸取

收稿日期: 2009-11-05 接受日期: 2010-01-18

共同第一作者

苏州大学医学发展基金资助项目(No.EE120603)

* 通讯作者。Tel: 0512-65880119, E-mail: zzy64@sina.com

少量精子悬液(终浓度为10^6~10^7个/ml)加入含对照或离心处理的COCs的CZB培养液中，培养4~6 h，洗去精子后移入新的CZB培养液中继续培养。

1.5 免疫荧光化学与激光共聚焦显微镜观察

免疫荧光细胞化学染色方法参见文献[10]。不同强度离心处理后的卵母细胞及孤雌激活卵置于3.7%多聚甲醛室温固定40 min。固定的卵在含有0.1% Triton X-100和0.3% BSA和的PBS中37 ℃温育30~40 min作打孔处理，用含0.01% Triton X-100的PBS清洗3次后，移入含150 mmol/L甘氨酸和0.3% BSA的PBS中，室温下温育2 h作封闭处理。样品与1∶100稀释的FITC标记的抗β微管蛋白抗体(monoclonal anti-β-tubulin-FITC, Sigma)共同温育，4 ℃过夜。清洗液洗涤3次，每次5 min，最后与10 μg/ml碘化丙啶(PI)温育10 min，进行核酸荧光染色。在洁净的载玻片上加一滴防荧光淬灭剂(DABCO, Sigma)，将处理好的卵转移其中，封片后置于暗盒，低温保存，尽快用激光共聚焦显微镜(confocal laser microscope, Leica TCS SP2, Germany)观察，取图，并统计纺锤体与PB1不同角度的比例以分析二者的相对距离。图像用Photoshop-CS处理。

1.6 统计分析

实验重复3次以上，所获数据用SPSS卡方检验作差异显著性分析。

2 结果

2.1 离心对纺锤体定位的影响

微管的免疫荧光染色显示对照组小鼠MII期卵母细胞纺锤体位于卵皮层，具有双极桶状构像，纺锤体相对于PB1的位置不确定(图1A，图1B)，35% (28/80)的卵纺锤体与PB1的夹角小于30° (表1)。1 000、3 000或5 000 r/min离心处理均保持了纺锤体的正常构像，与对照相比定位更靠近卵皮层边缘，且多数卵纺锤体与PB1之间的相对距离缩短(图1C~图1E)。1 000和3 000 r/min离心的卵分别有66.7% (70/105)和72.4% (63/87)的纺锤体与PB1的夹角小于30° (表1)，均与对照组差异显著(P<0.05)，表明离心改变了纺锤体的卵胞质定位。

2.2 离心对孤雌激活卵的纺锤体动态和功能的影响

对照组卵母细胞孤雌活化1~2 h发生染色体分离，4~5 h完成第二次减数分裂，在极体排放被抑制的情况下形成2个原核(图2A~图2C)。3 000 r/min离心处理的卵孤雌激活后的纺锤体动态和减数分裂进程与对照一致(图2D~图2F)，表明离心处理不影响纺锤体的正常构像和功能。

2.3 离心对小鼠卵孤雌激活或体外受精后的早期发育潜力的影响

离心处理后的小鼠卵母细胞进行孤雌激活，结果发现激活率、2-细胞率及桑囊胚率与对照组相比无显著性差异(P>0.05) (表2)。离心处理的卵进行体外受精后的受精率、2-细胞率及桑囊胚率与对照相比也无显著差异(P>0.05) (表3)。这些结果表明离心对小鼠卵孤雌激活或体外受精后的早期发育潜力没有不利影响。

Table 1 Effects of centrifugation of mouse oocytes on the localization of meiotic spindle

Group	Total oocytes	Angle (°) of spindle with regard to PB1				
		< 15	15–30	30–45	45–60	> 60
Control	80	11 (13.8)	17 (21.2)	28 (35.0)	15 (18.8)	8 (10.0)
1 000 r/min	105	37 (35.2) [a]	33 (31.4) [a]	23 (21.9) [a]	10 (9.5) [a]	2 (1.9) [a]
3 000 r/min	87	30 (34.5) [a]	33 (37.9) [a]	17 (19.5) [a]	7 (8.0) [a]	0 (0.0) [a]

The label a indicates significant difference compared with control (P<0.05).

Table 2 Early development of parthenogenetic embryos derived from centrifuged MII mouse oocytes

Group	Total oocytes	Activated oocytes (%)	Number (%) of parthenogenetic embryos to		
			2-cell	4-cell	Mol-Bla
Control 1	132	124 (93.9)	113 (85.6)	61 (46.2)	37 (28.0)
1 000 r/min	181	174 (96.1)	161 (88.9)	89 (49.2)	55 (30.1)
Control 2	71	60 (84.5)	58 (81.7)	36 (50.1)	24 (33.8)
3 000 r/min	134	110 (82.1)	102 (76.1)	64 (47.8)	42 (31.3)
Control 3	89	78 (87.6)	71 (79.8)	40 (44.9)	19 (21.3)
5 000 r/min	124	110 (88.7)	101 (81.5)	58 (46.8)	25 (20.2)

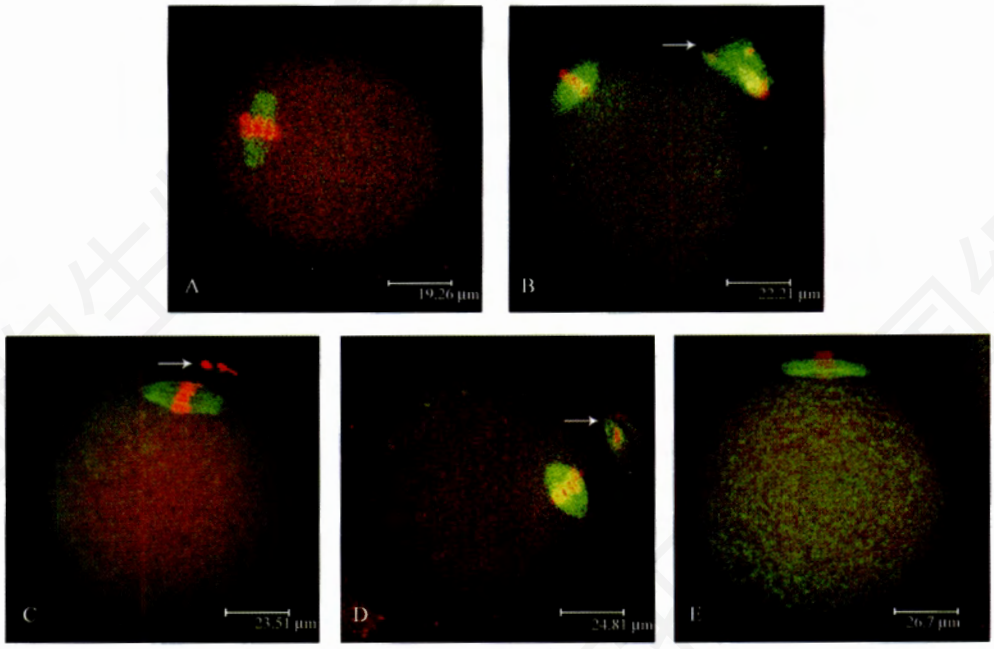

Fig.1 Configuration and localization of meiotic spindle of MII mouse oocytes after centrifugation (green: microtubules; red: chromatin; arrow shows PB1)

A, B: control; C–E: centrifugation at 1 000, 3 000, and 5 000 r/min for 10 min, respectively.

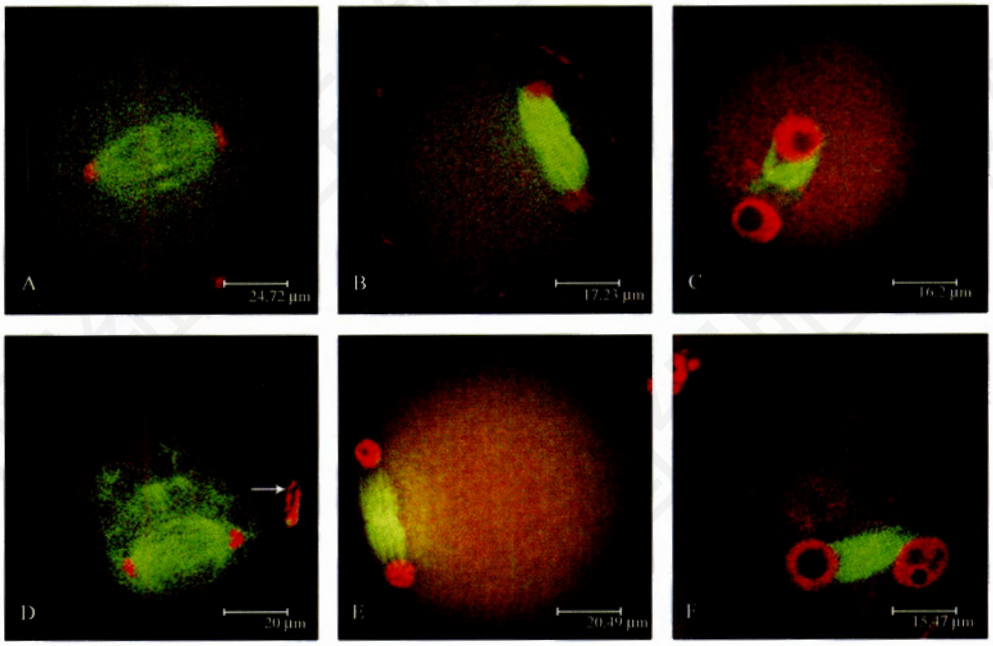

Fig.2 Spindle dynamics of parthenogenetic mouse oocytes treated with centrifugation (green: microtubules; red: chromatin; arrow shows PB1)

A–C: control oocytes 1–2 h, 2–3 h, and 4–5 h after parthenogenetic activation, respectively; D–F: centrifuged oocytes at 3 000 r/min 1–2 h, 2–3 h, and 4–5 h after parthenogenetic activation, respectively.

3 讨论

哺乳动物成熟的卵母细胞通常阻滞于MII期, 减数分裂纺锤体定位于卵皮层区域。动物克隆技术一般采用去核的MII卵母细胞为核移植受体, 去核的卵胞质才能支持供体核的重编程和重构胚的发育, 因此卵母细胞的去核是克隆技术的关键步骤之一[11,12]。

Table 3 Early development of fertilized embryos *in vitro* derived from centrifuged mouse oocytes

Group	Total oocytes	Fertilized oocytes (%)	Number (%) of IVF embryos to		
			2-cell	4-cell	Mol-Bla
Control 1	142	78 (54.9)	53 (35.9)	28 (19.7)	13 (9.2)
1 000 r/min	134	69 (51.5)	43 (32.1)	24 (17.9)	11 (8.2)
Control 2	161	72 (44.7)	47 (29.2)	22 (13.7)	10 (6.2)
3 000 r/min	183	84 (45.9)	56 (30.6)	28 (15.3)	11 (6.0)

目前的去核大多采用盲吸法，其原理是假定纺锤体-染色体复合物定位于PB1下方的卵皮层区域[13]。但实际上PB1在卵周隙中的位置是可变化的，因此纺锤体相对于PB1的定位具有不确定性[2]，依据PB1的位置盲目去核不可避免地造成去核的不彻底，这也是限制克隆效率的重要因素之一。早期有研究将牛卵离心后去核用于胚胎细胞克隆[14]。最近在牛和猪的体细胞核移植研究中也开展了离心辅助去核的尝试，结果均显示离心应用于牛或猪卵的去核可大大提高去核效率，且支持植入前发育，甚至发育潜力也得到提高[6~9]。小鼠卵母细胞核借助霍夫曼(Hoffmann)镜头或微分干涉(DIC)镜头可以清晰显示，但缺乏这些镜头的情况下依据PB1的位置去核带有较大的盲目性。我们对小鼠MII卵母细胞进行了不同强度的离心处理，结果显示纺锤体构像正常，定位更靠近卵皮层边缘，且与PB1的相对距离缩短，这与牛卵离心处理的结果相似[7]。表明适当的离心处理可导致纺锤体定位发生改变，与PB1更靠近，从而改善以PB1为指示的盲吸法去核的效率。而且这种离心辅助去核可以有效地减少卵胞质损失，避免卵胞质大量吸出对重构胚发育的不利影响[15]。

离心处理导致小鼠卵纺锤体定位的变化，但保持了其正常构像。孤雌激活后的纺锤体动态和减数分裂进程与对照组一致，表明离心不影响卵母细胞正常的纺锤体构像和功能。我们的研究表明离心处理也不影响小鼠卵孤雌激活或体外受精后的早期发育潜力，这与成熟牛卵离心的结果一致[16]。另有报道显示猪、小鼠和牛的受精卵离心处理不影响其后续发育[17~19]。这些结果表明虽然离心可导致卵母细胞或受精卵胞质的细胞器包括纺锤体的位置发生改变，但决定卵母细胞激活和早期胚胎发育的生化基础未受破坏，甚至离心处理的牛卵胞质更支持核移植重构胚的早期发育[6]。

我们的研究提示小鼠卵母细胞离心处理不影响其早期发育潜力，而纺锤体定位的变化有助于更准确地进行盲吸法去核。推测离心处理可作为小鼠核移植去核的前处理，从而改善没有霍夫曼镜头情况下的盲吸法去核效率。

感谢苏州大学大学生创新性实验计划项目的资助和本科生管文彩、林晓燕和沈立军等同学的大力协助。

参考文献(References)

1 Zhu ZY, Jiang MX, Yan LY, Huang JC, Lei ZL, Jiang Y, *et al.* Cytoskeletal and nuclear organization in mouse embryos derived from nuclear transfer and ICSI: a comparison of agamogony and syngamy before and during the first cell cycle. Mol Reprod Dev 2007; 74(5): 655-63.

2 Silva CP, Kommineni K, Oldenbourg R, Keefe DL. The first polar body does not predict accurately the location of the metaphase II meiotic spindle in mammalian oocytes. Fertil Steril 1999; 71(4): 719-21.

3 Cran DG. The distribution of organelles in mammalian oocytes following centrifugation prior to injection of foreign DNA. Gamete Res 1987; 18(1): 67-76.

4 Murakami M, Otoi T, Sumantri C, Suzuki T. Effects of centrifugation and lipid removal on the cryopreservation of in vitro produced bovine embryos at the eight-cell stage. Cryobiology 1998; 36(3): 206-12.

5 Hara K, Abe Y, Kumada N, Aono N, Kobayashi J, Matsumoto H, *et al.* Extrusion and removal of lipid from the cytoplasm of porcine oocytes at the germinal vesicle stage: centrifugation under hypertonic conditions influences vitrification. Cryobiology 2005; 50(2): 216-22.

6 Li GP, Bunch TD, White KL, Rickords L, Liu Y, Sessions BR. Denuding and centrifugation of maturing bovine oocytes alters oocyte spindle integrity and the ability of cytoplasm to support parthenogenetic and nuclear transfer embryo development. Mol Reprod Dev 2006; 73(4): 446-51.

7 Hua S, Zhang Z, Zhang C, Zhang Y. An improved enucleation method of bovine somatic cell nuclear transfer. J Genet Genomics 2007; 34(6): 491-6.

8 Savard C, Novak S, Saint-Cyr A, Moreau M, Pothier F, Sirard MA. Comparison of bulk enucleation methods for porcine oocytes. Mol Reprod Dev 2004; 67(1): 70-6.

9 Fahrudin M, Kikuchi K, Kurniani Karja NW, Ozawa M, Maedomari N, Somfai T, *et al.* Development to the blastocyst stage of porcine somatic cell nuclear transfer embryos reconstructed by the fusion of cumulus cells and cytoplasts prepared by gradient centrifugation. Cloning Stem Cells 2007; 9(2): 216-

28.
10 张军超, 叶 荣, 鲍柳君, 张焕相, 朱子玉。低温对小鼠卵母细胞纺锤体损伤和发育潜力的影响。细胞生物学杂志 2009; 31(3): 407-13.
11 Li GP, White KL, Bunch TD. Review of enucleation methods and procedures used in animal cloning: state of the art. Cloning Stem Cells 2004; 6(1): 5-13.
12 Fulka J Jr, Loi P, Fulka H, Ptak G, Nagai T. Nucleus transfer in mammals: noninvasive approaches for the preparation of cytoplasts. Trends Biotechnol 2004; 22(6): 279-83.
13 Prather RS, Barnes FL, Sims MM, Robl JM, Eyestone WH, First NL. Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. Biol Reprod 1987; 37(4): 859-66.
14 Tatham BG, Dowsing AT, Trounson AO. Enucleation by centrifugation of *in vitro*-matured bovine oocytes for use in nuclear transfer. Biol Reprod 1995; 53(5): 1088-94.
15 Dominko T, Chan A, Simerly C, Luetjens CM, Hewitson L, Martinovich C, *et al*. Dynamic imaging of the metaphase II spindle and maternal chromosomes in bovine oocytes: implications for enucleation efficiency verification, avoidance of parthenogenesis, and successful embryogenesis. Biol Reprod 2000; 62(1): 150-4.
16 Chung JT, Downey BR, Casper RF, Chian RC. Effect of centrifugation on early embryonic development and parthenogenetic activation of bovine oocytes matured *in vitro*. Reprod Fertil Dev 2001; 13(5-6): 383-8.
17 Wall RJ, Pursel VG, Hammer RE, Brinster RL. Development of porcine ova that were centrifuged to permit visualization of pronuclei and nuclei. Biol Reprod 1985; 32(3): 645-51.
18 Yanagimachi R, Yang CH. Preparation of nucleate and anucleate fragments of hamster and mouse eggs by centrifugation. J Exp Zool 1990; 253(2): 220-5.
19 Wall RJ, Hawk HW. Development of centrifuged cow zygotes cultured in rabbit oviducts. J Reprod Fertil 1988; 82(2): 673-80.

Effects of Centrifugation of Mouse Oocytes on the Localization of Meiotic Spindle and the Early Developmental Potential

Jun-Chao Zhang#, Rong Ye#, Pei-Ming Gu, Liu-Jun Bao, Huan-Xiang Zhang, Zi-Yu Zhu*
(*Department of Cell Biology, Medical College of Soochow University, Suzhou 215123, China*)

Abstract In order to evaluate the effects of centrifugation on the location of the meiotic spindle and early developmental potential of mouse oocytes, the metaphase-II mouse oocytes were centrifuged at 1 000, 3 000 and 5 000 r/min for 10 minutes. The location of the meiotic spindles of centrifuged oocytes was detected by confocal microscopy, and their early developmental potential was analyzed after parthenogenetic activation and *in vitro* fertilization (IVF). The results displayed that the meiotic spindles of the centrifuged oocytes showed the normal configuration, and located at the edge of cortex, adjoining the PB1. The spindle dynamics and the meiotic progress of parthenogenetic activated oocytes treated with centrifugation were consistent with control, and centrifuged oocytes kept the normal early developmental potential after parthenogenetic activation or IVF. The present results imply that the centrifugation-pretreatment of mouse oocytes before enucleation operation may improve the efficiency of enucleation without Hoffmann objective during nuclear transfer.

Key words oocyte; centrifugation; spindle; early developmental potential

Received: November 5, 2009 Accepted: January 18, 2010
#Jun-Chao Zhang and Rong Ye contributed equally to this work
This work was supported by the Medical Development Foundation of Soochow University (No.EE120603)
*Corresponding author. Tel: 86-512-65880119, E-mail: zzy64@sina.com