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Research Article

Is nucleo-cytoplasmic incompatibility the reason of acceleration polar body extrusion?

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Abstract

Timing extrusion of the first polar bodies (PBs) is significantly accelerated of fully grown germinal vesicle (GV) cytoplasts reconstituted with embryonic/somatic nuclei during maturation through unknown mechanism. The aim of the present study was to determine timing extrusions of PBs throughout combinations of recipient cytoplasts with donor nuclei and enucleolation of GV oocytes. The recipient cytoplasts usd were cytoplasts of fully grown germinal and metaphase I (MI) oocytes. The donor nuclei used were karyoplasts of growing and fully grown germinal vesicle oocytes; nucleolated and anuclolated G2 nuclei of 1/2-blastomeres; G2 and M-phase nuclei of 1/4-blastomeres. In addition, fully grown germinal vesicle oocytes were enucleolated. Timing of germinal vesicle breakdown and polar body extrusion and nuclear morphology were monitored upon introducing the donor nuclei into recipient cytoplasts. The results indicated that germinal vesicle breakdown (GVBD) and polar body extrusion were extended/delayed of GV cytoplasts reconstructed with GVgr karyoplasts (karyoplasts of growing oocytes) compared with those reconstructed with GV karyoplasts (karyoplasts of fully grown oocytes) as well as control and enucleolated oocytes. Timing extrusions of PBs were not differed between GV cytoplasts reconstituted either with nucleolated or anucleolated donor nuclei.Timing extrusions of PBs and percentage of maturation (17h of maturation) were differed between G2 and M-phase donor nuclei (1/4-blastomere) which introduced into MI (metaphase I) cytoplasts. It might be concluded that nucleo-cytoplasmic incompatibility might be the reason of acceleration of PB extrusions.

Keywords: *Nucleo-cytoplasmic; enucleolation; polar body; acceleration; extrusion*

1. Introduction

Embryonic/somatic nuclear transfer has been performed in mammals (**Choi et al. 2013**) with low efficiency. Recently, embryonic/somatic nuclei were transferred into enucleated GV oocytes for meiotic maturation of the reconstructed oocytes **(Chang et al., 2004; Grabarek et al., 2004; Polanski et al., 2005; Nan et al., 2007).** Our results (**Mohammed et al., 2008; Mohammed et al., 2010;**) and the previous ones demonstrated that meiotic maturation of GV cytoplasts reconstructed with embryonic/somatic nuclei were associated with abnormalities in cytokinesis and chromosome condensation as well as earlier extrusions of PBs.

The extrusion of the first polar bodies is significantly accelerated of GV cytoplasts reconstituted with embryonic/somatic nuclei in comparison with those reconstituted with GV nuclei which might affect in chromosomal condensation and their alignment over the metaphase spindle which disrubted **(Chang et al., 2004; Grabarek et al., 2004; Polanski et al., 2005; Nan et al., 2007; Mohammed et al., 2008; Mohammed et al., 2010)**.

It is unclear what caused such acceleration. **Chang et al. (2004)** suggested that the acceleration might due to MPF and MAK kinases of the donor nuclei at G2/M stage whereas **Mohammed et al., (2010)** found acceleration of polar body extrusion of GV cytoplasts reconstituted with G1, S-phase and G2 stages donor nuclei and the timing of PBs extrusions did not differ between them.

The acceleration might be due to GV-associated factors. **Mohammed et al., (2008)** found acceleration of PB extrusion with GV cytoplasts which their nuclear contents released into the cytoplasm with selective enucleation compared with those did not contain. One of the GV-associated karyoplast factors might be the nucleolus. Another factor of acceleration might be nucleo-cytoplasmic incompatibility of embryonic/somatic donor nuclei with GV recipient cytoplasts. Therefore, the aim of the current study was to determine timing extrusions of PBs throughout; 1) combinations of recipient cytoplasts and donor nuclei and 2) enucleolation of GV oocytes

2. Materials and methods

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Reagents and culture media

All inorganic and organic compounds used in this study were purchased from Sigma (Sigma Chemical Co., St. Louis, MO), unless otherwise stated. All media were prepared fresh and sterilized by filtering through a 0.22-µm filter (Acrodisc; Pall Gelman Laboratory, Ann Arbor, MI).

Animals. Young (1 week; 6-8 week old) and mature mice originated from the mouse colony which is bred in the Department of Experimental Embryology. Mice were kept under a 12h light/12h dark cycle starting at 7 a.m. Food (Labofeed H, Poland) and water were available *ad libitum*. Donor females were killed by cervical dislocation.

Recipient cells. Fully grown germinal vesicles oocytes were used as recipients of donor nuclei. For collection of germinal vesicle (GV) oocytes, 6- to 8-week old F1 (C57Bl10xCBA/H) females were injected with 7.5 IU of pregnant serum gonadotrophin (PMSG; Folligon, Intervet, Holland). Ovaries were removed from the donor females 44-48 h after PMSG injection. Antral follicles were punctured by 30-ga needles, and cumulus - GV oocyte complexes were released into Hepes-buffered M2 medium containing 0.2 mM dibutyryl cyclic adenosine 3',5'-monophosphate (dbcAMP) to inhibit GV breakdown. Cumulus cells were removed by gentle, repeated pipetting of oocytes

Donor cells. As the donors, karyoplasts of growing and fully grown GV oocytes and cleaving embryos were used. Fully grown GV oocytes were collected as was described above.

Growing oocytes. The ovaries of 7 day old MIZ (Swiss albino) females were placed in 3.5 ml of PBS containing 200 IU/ml hyaluronidase for 30 min. Oocyte–granulosa cell complexes were freed of the pre-antral follicles and the granulosa cells were removed by pipetting. The oocytes were then placed in phosphate-buffered saline containing 0.5% pronase to further remove the zona pellucida.

Cleaving embryos. Embryos were collected from mature MIZ (Swiss albino) female mice approximately 3 months old. Females were superovulated by injection of 7.5 IU of pregnant mare serum gonadotropin (PMSG; Folligon, Intervet, Holland) followed by 7.5 IU of human chorionic gonadotropin (hCG, Chorulon, Intervet, Holland) 48 h later and mated with F1 (C57Bl10xCBA/H) males. Two-cell stage embryos obtained from intact and enucleolated oocytes after *in vitro* maturation and fertilization were used as a source of nucleolated and anucleolated nuclei at time corresponding to their G2 stage, respectively. Four-cell stage embryos were collected from the oviducts 56-58 h after hCG injection and used as a source of G2 stage nuclei or were cultured in KSOM medium at 37 °C and used as a source of M- phase stage nuclei.

Isolation of 1/2 and 1/4 blastomeres. *Zonae pellucidae* were removed from cleaving embryos by treatment with 0.5% pronase in PBS for 3-5 min. After rinsing the embryos in M2 medium they were transferred to Dulbecco's salt solution (without Ca and Mg ions) for 15 min. After this treatment, the embryos were placed in M2 medium and were disaggregated into single blastomers by repeated pipetting with a flame-polished narrow-bore pipette.

Micromanipulations tools and equipment. All manipulations were performed under inverted Leitz Fluowert microscope (Leitz, Germany) equipped with Nomarski differential-interferential contrast (DIC) and connected with Leitz (Germany) mechanical micromanipulators. Beaudouin Alcatel (France) and Cell Tram Air (Eppendorf, Germany) micropumps were connected with micromanipulation and holding pipettes, respectively. Pipettes were prepared from thin-walled borosilicate glass capillaries of an external diameter 1 mm (GC 100T-15, Harvard Apparatus Ltd, Kent, Great Britain). Enucleation and nuclear injection pipettes were prepared using PB-7 vertical puller (Narishige, Japan). After pulling, the end of pipettes was broken at the appropriate point using a MF-79 micro-forge (Narishige, Japan) and bevelled on an EG-4 grinding wheel (Narishige, Japan) to obtain 45° cut. To facilitate penetration of the *zona pellucida* and to minimalize the damage of the oolemma, a spike was formed (by means of the MF-79 Narishige micro-forge) at the tip of enucleation and injection pipettes and the tools were bent with micro-forge (Alcatel, France). For enucleolation, the narrow conical micropipettes were pulled out on M-97 micropipette puller (Sutter Instrument Co., USA). The ends of pipettes were bent twice in order to adopt them to the micromanipulation chamber used.

Complete enucleation of GV oocytes. Complete enucleation (CE) of GV stage oocytes was performed as described by **Grabarek et al., (2004)** (**see Mohammed et al., 2008**). Briefly, GV with a smallest possible amount of surrounding cytoplasm was removed by smooth suction. In some experiments GV karyoplasts were saved to be used for oocyte reconstruction.

Enucleolation of GV oocytes. The enucleolation of mouse GV oocytes was performed according to the method described by **Fulka et al., (2003)** for enucleolation of pig oocytes (see **Mohammed et al., 2008**).

Nuclear transfer. Blastomeres isolated from 2 - and 4-cell stages were preincubated in M2 medium supplemented with CB (5µg/ml) for 20-30 min before being placed in a micromanipulation chamber filled with the enucleation medium. In the case of 1/4 blastomeres, the whole cell was introduced under the *zona pellucida*. When ½ blastomeres were used the whole blastomere was partially aspirated into the micropipette with its nucleus always located inside the pipette. The reduction of the blastomere (cytoplasm) volume was achieved by "cutting off" the non-aspirated part of the blastomere during penetration of the pipette through the *zona pellucida.* Karyoplasts originated from ½ blastomeres comprised 15-20% of the volume of the oocyte. They were introduced into the perivitelline space of ooplasts through the slit in the *zona pellucida* made during enucleation. After insertion of a donor cell into the perivitelline space the pairs were washed out of enucleation solution and were placed in M2 with dbcAMP to inhibit maturation, and were incubated until electrofusion. Then, the karyoplast – oocyte complexes were washed twice in electrofusion solution (0.3 M mannitol suplpplemented with 0.1 mM

$CaCl_2$, and 0.1 mM $MgSO_4$) and were placed between two parallel platinum electrodes in an electrofusion chamber filled with the same solution. Complexes were exposed to 2 DC pulses (50-60 µsec each, 1.8–2.5 $kV.cm^{-1}$) generated by the BTX 2001 ElectroCell Manipulator (Genetronics, Inc., San Diego, CA). Fusion usually occurred within 30 min. Fused pairs were then washed in M2 and were cultured in maturation medium up to 17 hrs.

Germinal vesicle nuclear transfer. For nuclear transfer, the previously obtained GV karyoplasts from fully grown oocytes or growing germinal vesicle oocytes were placed in a small drop of enucleation medium under paraffin oil in a micromanipulation chamber, close to the recipient cytoplasts. The small growing GV oocyte was wholly aspirated whereas the large growing GV oocyte was partially aspirated into the micropipette with its nucleus always located inside the pipette. The reduction of the oocyte (cytoplasm) volume was achieved by "cutting off" as previously indicated. The karyoplasts were introduced into the perivitelline space of enucleated oocytes through the slit in the *zona pellucida* made during enucleation. For fusion they were transferred into fusion chamber (BTX Genetronics, San Diego, USA) filled with the fusion medium (0.1 mM $CaCI_2$ and 0.05 mM $MgSO_4$ in HTF [Human Tubal Fluid (**Liu et al., 1999**)] and placed between two parallel platinum electrodes connected to the pulse generator (ElectroCell Manipulator 2001, BTX Genetronics, San Diego, USA). After manual alignment (cell contact plane perpendicular to the electric field vector), the GV karyoplast – enucleate oocyte complexes were submitted to 2 direct current (DC) pulses of 50 µs each and with the strength of 1.5-2.1 $kV.cm^{-1}$. After electric field treatment, complexes were washed twice in M2 medium + dbcAMP (0.2 mM), placed in the same medium, kept at 37°C and checked every 10 min. Fusion usually occurred within 10-30 min**.**

***In vitro* maturation of oocytes.** For *in vitro* maturation**,** oocytes were transferred into 50 µl droplets (10 oocytes/cytoplasts per droplet) of pre-equilibrated IVM medium (TCM199 medium supplemented with 10% fetal calf serum, 75 µg/ml penicillin G potassium salt and 50 µg/ml streptomycin sulfate) overlaid with light mineral oil and were cultured at 37 °C in an atmosphere of 5% CO2 in air with the saturated humidity.

Cytological studies during maturation. The control and enucleolated oocytes and the cytoplasts reconstructed with GV/GVgr were examined for GVBD under inverted microscope. To examine the nuclear morphology at 17 h postfusion for the MII stage, oocytes were fixed by transferring them into Heidenhein's fixative solution and stained for DNA with 10 µg/ml Hoechst. The GVBD was confirmed under regular light microscope while nuclear morphology of the MII stage as well as presence of the first polar body were analyzed under an epifluorescence microscope. Whole-mount preparations were made for some of matured oocytes according to the method decribed by **Tarkowski and Wróblewska (1967).**

3. Results

Germinal vesicle breakdown of enucleolated and reconstructed oocytes with GVgr karyoplasts. Germinal vesicle breakdown occurred 2-3 h after starting maturation in the control and enucleolated oocytes and in GV cytoplasts reconstructed with GV karyoplasts, whereas it started at 3h and extended till **>**5h post fusion with the ones received GVgr karyoplasts (**Table 1**).

Timing extrusions of polar bodies. In control and enucleolated oocytes, extrusions of the first polar bodies (PBs) were oocurred 9-12 h after the onset of maturation (**Table 2**). When GV cytoplasts were reconstituted with GV or GVgr, the extrusions of PBs were started two hours later than in the control and enucleolated oocytes and more extended with those reconstructed with GVgr nuclei (**Table 2**). When 1/2-blastomere nuclei were used to reconstruct GV cytoplasts; timing extrusions of polar bodies were not differed between reconstituted GV cytoplasts with either anucleolated (1/2-blastomere nuclei originated from enucleolated GV oocytes) or nucleolated ones (1/2-blastomere nuclei originated from GV oocytes) (**Table 3**). Therefore, timing extrusion of polar body is not nucleolus dependent and might relate to incompatible nucleo-cytoplasmic complex. This suggestion has been tested using 1/4 blatomeres at either G2 or M-phase which transferred into MI cytoplasts. Timing extrusions were differed between reconstituted oocytes (**Table 4**).

Nuclear morphology upon nuclear transfer. After 17 h of culture, nuclear morphology was observed in cytoplasts reconstructed with 1/4-blastomeres at G2 or M-phase. Nuclear anomalies were slightly decreased with those reconstructed with 1/4-blastomere nuclei at M-phase (**Table 5**).

Table 1. Germinal vesicle breakdown of enucleolated and reconstructed oocytes with GVgr karyoplasts

Oocyte/Cytoplast	Karyoplast	No. examined oocytes	Time post fusion
			GVBD
Control		40	2-3 h
Enucleolated	-	31	2-3 h
GV	GV	35	2-3 h
GV	GVgr	25	3->5 h

GV; fully grown germinal vesicle cytoplast/karyoplast , GVgr; germinal vesicle karyoplast of growing oocytes, GVBD; germinal vesicle breakdown

Table 2. Timing extrusions of polar bodies of enucleolated and reconstructed GV cytoplasts with GVgr karyoplasts

Oocyte/ Cytoplast	Karyoplast	No. oocytes	Timing extrusion, % (n)						
			9h	10h	11h	12h	13h	14h	15h
Control	-	40	25.0	70.0	90.0	100			
Enucleolated	-	31	45.2	74.2	87.1	96.8	100		
GV	GV	33			33.3	78.8	100		
GV	GVgr	20			10.0	25.0	45.0	65.0	100

GV; fully grown germinal vesicle cytoplast/karyoplast
GVgr; germinal vesicle karyoplast of growing oocytes

Table 3. Timing extrusions of polar bodies (PBs) of GV cytoplasts reconstructed with anucleolated G2 nuclei of 1/2-blastomere

Donor nuclei	No oocytes	Timing extrusion, % (n)		
		6h	7h	8h
Anucleolated	10	6 (60)	9 (90)	10.0 (100)
Nucleolated	25	68.0(17)	84.0(21)	100.0(25)

GV; fully grown germinal vesicle cytoplast

Table 4. Timing extrusion of MI cytoplasts reconstructed with 1/4-blastomeres at G2 and M-phase stages

Donor nuclei	No oocytes, % (n)	Timing extrusion (h)				
		3h	4h	5h	6h	7h
G2	5	60.0 (3)	40.0 (2)			
M-phase	14	28.6 (4)	14.3 (2)		35.7 (5)	21.4 (3)

Table 5. Meiotic maturation and nuclear morphology of MI cytoplasts reconstructed with 1/4-blastomeres at G2 and M- phase stages

Donor nuclei	No. fused oocytes	Meiotic maturation, %(n)	Nuclear morphology, % (n)	
			MII spindle	Unven distribution of chromosomes
G2	12	41.6 (5)	60.0 (3)	40 (2)
M-phase	20	70.0 (14)	71.4 (10)	28.6 (4)

4. Discussion

Timing extrusions of polar bodies were investigated with combinations of recipient cytoplasts and donor nuclei as well as enucleolation of GV oocytes. The results indicated that timing extrusions of PBs were not differed between; 1) enucleolated and intact GV oocytes, 2) GV cytoplasts reconstituted with nucleolated and anucleolated donor nuclei and 3) GV cytoplasts reconstituted with nuclei of growing (GVgr) and fully grown (GV) oocytes. Therefore, earlier extrusions of PBs are not nucleolus dependent. On the other hand, timing extrusions of PBs were affected by nucleo-cytoplasmic synchronization where they were significantly delayed of MI cytoplasts reconstructed with MI donor nuclei (MI- MI cytoplasts) - in comparison to MI cytoplasts reconstructed with G2 donor nuclei (G2 - MI cytoplasts).

Accleration of PB extrusion was observed when embryonic and somatic nuclei (**Grabarek et al., 2004; Chang et al., 2004; Polanski et al., 2005; Nandi et al., 2007; Mohammed et al., 2008; Mohammed et al., 2010**) were introduced into enucleated GV oocytes. It is unclear what caused such a hastening of the cell-cycle progression. It has been suggested that G2/M provided the MPF/MAPK that induced the enucleated GV oocytes to enter MI at a faster pace (Chang et al., 2004). With selective enucleation of GV oocyte where the nuclear contents were released back to the cytoplasm, timing extrusions of PBs were accelerated after transplantation of GV nuclei (in comparison to GV-GV oocytes) but was exactly the same as in the control oocytes (**Mohammed et al., 2008**). It has been reported that GV-associated factors control the timing of the first meiotic division (**Polanski et al., 1998**). Because the nucleoli of embryonic/somatic cells and growing germinal vesicle oocytes are different than those in fully grown germinal vesicle oocytes, the hastening of PB extrusions might be related to nucleolar factors. In contrast, timing extrusions of PBs were not differed between oocytes reconstituted with; 1) karyoplasts of growing and fully grown oocytes; 2) nucleolated and anucleolated donor nuclei indicating that earlier extrusions of PBs are not nucleolus dependent. On the basis of the nucleolus– chromatin association, two distinct groups of fully grown oocytes can be aspirated from large antral ovarian follicles. In the first group, the nucleolus is surrounded by a heavily condensed chromatin, whereas in the second group, the chromatin is dispersed in the oocyte nucleus (germinal vesicle). These two groups of oocytes differ in the speed and extent of maturation — oocytes from the first group are more developmentally competent

(**Combelles et al., 2002; Miyara et al., 2003**). Therefore, the hastening of the extrusion of PBs is not nucleolus dependent but it is chromatin dependent. When 1/4-blastomeres at M-phase were introduced into MI cytoplasts, the timing of the extrusions of PBs was prolonged and ended 2-3 h later than in the same cytoplasts reconstructed with G2 1/4-blastomeres, reflecting the uncompatability of donor nuclei and recepient cytoplasts as a possible factor of the hastening of PBs extrusions. This was confirmed with meiotic maturation and organization of chromosomes in like MII-spindle which slightly increased. The enormous length of the first meiotic M-phase in the mouse oocyte (10 h) as compared to first mitosis of the fertilized egg (**Howlett and Bolton, 1985; Polanski, 1986, 1997; Ciemerych et al., 1999**) or of a typical somatic cell (30-60 min) might be the reason of hastening. It might be concluded that nucleo-cytoplasmic incompatibility might be the reason of acceleration of PB extrusions.

References

Chang, C.C., Nagy Z.P., Abdelmassih R., Yang X. and Tian X.C. (2004), Nuclear and microtubule dynamics of G2/M somatic nuclei during haploidization in germinal vesicle-stage mouse oocytes. *Biology of Reproduction*, 70, 752-758

Choi, I., Zhu, J., & Campbell, K. H. (2013), The combined treatment of calcium ionophore with strontium improves the quality of ovine SCNT embryo development. *Zygote* (Cambridge, England), 21(2), 139-150.

Ciemerych, M.A., Maro B. and Kubiak J.Z. (1999), Control of duration of the first two mitoses in a mouse embryo. *Zygote*, 7, 293-300

Combelles, C.M.H., Cekleniak N.A., Racowsky C. and Albertini D.F. (2002), Assessment of nuclear and cytoplasmic maturation in in-vitro matured human oocytes. *Human Reproduction*, 17, 1006–1016

Fulka, J. Jr., Moor R.M., Loi P. and Fulka J. (2003), Enucleolation of porcine oocytes. *Theriogenology*, 59, 1879–1885

Grabarek, J.B., Plusa B., Modlinski J.A. and Karasiewicz J. (2004), Reconstruction of enucleated mouse germinal vesicle oocytes with blastomere nuclei. *Zygote* 12 163-172

Howlett, S.K. and Bolton V.N. (1985) Sequence and regulation of morphological and molecular events during the first cell cycle of mouse embryogenesis. *Journal of Embryology and Experimental Morphology*, 87, 175-206

Liu, H., Wang C.W., Grifo J.A., Krey L.C. and Zhang J. (1999), Reconstruction of mouse oocytes by germinal vesicle transfer: maturity of host oocyte cytoplasm determines meiosis. *Human Reproduction*, 14, 2357–2361

Miyara, F., Migne C., Dumont-Hassan M., LeMeur A., Cohen-Bacrie P., Aubriot F.X., Glissant A., Nathan C., Douard S., Stanovici A. and Debey P. (2003), Chromatin configuration and transcriptional control in human and mouse oocytes. *Molecular Reproduction and Development*, 64, 458–470

Mohammed, A.A., Karasiewicz J., Modlinski J.A. (2008), Developmental potential of selectively enucleated immature mouse oocytes upon nuclear transfer. *Molecular Reproduction Development*, 75 1269-1280

Mohammed, A.A., Karasiewicz J., Kubacka J., Gręda J.Modlinski J.A. (2010), Enucleated GV Oocytes as Recipients of Embryonic Nuclei in the G1, S, or G2 Stages of the Cell Cycle. *Cellular Reprogramming*, 12, 427-435.

Murray, A.W. and Hunt (1993), The cell cycle, An introduction, Freeman, New York.

Nan, C-L., Ouyang Y-C., Zhao Z-J., Jiang Y., Lei Z-L., Huang J-C., Song X-F., Sun Q-Y., Chen D-Y. (2007), Time course of meiotic progression after transferring primary spermatocyst into ooplasm at different stages. *Molecular Reproduction Development*, 74: 1072-1080.

Polanski, Z. (1986), In-vivo and in-vitro maturation rate of oocytes from two strains of mice. *Journal of Reproduction and Fertility*, 78, 103-109

Polanski, Z. (1997), Genetic background of the differences in timing of meiotic maturation in mouse oocytes: a study using recombinant inbred strains mice. *Journal of Reproduction and Fertility*, 109, 109-114

Polanski, Z., Hoffmann S. and Tsurumi C. (2005), Oocyte nucleus controls progression through meiotic maturation. *Devlopmental Biology*, 281, 184-195

Polanski, Z., Ledan E., Brunet S., Louvet S., Verlhac M.H., Kubiak J.Z. and Maro B. (1998), Cyclin synthesis controls the progression of meiotic maturation in mouse oocytes. *Development*, 125, 4989– 4997

Tarkowski, AK and Wroblewska J (1967), Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. *Journal of Embryology and Experimental Morphology*, 18, 155-180.