Reprod. Nutr. Dev. 38 (1998) 665–670

Original article

Mouse oocyte maturation: the effect of modified nucleocytoplasmic ratio

Lenka Kárníková[a]*, František Urban[a], Robert Moor[b], Josef Fulka Jr[a]

[a] Institute of Animal Production, POB 1, CS-104 01 Prague 10, Czech Republic
[b] The Babraham Institute, Developmental Genetics Programme, Babraham, Cambridge CB2 4AT, UK

(Received 1 October 1998; accepted 13 October 1998)

Abstract — Fully grown mouse oocytes isolated from large antral follicles and cultured in vitro complete their maturation up to the second metaphase with extrusion of the first polar body (1PB) with a 40/50 proportion (80 %). When their cytoplasmic volume is, however, reduced before the onset of culture, the frequency of oocytes completing maturation gradually decreases. In the half oocytes, 66 % (33/50) extruded 1PB, while in third oocytes the proportion was 57 % (28/49) and in quater oocytes no polar bodies were extruded. The time course of germinal vesicle breakdown was also delayed in comparison to the decreased cytoplasmic volume. Moreover, the isolated germinal vesicles surrounded with a thin cytoplasmic rim only remained intact after a prolonged culture. The full competence of complete maturation can be restored by fusion of an additional cytoplast to the manipulated nucleate parts. We postulate that a critical nucleocytoplasmic volume ratio is absolutely necessary for normal maturation in mammalian oocytes. © Inra/Elsevier, Paris.

mouse / oocyte / germinal vesicle / cytoplasm

Résumé — Maturation de l'ovocyte de souris : effet d'un rapport nucléo-cytoplasmique réduit. Des ovocytes murins ayant achevé leur croissance et extraits de grands follicules à antrum achèvent leur maturation in vitro jusqu'à la métaphase II avec émission du premier globule polaire (GPI) dans la proportion 40/50, soit 80 %. Cependant, quand leur volume cytoplasmique est réduit avant le début de la culture, la fréquence des ovocytes qui terminent leur maturation décroît corrélativement. Dans des demi ovocytes, 66 % (33/50) expulsent le GPI, dans les tiers d'ovocytes la proportion est 28/49 (57 %) et dans les quarts d'ovocytes il n'y a pas d'émission de GP. La rupture de la vésicule germinative est aussi retardée en relation avec la réduction du cytoplasme ; les vésicules germinatives isolées, entourées seulement d'une fine couche de cytoplasme restent intactes après culture prolongée. La compétence complète à la maturation peut être restaurée par la fusion d'un cytoplaste aux fractions nuclées. Nous suggérons qu'un rapport nucléo-cytoplasmique critique est absolument nécessaire pour la maturation normale des ovocytes de mammifères. © Inra/Elsevier, Paris.

souris / ovocyte / vésicule germinale / cytoplasme

* Correspondence and reprints
E-mail: gott@vuzv.cz

1. INTRODUCTION

The relationship between the nucleus and the cytoplasm is of critical importance in cell-cycle control at least in oocytes and early embryonic cells. Thus it has been shown that the maturation promoting factor (MPF) is activated in anucleate oocyte fragments [1, 13]. Its stability, however, is much lower than in nucleate parts [2, 7, 12]. Although MPF is activated in anucleate oocyte halves, the transition between metaphase I to metaphase II (decrease in MPF activity), typical for intact oocytes, is typically undetected and halves are arrested in a metaphase I-like stage with slowly decreasing MPF activity. These results clearly suggest that the nucleus and cytoplasm have complementary roles and that both these components are absolutely necessary for the normal cell-cycle course [10]. These roles are not, however, fully understood. At present, different manipulation procedures are commonly used which change the nucleocytoplasmic ratio. It is not fully known how these changes modify the cell cycle and results are still conflicting. In our model system we modified the nucleocytoplasmic ratio in immature mouse oocytes and examined how this manipulation influences the maturation of oocytes in vitro.

2. MATERIALS AND METHODS

Fully grown mouse oocytes were isolated from large antral follicles of pregnant mare serum gonadotrophin (PMSG) (5 IU) stimulated ICR females. The oocytes were manipulated in M2 medium supplemented with dbcAMP (150 $\mu g \cdot mL^{-1}$). Cumulus cells were removed by extensive pipetting and only those oocytes with clearly visible germinal vesicles (GV) were used. Their zonae pellucidae were removed by pronase (0.5 % in PBS) and cells were incubated in M2 medium containing cytochalasin B (5 $\mu g \cdot mL^{-1}$) and nocodazole (3 $\mu g \cdot mL^{-1}$) for 30 min. The oocytes were then elongated in a very narrow pipette and bisected with a glass needle exactly as described by Tarkowski [14] into half, third and quarter oocytes and GV surrounded by a very thin rim of cytoplasm (GV-plasts). The oocyte parts containing GV were extensively washed in dbcAMP-free medium and cultured in M199 supplemented with Na-pyruvate (0.2 mM), gentamicin (25 $\mu g \cdot mL^{-1}$) and BSA (4 $mg \cdot mL^{-1}$) at 37 °C in a 5 % CO_2 air atmosphere for different time intervals (see Results). Some cytoplasts were kept in the medium supplemented with dbcAMP before being fused to GV-plasts in order to test if the restored oocyte had the same ability to mature as an intact cell. Both components (GV-plast × cytoplast) were incubated in PBS containing phytohemagglutinin (PHA; 200 $\mu g \cdot mL^{-1}$) and the close contact between them was achieved in a very narrow pipette. The agglutinated parts were then exposed to one electric pulse (1 kV, 100 µs) in an isotonic glucose solution. The treated cells were cultured as described earlier. In all experiments, cells were periodically observed before being fixed in acetalcohol, stained with aceto-orcein and examined under a phase contrast microscope. To verify the presence and morphology of GV in GV-plasts, some samples ($n = 5$) were prepared for electron microscope evaluation and their morphology was compared to intact immature oocytes ($n = 5$). GV-plasts and immature oocytes were fixed for 60 min in 2.5 % glutaraldehyde and 0.6 % paraformaldehyde in 0.06 cacodylate buffer (pH 7.2), postfixed for 60 min in 1 % OsO_4, dehydrated in ethanol and embedded in EPON. The sections were examined under a JEOL JEM CX II 100 electron microscope. Each experiment was repeated at least five times. All chemicals were purchased from Sigma, Prague, Czech Republic.

3. RESULTS

Under our culture conditions, germinal vesicle breakdown (GVBD) in mouse oocytes occurred within 60 min, well formed metaphases I were observed after 7 h and between 8 and 9 h anaphase-telophase transition was detected. Fully formed metaphases II were noted after 10 h of culture. When the time course of GVBD has been compared in intact, half, third and quarter oocytes, an evident delay in GVBDs was observed concomitantly to the decreased oocyte size (*table I*). Thus, for example, in intact oocytes 96 % (34/35) underwent GVBD within 1 h of culture, in half oocytes

82 % (36/47), in third oocytes 38 % (19/47) and in quarter oocytes only 20 % (11/45) contained condensed chromosomes. The percentage of manipulated oocytes undergoing GVBD gradually increased with the culture interval and after 3 h 95 % (42/44), 94 % (44/47) and 87 % (41/45) of, respectively, half, third and quarter oocytes contained condensed chromatin. The results are summarised in *table I*. Interestingly, the isolated germinal vesicles (GV-plasts) surrounded only with a thin rim of cytoplasm remained intact and did not undergo GVBD even after a prolonged culture (20 h). The presence of intact GVs was confirmed when these GV-plasts were examined under the electron microscope. As in GVs in immature intact oocytes, the nuclear membrane was intact and a compacted nucleolus was clearly visible. Contrary to GVs from freshly isolated oocytes, the nucleolus in GV-plasts is surrounded with a condensed chromatin (*figures 1* and *2*). Next, it was important to know if the delayed GVBD also altered the frequency of oocytes maturing up to the second metaphase. In control oocytes 80 % (40/50) extruded the first polar body when evaluated after 14 h in culture. This frequency gradually decreased with the decreasing oocyte volume: half oocytes 66 % (33/50), third oocytes 57 % (28/49) and quarter oocytes did not extrude the polar bodies. The percentage of oocytes with polar bodies remained constant even after a prolonged culture. The oocytes without polar bodies were arrested in metaphase I with heavily condensed chromosomes organised on a normal spindle. In quarter oocytes the metaphase I groups were rather clumped and individual chromosomes could not be distinguished. The results are summarised in *table II*. Interestingly, the ability of oocytes to complete maturation could be restored in quarter oocytes when, for example, an additional cytoplast was fused to then. The oocytes were bisected and corresponding parts were fused together. Fused cells were then cultured in normal medium. GVBD occurred within 1 h after fusion and 76 % (19/25) of the fused cells completed maturation. Our results show that a critical cytoplasmic volume is absolutely essential for the completion of normal oocyte maturation to metaphase II.

4. DISCUSSION

At the present, a wide variety of different manipulation procedures is commonly used in embryological studies and in assisted reproduction. These procedures mainly involve the production of clones in farm animals and mice [5], cytoplasmic transfers

Table I. The frequency and time course of germinal vesicle breakdown (GVBD) in oocytes with modified nucleocytoplasmic ratio.

Frequency of GVBD after	Nucleocytoplasmic ratio GVBD/Total no. of oocytes (%)									
	Controls		1/2		1/3		1/4		GV	
1 h	34/35	(96)	36/44	(82)	19/47	(38)	11/45	(20)	0/75	(0)
2 h	34/35	(96)	42/44	(95)	43/47	(89)	18/45	(36)	0/75	(0)
3 h	34/35	(96)	42/44	(95)	44/47	(94)	41/45	(87)	0/75	(0)
Arrested GV*	1	(4)	2	(5)	3	(6)	4	(9)	60	(80)

Controls: intact oocytes; 1/2, 1/3, 1/4: half, third, quarter oocytes; GV: isolated germinal vesicles.
* Final evaluation after 20 h in culture.

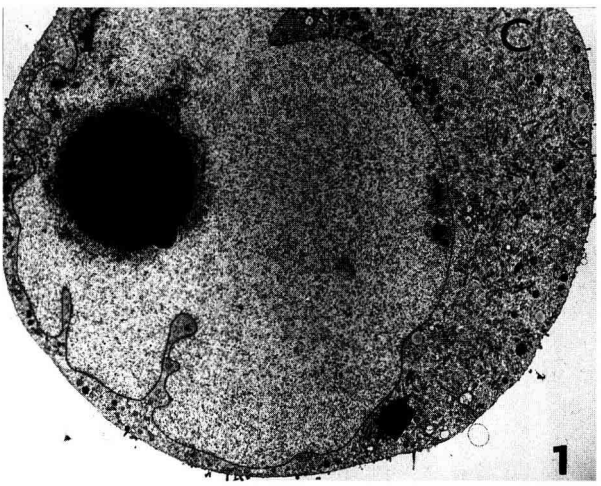

Figure 1. Ultrastructure of the germinal vesicle surrounded by a very thin layer of cytoplasm (GV-plast) cultured for 20 h. Note an intact germinal vesicle envelope (arrow) and a compact nucleolus surrounded by condensed chromatin (N). The GV-plast contains a minimum amount of cytoplasm (C) (× 2 400).

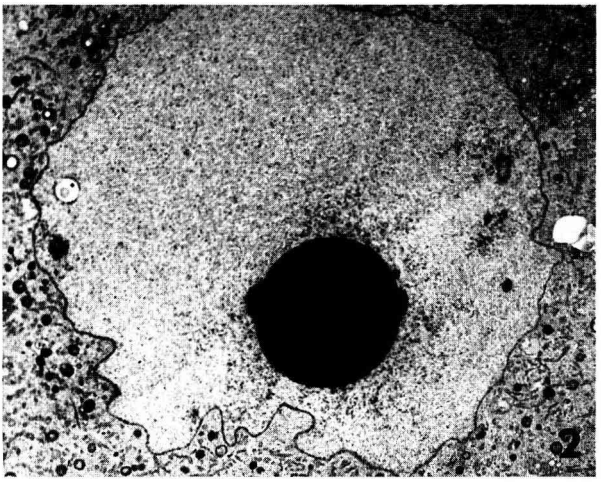

Figure 2. Ultrastructure of the germinal vesicle (GV) in a freshly isolated intact mouse oocyte is almost similar to the GVs in cultured GV-plasts (× 2 400).

into human oocytes [4] and potential nuclear exchanges. It is logical to assume that in some cases the nucleocytoplasmic ratio must be changed. However, how these eventual changes influence the development of manipulated cells is only poorly understood and there are still conflicting results. Our model system showed that the decrease of cytoplasmic volume influences the time course of GVBD and the ability of oocytes to extrude the first polar body. Similarly, in one-cell staged embryos, the modified nucleocytoplasmic ratio influences the developmental programme in manipulated cells [6, 9]. The most commonly used procedure in which the nucleocytoplasmic ratio may sig-

Table II. Reduced cytoplasmic volume decreases the frequency of polar body extrusion.

Type of oocyte	Total no. of oocytes	Stage of maturation after 14 h in culture (%)							
		GV		MI		PB		Degenerated	
Controls	50	1	(2)	9	(18)	40	(80)	0	(0)
1/2	50	2	(4)	11	(22)	33	(66)	4	(8)
1/3	49	2	(3)	19	(39)	28	(58)	0	(0)
1/4	78	9	(12)	61	(77)	0	(0)	8	(11)
GV*	75	60	(80)	10	(13)	0	(0)	5	(7)

GV: germinal vesicle; MI: metaphase I; PB: metaphase II - polar body extruded; controls: intact oocytes; 1/2, 1/3, 1/4: half, third and quarter oocytes; * GV: isolated germinal vesicles. Isolated GV did not undergo germinal vesicle breakdown even after 20 h in culture.

nificantly influence the overall success involves the cloning of mammalian embryos. Yet even here the results are still conflicting. Bordignon and Smith [3] enucleated bovine oocytes during the second telophase so that only the spindle with chromosomes was removed with a minimum of cytoplasm. These authors reported much better development than in the currently used method of removing a large volume of cytoplasm with the metaphase II chromosomes (38 versus 16 %). Similar results were reported by Zakhartchenko et al. [16] when significantly poorer development was observed after a substantial amount of cytoplasm was removed at enucleation. In contrast, some earlier results do not support these results [15]. When interpreting the cloning results the nucleus size after transfer must also be estimated, depending on the procedure used. Thus, the nucleus transferred into a cytoplast containing the MPF with concomitant or delayed activation increases its size (swelling). On the other hand, after the nuclear transfer into the activated cytoplast, the swelling is not as intensive [8]. Therefore, the original size of the nucleus may be significantly changed shortly after it is introduced into the cytoplast and this logically changes the originally proposed nucleocytoplasmic ratio. Clearly, more precise definition and control in nuclear transfer experiments must be achieved. It is difficult to explain why the process of oocyte maturation is influenced by the modified nucleocytoplasmic ratio. The extrapolation from early embryonic studies is rather risky because the cell-cycle regulation is not absolutely the same in meiotic and mitotic cells. It has been shown that the basic cell-cycle molecules are already present in fully grown oocytes. The activity of a central cell-cycle regulator cyclin B/Cdc2 is controlled by phosphorylation/dephosphorylation. Dephosphorylation is carried out by Cdc25C phosphatase. Both regulators are cytoplasmically localised and just before nuclear membrane breakdown cyclinB/Cdc2 is translocated into the nucleus [11]. Thus, the removal of a certain part of a cytoplasmic oocyte compartment may actually decrease the concentration of central cell cycle regulators. The remaining molecules are thereafter unable to drive the oocyte maturation as in oocytes that are not manipulated. It must be noted, however, that the previous explanation is greatly simplified and certainly some other factors are involved. Unless more detailed studies in manipulated oocytes are performed, the explanation as

to why manipulated oocytes have a reduced capacity to fully mature will remain rather speculative. Nevertheless, we do believe that our preliminary observations clearly indicate that under some conditions the oocyte manipulation procedures can influence the ability of mammalian oocytes to fully mature.

ACKNOWLEDGEMENTS

This experiment was in part (J.F. Jr) supported by a fellowship under OECD Co-operative Research Programme: Biological Resource Management for Sustainable Agricultural Systems. The support of the laboratory of J.F. Jr from GACR 524/96/K162 and NAZV is also gratefully acknowledged.

REFERENCES

[1] Balakier H., Czolowska R., Cytoplasmic control of nuclear maturation in mouse oocytes, Exp. Cell Res. 110 (1977) 466–469.

[2] Balakier H., Masui Y., Chromosome condensation activity in the cytoplasm of anucleate and nucleate fragments of mouse oocytes, Dev. Biol. 113 (1986) 155–159.

[3] Bordignon V., Smith L.C., Telophase enucleation: an improved method to prepare recipient cytoplasts for use in bovine nuclear transfer, Biol. Reprod. 49 (1998) 29–36.

[4] Cohen J., Scott R., Alikani M., Schimmel T., Munne S., Levron J., Wu L., Brenner C., Warner C., Willadsen S., Ooplasmic transfer in mature human oocytes, Mol. Hum. Reprod. 4 (1998) 269–280.

[5] DiBerardino M.A., Genomic Potential of Differentiated Cells, Columbia University Press, New York, 1997.

[6] Evsikov S.V., Morozova L.M., Solomko A.P., The role of the nucleocytoplasmic ratio in development regulation of the early mouse embryo, Development 109 (1990) 323–328.

[7] Fulka Jr J., Ouhibi N., Fulka J., Kanka J., Moor R.M., Chromosome condensation activity (CCA) in bisected C57BL/6J × CBA mouse oocytes, Reprod. Fert. Develop. 7 (1995) 1123–1127.

[8] Fulka Jr J., First N.L., Moor R.M., Nuclear transplantation in mammals: remodelling of transplanted nuclei under the influence of maturation promoting factor, BioEssays 18 (1996) 835–840.

[9] Howlett S.K., Barton S.C., Surani M.A., Nuclear cytoplasmic interactions following nuclear transplantation in the mouse embryos, Development 101 (1987) 915–925.

[10] Iwashita J., Hayano Y., Sagata N., Essential role of germinal vesicle material in the meiotic cell cycle of *Xenopus* oocytes, Proc. Natl. Acad. Sci. USA 95 (1998) 4392–4397.

[11] Kishimoto T., Okamura E., In vivo regulation of the entry into M-phase: initial activation and nuclear translocation of cyclin B/Cdc2, in: Meijer L., Guidet S., Philippe M. (Eds.), Progress in Cell Cycle Research, Plenum Press, New York, 1997, pp. 241–249.

[12] Kubiak J.Z., Weber M., de Pennart H., Winston N., Maro B., The metaphase II arrest in mouse oocytes is controlled through microtubule-dependent destruction of cyclin B in the presence of CSF, EMBO J. 12 (1993) 3773–3778.

[13] Matiolli M., Galeati G., Bacci M.L., Barboni B., Changes in maturation promoting factor activity in the cytoplasm of pig oocytes throughout maturation, Mol. Reprod. Dev. 30 (1991) 119–125.

[14] Tarkowski A.K., In vitro development of haploid mouse embryos produced by bisection of one-cell fertilised eggs, J. Embryol. Exp. Morphol. 38 (1977) 187–202.

[15] Westhusin M.E., Collas P., Marek D., Sullivan E., Stepp P., Pryor J., Barnes F., Reducing the amount of cytoplasm available for early embryonic development decreases the quality but not quantity of embryos produced by in vitro fertilization and nuclear transplantation, Theriogenology 46 (1996) 243–252.

[16] Zakhartchenko V., Stojkovic M., Brem G., Wolf E., Karyoplast-cytoplast volume ratio in bovine nuclear transfer embryos: effect on developmental potential, Mol. Reprod. Dev. 48 (1997) 332–338.