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CUMULUS CONTRIBUTIONS IN TIMING OF MOUSE OOCYTES MATURATION AND DEVELOPMENTAL POTENTIAL.*

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Abstract: A mandatory step in performing micromanipulation techniques, studying sperm-oocyte interactions after fertilization and evaluating morphological aspects of oocyte quality is the removal of cumulus cells from oocytes or zygotes at various stages. In mice, cumulus removal before maturation of oocytes *in vitro* inhibits sperm penetration rates. The aim of the study was to investigate timing of maturation and pronuclei formation and developmental competence of oocytes matured *in vitro* in the presence of loose cumulus cells. Germinal vesicle breakdown (2-3h) and timing extrusions of first polar bodies (9-13h) occurred approximately at the same time of the starting maturation regardless of the presence or absence of cumulus cells (+/-). Furthermore, timing extrusions of second polar bodies (1.30-2.45h) and pronuclei formation (3.30-4.30h) occurred from the starting fertilization at the same time (+/-). Cumulus cells increased markedly the cleavages and development of zygotes to the blastocyst stages upon activation.

Key words: Cumulus, maturation, development.

Introduction

Cumulus-enclosed oocytes resume meiosis when cultured in medium containing only glucose as a source of energy whereas cumulus-free oocytes do not. Nevertheless, addition of pyruvate to the oocyte culture medium provides support for the resumption of meiosis by denuded oocytes (Donahue and Stern 1968). This evidence indicates that cumulus cells take up and metabolize glucose to products that can be used by oocytes for the energy metabolism necessary to support meiotic maturation (Leese and Barton, 1985). Metabolic cooperativity of oocytes by cumulus cells involves the uptake of some amino acids, such as L-alanine, which are poorly

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transported into mouse oocytes and require uptake first by cumulus cells and then transfer to the oocyte via gap junctions (Colonna and Mangia, 1983). When oocytes are cultured with radiolabeled L-alanine, the amount of radioactivity detected in the cumulus-enclosed oocytes is greater than in cumulus-free oocytes (Colonna and Mangia, 1983). Blocking the function of gap junctions in cumulus enclosed oocytes abrogates this difference (Haghighat and Van Winkle, 1990). Recent study showed that oocytes control glycolysis in granulosa cells by regulating expression levels of genes encoding glycolytic enzymes (Sugiura *et al.*, 2005). Therefore, oocytes promote a key metabolic function of cumulus cells that is necessary for oocyte meiotic maturation and further embryonic developemnt. Moreover, coculture with cumulus cells may assist the oocyte to avoid undergoing DNA fragmentation (Wongsrikeao *et al.*, 2005). Moreover, Fatehi *et al.* (2002) and Yamazaki *et al.* (2001) found that the presence of loose cumulus cells partially restored the effect of denudation prior to in vitro fertilization. Collectively, the previous results suggest that cumulus-derived factors influencing cytoplasmic maturation reach the oocytes both through the gap junction coupling pathway and by diffusion. Therefore, the aim of the study was to investigate timing of oocytes maturation and pronuclei formation in the presence of loose cumulus cells and their developmental competence.

Materials and Methods

All organic and inorganic compounds used in the present study obtained 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).

Eighteen females (6- to 8-week old) and three males (3-6 months) were used during the study. All 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*. Animals were killed by cervical dislocation.

For collection of germinal vesicle (GV) oocytes, hybrid 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 (Grabarek *et al.*, 2004).

Oocytes maturation and experimental groups

After GV oocytes collection, they were transferred into 50 µl droplets (10 oocytes per droplet) of pre-equilibrated *in vitro* maturation (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 for 17h at 37 °C in an atmosphere of 5% CO2 in air with the saturated humidity. Loose cumulus cells stripped previously from the GV oocytes were added to the maturation medium (cumlus+) or not (cumulus-). Timing of germinal vesicle breakdown [2-3h (cumlus+ vs. cumulus-)] and 1st polar body extrusions [8-13h (cumlus+ vs. cumulus-)] were investigated from the starting maturation.

Upon maturation, the matured oocytes were either fertilized by spermatozoa obtained from male mice to investigate timing of 2nd polar body extrusions [1.30h-2.45h (cumulus+ vs. cumulus-)] and pronuclei formation [3.30h-4.30h (cumulus+ vs. cumulus-)] or activated by alcohol to investigate the developmental potential of the zygotes to the morula/blastocyst stage [120h (cumulus+ vs. cumulus-)].

Oocytes fertilization

For fertilization of the matured oocytes, drilling the hardened zona pellucida and spermatozoa collection and capacitation were carried out firstly. Tools and equipments were used to drill the zona. Drilling the zona pellucida was 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). 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 polished. The ends of pipettes were bent twice in order to adopt them to the micromanipulation chamber used. Drilling the zona pellucida was performed by using the acidified Tyrode's solution (pH = 2.5) supplemented with

polivinylpyrrolidone (m.w. 40.000). In order to obtain the small hole in the zona pellucida (*zona*-drilled oocytes) the tip of micropipette filled with acidified Tyrode's solution was placed in the vicinity of the zona and the solution was slowly blown off until the opening in the zona has been made.

To collect and capacitate spermatozoa, cauda epididymides were cut off from mature males of proven fertility after cervical dislocation. Spermatozoa were squeezed out from cauda epididymiae into 1.0 mL of human tubal fluid (HTF) medium. Spermatozoa were allowed to disperse and capacitate for 1.5 h at 37 °C, 5% CO_2 in air (Mohammed *et al.*, 2008). The zona-drilled oocytes were inseminated in 50 µl microdroplets (10 oocytes/droplet) of sperm suspension in IVF medium (HTF). Prior to insemination small opening was made in the zona (zona – drilled oocytes). For the fertilization of zona - drilled oocytes the concentration of about 0.5×10^6 of motile sperm/ml was used, respectively. Inseminated oocytes were kept 3h with the sperm suspensions. After insemination, oocytes were washed twice in pre-heated KSOM medium and were transferred into KSOM medium for further culture (37°C, 5% CO_2 in air) till the remaining period of pronuclei formation.

Oocytes activation and developmental potential

For investigating the developmental potential of matured oocytes (cumulus+ vs. cumulus-) till the morula/blastocyst stage, the matured oocytes were activated by 5 min. exposure to the medium M2 containing 7% ethanol at room temperature and were cultured in KSOM medium containing 5 µg/ml cytochalasin B (CB) for 5-6 h (Mohammed 2006) followed by culturing in KSOM medium without CB till the morula/blastocyst stage (120h).

RESULTS

Germinal vesicle breakdown were occurred approximately at the same time (2-3h) from the starting maturation regardless of the presence or absence of loose cumulus cells and also timing extrusions of first polar bodies (9-13h). Upon maturation, the zona-drilled oocytes were fertilized and observed for timing extrusions of second polar bodies and pronuclei formation. Neither timing of second polar body extrusions (1.30-2.45h) nor pronuclei formation (3.30-4.30h) was differed in the presence or absence of cumulus cells. (Table 1).

Table(1): Effects of cumulus cells on timing of mouse oocytes maturation and pronuclei formation after *in vitro* fertilization.

Items	No. oocytes	Germinal vesicle breakdown % (n)*					
		2h			3h		
Cumulus-	37	91.9 (34/37)			8.1 (3/37)		
Cumulus+	37	86.5 (32/37)			13.5 (5/37)		
		Extrusion of 1st polar body % (n)*					
		8h	9h	10h	11h	12h	13h
Cumulus-	32	0	21.8 (7/32)	46.8 (15/32)	21.8 (7/32)	9.4 (3/32)	0
Cumulus+	32	6.2 (2/32)	18.7 (6/32)	40.6 (13/32)	9.4 (3/32)	12.5 (4/32)	12.5 (4/32)
		Extrusion of 2nd polar body % (n)**					
		1.30h	1.45h	2.0h	2.15h	2.30h	2.45h
Cumulus-	34	2.9 (1/34)	26.5 (9/34)	35.3 (12/34)	17.6 (6/34)	9.4 (3/34)	9.4 (3/34)
Cumulus+	34	5.9 (2/34)	35.3 (12/34)	35.3 (12/34)	17.6 (6/34)	5.9 (2/34)	0
		Pronuclei formation % (n)**					
		3.30h		4.0h		4.30h	
Cumulus-	36	33.3 (12/36)		44.4 (16/36)		22.2 (8/36)	
Cumulus+	36	25.0 (9/36)		25.0 (9/36)		50.0 (18/36)	

Cumulus-: loose cumulus cells were not added to the maturation medium

Cumulus+: loose cumulus cells were added to the maturation medium

*: germinal vesicle breakdown and extrusion of 1st polar body of the starting maturation

**: extrusion of 2nd polar body and pronuclei formation of the starting fertilization h: hour

Furthermore, although the percentages of activated oocytes in the presence or absence of cumulus cells during maturation were not differed, the cleavages to 2-cell and 4-cell stage embryos were markedly increased in the presence of cumulus cells as well as with the further development to the morula/blastocyst stages (Table 2).

Table(2): Effects of cumulus cells on developmental competence upon activation of mouse oocytes

Items	No. oocytes	% (n) Activated oocytes	% (n) 2 cells embryos	% (n) 4 cells embryos	% (n) Morula/ balstocyst
Cumulus-	33	90.9 (28/33)	66.6 (22/33)	36.4 (12/33)	9.1 (3/33)
Cumulus+	28	92.8 (26/28)	85.7 (24/28)	67.8 (19/28)	42.8 (12/28)

Cumulus-: loose cumulus cells were not added to the maturation medium
Cumulus+: loose cumulus cells were added to the maturation medium
M: morula, Bl: blastocyst

Discussion

The interactions between the GV nucleus, the cytoplast and the surrounding cumulus cells are of the fundamental questions in the field of assisted reproductive technology for treatment of infertility and embryonic/somatic cloning. Immature oocytes seem to be an interesting model for studying the mechanisms of meiotic maturation. As mentioned previously, a mandatory step in performing micromanipulation technique is the removal of cumulus cells. Therefore, the present study started the investigation from germinal vesicle breakdown (GVBD). Timing of GVBD and 1st PBs occurred 2-3h and 8-13h respectively of the starting maturation in both groups (+/-). These results were in agreement with Grabarek *et al.* (2004). It seems that the factors secreted from the cumulus cells did not affect in timing of oocytes maturation. The reported results by Mohammed *et al.* (2005) indicated that addition of bovine follicular fluid (partially secreted from cumulus cells) to the maturation medium of bovine oocytes delayed timings of first cleavages in comparison of fetal calf serum. Recent work (Mohammed *et al.*, 2008) showed that even the removal of nucleolus did not affect in timing of oocytes maturation.

With fertilization, the timing of 2nd PBs extrusions and pronuclei formation occurred 1.30-2.45h and 3.3-4.30h respectively post fertilization in both groups (+/-). It seems possible that the technique of drilling the zona pellucida fasten the penetration of sperm into the oocytes where the timing of timing 2nd PBs and pronuclei formation were accelerated compared with zona-intact oocytes (personal observation). Although the added cumulus cells did not affect the timing of 2nd PBs and pronuclei formation; cleavages and further development to the blastocyst stage were increased (data not shown).

Upon activation with alcohol, the percentages of cleavages and further development to the blastocyst stage were markedly increased in the presence of cumulus cells during maturation. Yamazaki *et al.* (2001) and Fatehi *et al.* (2002)found that the presence of loose cumulus cells during maturation partially restored the effect of cumulus removal.

Wongsrikeao *et al.* (2005) suggested that addition of cumulus cells may assist the oocyte to avoid undergoing DNA fragmentation. On the other hand, Mori *et al.* (2000) reported that the contact of cumulus cells and oocytes is important for male pronucleus formation and when gap junctional coupling was

chemically inhibited; normal pronucleus formation was significantly inhibited. Collectively, the previous results suggest that cumulus-derived factors influencing cytoplasmic maturation reach the oocytes both through the gap junction coupling pathway and by diffusion. The results lead to conclude that the presence of cumulus cells around oocytes is not essential for nuclear maturation *in vitro* whereas it is beneficial for fertilization and developmental competence of oocyte to the balsocyst stage.

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دور خلايا الركام في أوقات نضج بويضات الفئران وتطورها.*

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الخطوة الإجبارية في أداء تقنيات الممارسة الدقيقة أو لدراسة كلا من أنوية الحيوان المنوي والبويضة بعد الإخصاب أو التقييم المورفولوجي للبويضات هي إزالة الخلايا الركامية من البويضات أو الزيجوت في المراحل المختلفة. ولقد وجد أن إزالة الخلايا الركامية قبل الإنضاج يثبط إخصاب البويضات في الفئران. ولذلك يهدف البحث إلي دراسة تأثير إضافة خلايا الركام أثناء الإنضاج علي وقت الإنضاج وكذلك تكوين الانوية الأولية وتطور البويضات إلي بلاستوسيست بعد الإخصاب/التنشيط. ولقد أظهرت النتائج أن انحلال أنوية البويضات (2–3 ساعة)، تكوين الجسم القطبي الأول (9–13 ساعة) والثاني (1.30–2.45 ساعة) وكذلك تكوين الانوية الأولية (3.30–4.30 ساعة) يحدث في نفس الوقت تقريبا للبويضات مع وجود أو عدم الخلايا الركامية أثناء الإنضاج. أما نسبة الانقسامات والتطور إلي مراحل المورولا/البلاستوسيست للبويضات فقد زادت مع وجود الخلايا الركامية بعد التنشيط.

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