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REVISED

A THREE-DIMENSIONAL ALGINATE SYSTEM FOR IN VITRO CULTURE OF CUMULUS-DENUDED FELINE OOCYTES

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~~*Abstract*~~*Contents*

In the case of high valuable individuals with very precious genetic material, widening the genetic pool including gametes with poor morphological characteristics, as cumulus-denuded oocytes (CDOs), could be an option.

To improve the in vitro culture of low-competence feline CDOs, an enriched three-dimensional (3D) system in association with competent cumulus-oocyte complexes (COCs) was developed. For this purpose, domestic cat CDOs were cultured with or without companion COCs in the 3D barium alginate microcapsules. The overall viability and the meiotic progression of feline CDOs co-cultured with COCs or cultured separately in 3D or in 2D (traditional microdrops) system were compared. The 3D system was able to support viability and meiotic resumption of the feline oocytes, as well as the 2D microdrops. In 3D microcapsules, the presence of COCs resulted in a higher viability of CDOs (91.1%, $p < 0.05$), than that obtained without COCs or in 2D microdrops (71.2% and 67.3%, respectively), but the percentages of meiotic resumption were similar of those of CDOs cultured separately (55.4% vs. 40.4%, $p > 0.05$). It is notable that the presence of CDOs seemed to enhance the meiotic progression of the associated COCs.

In conclusion, the 3D barium alginate microcapsules are a suitable system for feline oocytes in vitro culture, but more specific enriched conditions should be developed to improve the CDOs full competence in vitro.

Keywords

Domestic cat; low competence oocytes; in vitro maturation; 3D system.

Abridged title: 3D in vitro culture of feline oocytes.

1 Introduction

Cumulus-denuded oocytes (CDOs) are generally not included in the in vitro procedures due to their poor nuclear and cytoplasmic competence caused by the lack of surrounding cumulus oophorus cells. These closely associated cumulus cells (CCs) form an intimate network with the oocyte; thus, the somatic-germinal two-way transfer of different small molecules is ensured through the highly specialized projections through the zona pellucida, that are gap junctions (Eppig 1982). The structural integrity of CCs and the functional coupling between the two compartments are of crucial importance for the successful subsequent embryo development (Fagbohun and Downs 1991; Tanghe et al. 2002; Luciano et al. 2004).

Several attempts have been made to improve the in vitro performances of oocytes with poor developmental potential, as well as oocytes deprived of CCs. The co-culture with companion cumulus-oocyte complexes (COCs) seemed to have beneficial effects on the CDOs' in vitro outcomes. In the bovine species, the presence of intact COCs during both in vitro maturation and fertilization promoted the restoration of CDOs' competence, although the blastocyst rates remained low (Luciano et al. 2005). Co-culture of feline CDOs with cumulus cells clumps enhanced the resumption of meiosis, although the frequency of complete nuclear maturation was lower than that of competent COCs (Chigioni et al. 2005).

In the case of high valuable individuals with very precious genetic material, widening the genetic pool including gametes with poor morphological characteristics, as CDOs, could be an option. Therefore, enriched conditions for the culture of these low-competence oocytes should be further developed.

The traditional culture systems for follicles and oocytes are based on microdrops of medium, but this condition seemed to lead to a non-physiological cells conformation and biological activity. To mimic more faithfully the in vivo follicular architecture and cellular spatial arrangement, bioengineering and nanotechnology researches have been focused on

developing different in vitro conditions. With the support of natural or synthetic polymers, three-dimensional (3D) innovative culture systems were developed to enhance the adhesion, the proliferation and the release of secreted factors by cultured cells (Desai et al. 2010; Antoni et al. 2015). The 3D environment also resulted in cell behaviour, signalling and gene expression profiles that most resemble those observed in living cells (Cukierman et al. 2002). It has been demonstrated that the encapsulation of follicles and oocytes in biocompatible 3Dthree-dimensional systems allows the maintenance of their physiological structure and functional integrity in different species (mouse, Pangas et al. 2003; human, Combelles et al. 2005;, pig, Munari et al. 2007).

The domestic cat is an excellent animal model for wild felids reproductive biotechnologies, but in this species, only few studies were focused on the improvement of the in vitro performances of low- competence oocytes and on the use of 3D systems for oocyte culture (Godard et al. 2009; Fujihara et al. 2012).

Codice campo modificato

Thus, this e present study was performed to investigate: (ia) the suitability of a 3D system (barium alginate microcapsules) for feline oocytes in vitro culture, and (iib) whether 3D system would improve in vitro maturation of CDOs co-cultured with COCs or cultured separately.

2 Materials and methods

The study was approved by the Ethical Committee of the Università degli Studi di Milano (December 9th, 2014), and all animals were enrolled following written consent by the owner.

2.1 Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

105 *2.2 Animals and collection of feline oocytes*

106

107 Ovaries (n. 131) from domestic cats were harvested at random stages of the oestrous cycle
108 during routine ovariectomy at veterinary clinics. After surgery, ovaries were immediately
109 placed in a phosphate-buffered saline (PBS) with a mixture of antibiotics (AB) and
110 antimycotics (100 IU/ml of penicillin G sodium, 0.1 mg/ml of streptomycin sulphfate, and
111 0.25 µg/ml of amphotericin B), and transported to the laboratory at room temperature (RT)
112 where they were processed.
113 Feline COCs (n. 402) were obtained by mincing of the ovaries in PBS and AB with 0.1%
114 (w/v) polyvinyl alcohol (PVA), and only grade I COCs were selected for the experiments.
115 The CDOs were obtained by mechanical deprivation, with a small bore pipette, of COCs'
116 cumulus cells.

117

118 *2.3 In vitro maturation in 3D and 2D systems*
119 The feline oocytes were matured in vitro for 24 hr in a controlled atmosphere (38.5°C and 5%
120 CO_2 in air) in modified Kreb's-Ringer bicarbonate-buffered salt solution with AB (b-
121 mKRB) supplemented with 3 mg/mlL of bovine serum albumin (BSA), 0.5 IU/mlL of equine
122 chorionic gonadotropin (eCG), 1 IU/mlL of human chorionic gonadotropin (hCG), 10 ng/mlL
123 of epidermal growth factor (EGF) and 0.6 mM cysteine (complete maturation medium, c-
124 mKRB).
125 For the 3D system, a two-steps encapsulation technique in barium alginate (BA) was
126 developed, as a modification of the protocol previously described for living cell (Conte et al.
127 1999; Vigo et al. 2004). The Na-alginate powder (0.5%) was dissolved into the different
128 solutions reported below, to obtain the melting solution (MS) at medium viscosity (3.500 cP,
129 centipose). A saturated solution of $BaCl_2$ was then added to an aliquot of a different medium
130 (see below) to obtain the dropping solution (DS) of $BaCl_2$ (40 mM) that was dropped at RT

with a 25-G needle into the MS maintained stirred for 30-40 minutes. The microcapsules were then collected, washed twice in PBS and suspended in the c-mKRB for immediate use, or maintained at 4°C in a petri dish with PBS until use.

To obtain the BA microcapsules, the following working conditions were tested: (i)1: MS with b-mKRB and DS with b-mKRB. (ii)2: MS with c-mKRB and DS with c-mKRB. (iii)3: MS with sterile water and DS with b-mKRB. (iv)4. MS with sterile water and DS with c-mKRB.

The feline oocytes were injected into the inner core of the microcapsule (Fig. 1) by a small bore pipette and subsequently immersed in the c-mKRB in a multiwell dish.

For the 2D culture system, traditional microdrops of c-mKRB (50-100µl) were placed in a petri dish and covered by mineral oil.

2.4 Assessment of viability and maturation rates

After 24 hr of in vitro maturation, COCs and CDOs were evaluated for overall viability and nuclear maturation rates. Sequential stainings with fluorescein diacetate/propidium iodide (FDA/PI) for viability and bis-benzimide (Hoechst 33342) for chromatin configuration were performed.

For the viability, the oocytes were maintained at dark in 50 µl of the staining solution (PI: 10 mg/ml; FDA: 5 mg/ml) for 5 min and then evaluated under a fluorescent microscope (Axiovert 100, Zeiss, Arese, Italy). The FDA maximum excitation wavelength was 490 nm and the emission wavelength was 520 nm, whereas the PI maximum excitation wavelength was 536 nm and the emission wavelength was 617 nm. This differential staining allowed the evaluation of viable (bright green fluorescence) or dead cells (red fluorescence).

After washing, CDOs and COCs (deprived of CCs cumulus cells by mechanical displacement with a small bore glass pipette) were placed on a slide with a minimum amount of c-mKRB, and then covered by 10 µl of Hoechst solution. After 5 min of incubation in the dark, the

Hoechst solution was removed and the oocytes were covered with an anti-fade reagent (Fluoromount™ Acqueous Mounting Medium, Sigma Chemical Company). The fixed oocytes were then observed under a fluorescent microscope at 400x magnification for nuclear evaluation. The Hoechst 33342 maximum excitation wavelength was 352 nm, and the emission wavelength was 461 nm.

The chromatin configurations were classified as follows (Bolamba et al. 1998; Hewitt and England 1999):

- Germinal vesicle (GV): identification of nucleolus and very fine filaments of chromatin;
- Germinal vesicle break down-Aanaphase I (GVBD–AI): identification of different patterns of chromatin condensation (GVBD) or identification of bivalents (AI);
- Telophase I-Mmetaphase II (TI–MII): identification of two groups of chromosomes moving to opposite ends of meiotic spindle (TI) or two sets of chromosomes clearly visible (MII);
- Degenerated: collapsed nucleus or irregular nuclear conformation.

2.5 Experimental design

2.5.1 Experiment I

ha formattato: Nessuna sottolineatura

To investigate the suitability of a 3D system for the oocyte in vitro culture, barium alginate (BA) microcapsules were prepared with different working conditions and a scoring method was applied to evaluate the physical properties of the obtained microcapsules.

Fresh feline COCs were in vitro cultured in the 3D system or in traditional microdrops of medium (2D system) for 24 hr. At the end of the in vitro maturation, viability and maturation rates of feline COCs were evaluated.

2.5.2 Experiment II

ha formattato: Nessuna sottolineatura

To verify whether the 3D system would improve the in vitro maturation of cumulus-denuded oocytes, fresh feline CDOs were co-cultured with COCs or cultured separately in 3D or 2D

system. After 24 hr, the viability and maturation rates of CDOs and COCs in co-culture, and of CDOs cultured separately were evaluated.

2.6 Statistical analysis.

Data for physical properties of the microcapsules were reported as mean value and standard deviation (SD), the viability and maturation rates of COCs and CDOs in coculture or cultured separately, CDOs(+), CDOs(-) and COCs(+) were analyzed by cChi-square test, and the level of significance was set at $p < 0.05$.

3 Results.

3.1 Experiment I

ha formattato: Nessuna sottolineatura

For the physical evaluation of the microcapsules, the following properties were recorded:

- dimensions (mm): length, width;
- shape: R (round), E (elongated);
- consistency: E (excellent), G (good), L (low).

The working condition 3 showed the best physical properties of BA microcapsules for encapsulation of feline oocytes encapsulation (Table 1). The dissolution of Na-alginate powder in sterile water and the dropping of $BaCl_2$ in b-mKRB was the proper combination. Although the dropping solution made with c-mKRB (working condition 4) allowed the formation of BA microcapsules, their shape and consistency were not as good as in working condition 3. On the other hand, the dissolution of Na-alginate powder in b- or c-mKRB (working conditions 1 and 2) did not allow the formation of any microcapsule.

The results of in vitro maturation of the COCs cultured in both 3D and 2D systems (Table 2) showed that they maintained a similar high viability (p > 0.05) and no differences were found in their meiotic resumption, nor full maturation (TI-MII stages) rates (p > 0.05).

3.2 Experiment II

The results presented in Table 3, showed that the overall viability was similar in 3D and 2D systems (p > 0.05). In 3D microcapsules, the presence of COCs resulted in a higher viability of co-cultured CDOs, than that obtained in CDOs cultured separately or in 2D microdrops (p = 0.007 and p = 0.002, respectively).

The 3D system was able to support the meiotic resumption of the feline oocytes, as well as the 2D microdrops (Table 4). The group of CDOs did not benefit from the co-culture in 3D microcapsules, as the percentages of meiotic resumption were similar of those of CDOs cultured separately. The highest values were reached by COCs in co-culture in both 3D and 2D system (p < 0.05). This group achieved better results of full maturation (TI-MII stages) than the associated CDOs (p = 0.005) and the CDOs cultured separately (p = 0.003).

4 Discussion

The present study was aimed at developing an enriched culture system to improve the in vitro performances of low-competence feline oocytes that lost their surrounding ~~cumulus cells~~CCs (cumulus-denuded oocytes, CDOs). At very low rates, these gametes reach the full cytoplasmic and nuclear competence because the functional and metabolic support of their somatic cells is missed. The subsequent in vitro fertilization and embryo development are~~is~~ also highly compromised (Tanghe et al. 2002; Luciano et al. 2005).

The enriched culture system used for CDOs in this work consisted ~~in a~~ of ~~three-dimensional~~3D microcapsules of barium alginate (BA) in association with fresh feline cumulus-oocytes complexes (COCs).

ha formattato: Nessuna sottolineatura

To obtain the 3D BA microcapsules, different working conditions were tested. The results showed that the dissolution of Na-alginate powder in sterile water and the subsequent dropping of $BaCl_2$ dissolved in the basic maturation medium (b-mKRB), whereas the best protocol to obtain round microcapsules with a solid inner core useful for encapsulation of feline oocytes encapsulation. The working conditions that involved media with hormones and growth factors supplementation (c-mKRB), as melting or dropping solution, seemed to inhibit the complete dissolution of Na-alginate powder and the ionic interactions with $BaCl_2$, compromising the effective creation and use of the microcapsules. However, the metabolic effect of nutrients, growth factors and hormones on the encapsulated oocytes was ensured by the immersion of the BA microcapsules in c-mKRB. The effective exchange of different molecules through these systems has been documented (Vigo et al. 2004).

A similar viability and maturation rate of feline COCs cultured in these proper BA microcapsules compared to those cultured in 2D microdrops, proved that the 3D system was a suitable culture condition for feline oocytes.

Many authors reported the efficiency of 3D systems for mammalian follicles and oocytes in vitro culture. In vitro growth and development (i.e. the proliferation and the differentiation of theca and granulosa cells, the steroid secretion and markers expression) of murine and human follicles were enhanced after in vitro culture in 3D alginate microcapsules, and the achievement of the full competence of the inner oocytes was also obtained (Xu et al. 2006; Xu et al. 2009; Shikanov et al. 2011). In addition, the encapsulation of human or swine oocytes in a collagen gel or in a BA capsules helped the in vitro meiosis progression until MII stage (Combelles et al. 2005; Munari et al. 2007).

In the present study, the CDOs co-cultured with COCs showed a higher viability in 3D than in 2D system, but no beneficial effects of this association was observed in meiosis resumption and full maturation (TI-MII stages) rates. Instead, the associated COCs had the highest viability and maturation rates in both systems.

Present data differed from previous studies in the domestic cat and in other species (Luciano et al. 2005; Ge et al. 2008; Godard et al. 2009) in which the presence of COCs during in vitro maturation and in vitro fertilization seemed to promote the achievement of MII stage and of the subsequent embryo development of CDOs.

In this study, fresh feline COCs were mechanically deprived of the surrounding ~~cumulus cells~~CCs to obtain the CDOs. This method, that differs from those of the aforementioned studies in which vortex or incubation with hyaluronidase were used, could have influenced the results. It remains to investigate how the oocytes without cumulus cells, and not denudated ad hoc, behave in the same culture conditions.

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It could also be hypothesized that the feline CDOs need different conditions to improve their performances in vitro, as the formulation of more specific maturation media.

It is notable that the presence of CDOs seemed to enhance the meiotic competence of the associated COCs. In bovine and murine model, this positive effect of denuded oocytes in the same culture condition as companion cells of COCs has been already reported (Hussein et al. 2006; Gilchrist et al. 2008). It is well known that the oocytes themselves produce some specific paracrine factors, known as the oocyte-secreted factors (OSFs), which act specifically on surrounding cumulus cells, regulating their differentiation, functional activity and gene expression. In the present study, these factors could have provided some beneficial support to ameliorate the maturation rates of COCs in both 3D and 2D culture conditions.

In conclusion, the 3D barium alginate microcapsules could support the in vitro culture of the feline oocytes, as well as the traditional 2D system. ~~Since~~ As the in vitro maturation rates of CDOs remain low, more specific and designing ad hoc in vitro conditions for these low-competence oocytes should be adopted.

The more physiological microenvironment, ~~i.e.~~that is the maintenance of oocytes architecture ensured by the 3D culture, represents an enriched condition that might also modulate the molecular expression of some oocyte quality markers, as the OSFs. The genetic expression of

these factors in oocytes cultured in 3D or 2D system should be investigated for improving the feline oocytes in vitro performances.

Author contributions

MGM, VG and GCL contributed to design the study, analysed the data and draft the ~~paper~~manuscript. MGM and SC performed the experiments. All authors have approved the final version.

Conflict of interest

None of the authors have any conflict of interest to declare. ~~of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.~~

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398 916-923.
399

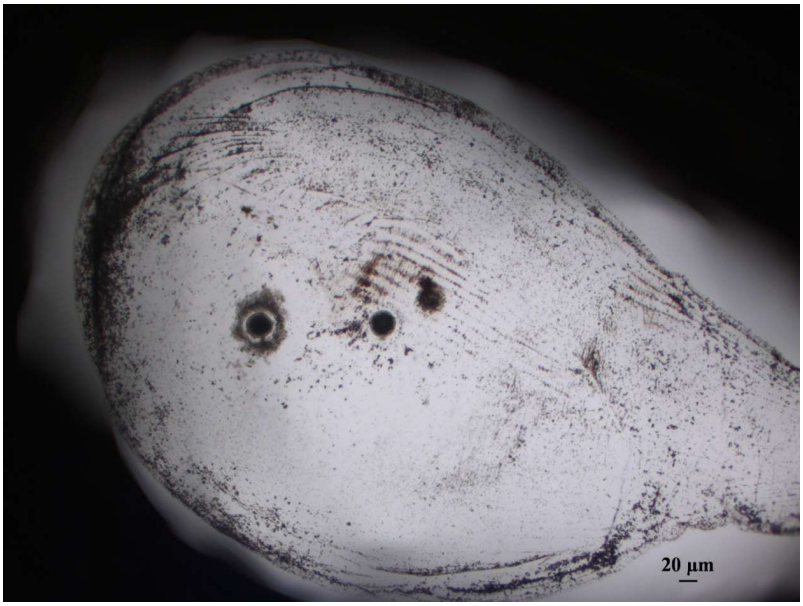
400 FIGURE AND TABLES

401

402 Figure 1

403 Domestic cat cumulus-denuded oocyte ~~(CDO)~~ co-cultured with cumulus-oocyte complex

404 ~~(COC)~~ in the three-dimensional ~~(3D)~~ barium alginate microcapsule.



405

406

407 Table 1.

408 Physical properties of the barium alginate (BA) microcapsules in different working

409 conditions.

410

Working conditions	Width, mm (mean±SD)	Lenght, mm (mean±SD)	Shape (R,E)	Consistency (E,G,L)
1				
MS with b-mKRB DS with b-mKRB	0	0	-	-
2				
MS with c-mKRB DS with c-mKRB	0	0	-	-
3				
MS with sterile water DS with b-mKRB	15.8 ± 1.81	24.12 ± 4.12	R	E
4				
MS with sterile water DS with c-mKRB	15.03 ± 2.47	31.52 ± 3.98	R and E	G and L

411

412 MS: melting solution of Na-alginate (0.5%); DS: dropping solution of $BaCl_2$ (40 mM); b-

413 mKRB: modified Krebs-Ringer bicarbonate-buffered salt solution with antibiotics; c-mKRB:

414 b-mKRB supplemented with 3 mg/ml of bovine serum albumin (BSA), 0.5 IU/ml of equine

415 chorionic gonadotropin (eCG), 1 IU/ml of human chorionic gonadotropin (hCG), 10 ng/ml of

416 epidermal growth factor (EGF) and 0.6 mM cysteine; Shape: R (round), E (elongated);

417 Consistency: E (excellent), G (good), L (low).

418

Table 2.

Viability and nuclear status of feline cumulus-oocyte complexes (COCs) after in vitro maturation in 3D or 2D system.

System	Viability	Immature (GV)	Meiotic resumption (GVBD-MII)	Full maturation (TI-MII)	Degenerated
	No. of oocytes (%)	No. of oocytes (%)	No. of oocytes (%)	No. of oocytes (%)	No. of oocytes (%)
3D	41/47 (87.2)	8/47 (17)	32/47 (68.1)	7/47 (14.9)	7/47 (14.9)
2D	37/44 (84)	9/44 (20.5)	26/44 (59.1)	7/44 (15.9)	9/44 (20.5)

GV, germinal vesicle; GVBD, germinal vesicle break down; TI, telophase I; MII, metaphase II.

No statistical differences.

3D: barium alginate microcapsules; 2D: microdrops of maturation medium.

Table 3.

Viability of feline cumulus-denuded oocytes (CDOs) and cumulus-oocyte complexes (COCs) cultured in 3D or 2D system.

Groups	Viability in 3D system	Viability in 2D system
	No. of oocytes (%)	No. of oocytes (%)
CDOs in co-culture	51/56 (91.1)^{a,x}	35/52 (67.3)^{b,x}
COCs in co-culture	45/47 (95.7)^{x}	44/48 (91.7)^{y}
CDOs cultured separately	37/52 (71.2)^{y}	46/56 (82.1)^{x,y}
Overall Viability	133/155 (85.8)	125/156 (80.1)

^{a,b}Different superscripts indicate significant differences within rows ($p < .05$).

^{x,y}Different superscripts indicate significant differences within columns ($p < .05$).

3D: barium alginate microcapsules; 2D: microdrops of maturation medium.

Table 4.

Nuclear status of feline cumulus-denuded oocytes (CDOs) and cumulus-oocyte complexes (COCs) cultured in 3D or 2D system.

Groups	Immature (GV)		Meiotic resumption (GVBD-MII)		Full maturation (TI-MII)		Degenerated	
	No. of oocytes (%)		No. of oocytes (%)		No. of oocytes (%)		No. of oocytes (%)	
	3D	2D	3D	2D	3D	2D	3D	2D
CDOs in co-culture	18/56 (32.1)^{x}	18/52 (42.9)^{x}	31/56 (55.4)^{x}	27/52 (51.9)^{x}	4/56 (7.1)^{x}	4/52 (7.7)^{x}	7/56 (12.5)	7/52 (13.5)
COCs in co-culture	3/47 (6.4)^{y}	5/48 (10.4)^{y}	39/47 (83)^{y}	40/48 (83.3)^{y}	19/47 (40.4)^{y}	25/48 (52.1)^{y}	5/47 (10.6)	3/48 (6.3)
CDOs cultured separately	21/52 (40.4)^{x}	20/56 (35.7)^{x}	21/52 (40.4)^{x}	23/56 (41.1)^{x}	3/52 (5.8)^{x}	2/56 (7.1)^{x}	10/52 (19.2)	13/56 (23.2)

GV, germinal vesicle; GVBD, germinal vesicle break down; TI, telophase I; MII, metaphase II.

No differences within rows.

^{x,y}Different superscripts indicate significant differences within columns ($p < .05$).

3D: barium alginate microcapsules; 2D: microdrops of maturation medium.