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Effect of culture methods on cumulus and oocyte morphology and meiotic competence of bovine oocytes from early antral follicles*

Abstract

The objective of the present study was to establish a culture system that would maintain the three-dimensional structure of bovine early antral follicles (EAF) or isolated cumulus-oocyte-granulosa complexes (COCGs) and increase the resulting portion of COCs with normal morphology for subsequent IVM. The morphological quality and meiotic competence of oocytes originating from early antral bovine ovarian follicles (0.2 to 0.7 mm and 0.4 to 0.7 mm diameter) were evaluated following culture in vitro for 14 and 7 d, respectively, and subsequent in vitro maturation. Growth culture modifications included culture in the well of the well (WOW) system; a microdroplet of collagen gel (2 x 5 µl vs. 2 x 400 µl; standard system) and culture of EAF in hanging drops of medium (inverted system). Significantly higher ($P<0.01$) proportions of COCs with normal morphology (60.4%) were obtained from COCGs compared to EAF (4.8%) grown in the WOW system. Embedding of COCGs in microdrops of collagen gel significantly increased proportion of COCs with normal morphology (63.2%) compared to those embedded in standard volume gels (35.3%). Recovery rate of COCs with normal morphology from cultured EAF was improved both by using microdrops of gel (44%) and by culture in the inverted system (39.3%) over that found for the standard system (8.5%).

Key Words: cattle, ovary, follicle, in vitro culture, oocyte quality and competence

Zusammenfassung

Titel der Arbeit: **Einfluss der Zellkulturmethode auf die Kumulus- und Oozytenmorphologie sowie auf die Meiosekompetenz von Rinderoozyten aus frühen Antralfollikeln**

Das Ziel der vorliegenden Untersuchungen war es ein Zellkultursystem zu etablieren, welches die dreidimensionale Struktur von frühen Antralfollikeln des Rindes (EAF) sowie von isolierten Cumulus-Oozyten-Komplexen mit anhaftender Granulosa (COCGs) gewährleistet. Ein weiteres Ziel war es, die Qualität der Oozyten in diesen Follikeln bzw. COCGs zu verbessern. Die Qualität der Oozyten wurde nach 14 bzw. 7 Tagen der Kultivierung sowie nach anschließender IVM bestimmt. Folgende Kultursysteme wurden verglichen: well of the well System (WOW), Mikrotropfen und Oberflächenbeschichtung aus Kollagengel, hängender Tropfen (Inverses System). Die Qualität der COCs nach Kultivierung von COCGs war signifikant erhöht (60,4%) im Vergleich zu Oozyten aus im WOW-Sytem kultivierten Follikeln (4,8%). Nach Einbettung der COCGs in Tropfen aus Kollagengel war der Anteil an morphologisch intakten COCs signifikant erhöht (63,2%) im Vergleich zu den Oozyten, die in oberflächenbeschichteten Kollagenschalen kultiviert wurden (35,3%). Der Anteil intakter COCs aus kultivierten Follikeln konnte sowohl nach Kultivierung der Follikel in Kollagentropfen als auch in hängenden Tropfen verbessert werden (44 und 39,3 vs. 8,5%).

Schlüsselwörter: Rind, Ovar, Follikel, in vitro Kultivierung, Oozytenqualität und Entwicklungsvermögen

Introduction

The mammalian ovary contains thousands of oocytes which are enclosed in preantral follicles. Since >99.9% of ovarian oocytes undergo atresia (ERICKSON, 1966; SAUMANDE, 1991) it would be of great practical benefit if these follicles could be rescued by growing them in culture, thus providing an abundant source of gametes and allowing for maximal utilization of the reproductive potential of the female. Many

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culture systems have been used to support follicle and oocyte growth in rodents (EPPIG and SCHROEDER, 1989; CARROLL et al., 1990; SPEARS et al., 1994; CAIN et al., 1995; EPPIG and O'BRIEN, 1996; O'BRIEN et al., 2003; WYCHERLEY et al., 2004). However, limited success has been achieved in farm animals (WANDJI et al., 1996; CECCONI et al., 1999, 2004; NEWTON et al., 1999; GUTIERREZ et al., 2000; KĄTSKA et al., 2000; KANITZ et al., 2001; WU et al., 2001; HIRAO et al., 2004; MATOS et al., 2004).

In cattle, HARADA et al. (1997) reported that 70% of bovine oocytes isolated from early antral follicles 0.5 to 0.7 mm in diameter increased their diameter during in vitro culture over 11 d. Using the same culture system, MIYANO et al. (1998) reported that only 5% of oocytes from follicles of this size showed developmental competence after IVM/IVF. Other attempts to culture bovine oocyte-granulosa cell complexes originating from early antral follicles on the flat substratum in medium supplemented with PVP allowed to improved efficiency of development to blastocyst stage to 12% (HIRAO et al., 2004). Small preantral follicles (diameter 35 to 100 μm) survived in culture for up to 6 or 7 d, however the quality of oocyte ultrastructure appeared to be unsatisfactory not only after culture but even immediately after follicle isolation (WANDJI et al., 1996; SAHA et al., 2000).

We have also found that late preantral and early antral follicles isolated from bovine ovaries can grow and survive in vitro (KĄTSKA and RYŃSKA, 1998; KĄTSKA et al., 2000). After 14 d of the growth culture of intact follicles, meiotic arrest was preserved in 71.9% of oocytes. The proportion of oocytes at the germinal vesicle (GV) stage did not differ significantly among oocytes evaluated immediately after recovery from the ovary, or those cultured for 6, 8, 11 or 14 d (KĄTSKA et al., 2000).

In most bovine follicle culture systems, intact follicles or cumulus oocyte complexes surrounded by parietal granulosa cells (COCGs) are cultured under a gase phase of 5% CO_2 in air (~ 20% oxygen), between two coats of collagen gel placed on the bottom of culture dish or well, which is covered by medium and, in some cases also an overlay of oil (TELFER, 1998; YAMAMOTO et al., 1999; ITOH et al., 2002). However, it has been observed that during long term culture in collagen gels that most granulosa cells migrate out of the follicle or follicle-like structures, and the oocyte becomes denuded of cumulus cells, which could drastically reduced its quality and competence (MIYANO et al., 1998; YAMAMOTO et al., 1999; KĄTSKA-KSIĄŻKIEWICZ, unpublished observations). Obtaining meiotically competent oocytes from cultured early antral follicles requires not only the survival of oocyte and the granulosa cells, but also maintenance of gap junctions and metabolic cooperation between these cells that are essential for oocyte growth and development. Unfortunately, some recent reports describing preantral follicle culture have given no information concerning oocyte quality and competence (GUTIERREZ et al., 2000; ITOH et al., 2002; WYCHERLEY et al., 2004). It is essential to characterize maturational competence of bovine oocytes originated from growing in vitro early antral ovarian follicles.

In this study our goal was to establish a culture system that would be able to maintain the three-dimensional structure of bovine early antral follicles or COCGs for a long period, reduce the migration of cumulus cells and increase the proportion of cumulus-oocyte complexes (COCs) with normal morphology, and as a consequence increase meiotic competence.

Material and Methods

All media and chemical reagents were purchased from Sigma, Germany.

Follicle recovery. The methods for follicle recovery were based on those described in previous experiments (KĄTSKA and RYŃSKA, 1998; KĄTSKA et al., 2000). Briefly, ovaries were collected from heifers and cows at local abattoirs and were transported to the laboratory within 2 h. The ovaries were washed 4 times in PBS supplemented with kanamycin (0.075 g/L), and thin cortical slices (approximately 1.0 mm of thickness) were cut from the ovarian surface with a razor blade. The slices were stored in a HEPES-buffered medium TCM 199 containing 5% FCS and antibiotics, at room temperature. Individual early antral follicles with diameter 0.2 to 0.7 mm were isolated by microdissection of the ovarian slices using fine sharp needles (30 g ½ inch) under a stereomicroscope. Follicle isolation was carried out over approximately 2 h of manipulation (KĄTSKA and RYŃSKA, 1998).

Follicle selection and in vitro growth. Intact early antral follicles were sorted into two size groups on the basis of their diameter: Group I with the diameter 0.2 to 0.4 mm and Group II with the diameter 0.4 to 0.7 mm. Group I follicles were cultured intact (EAF) for 14 d while in Group II cumulus-oocyte complexes with pieces of parietal granulosa (COCGs) were dissected and cultured for 7 to 14 d. Dissection of COCGs was carried out using technique presented by YAMAMOTO et al. (1999). Methods for culture of Group I and Group II follicles are presented in Experimental Design, below. The medium used for growth culture was TCM 199 with 3% BSA, ITS (insulin 6.25 µg/mL, transferrin 6.25 µg/mL and sodium selenite 6.25 ng/mL), 0.23 mM sodium pyruvate, 2 mM L-glutamine and 4 mM hypoxathine (KĄTSKA-KSIĄŻKIEWICZ and ALM, 2003). Medium in the dishes (half of the volume) was refreshed every 48 h.

Oocyte recovery, maturation and assessment of chromatin configuration. After the growth culture, cumulus-oocyte complexes (COCs) were recovered. Oocytes that lost cumulus cells and showed degeneration of cytoplasm were excluded; COCs showing normal morphology were used both for evaluation of chromatin configuration and for IVM. They matured in group 4 to 6 oocytes/10 µL drop of standard IVM medium, i.e. TCM 199 with 20% FCS and 5 µg ml^{-1} FSH for additional 24 h. For assessment of chromatin configuration oocytes freed of cumulus cells were fixed in buffered formol saline, stained with Hoechst 33258 and examined under a fluorescence microscope.

Experimental designs.

Experiment 1: Culture in the well of the well (WOW) system. The morphological quality and meiotic competence of oocytes originating from early antral bovine ovarian follicles were evaluated following in vitro growth and subsequent in vitro maturation. Both isolated follicles (EAF, Group I) and COCGs (Group II) were used for comparisons. They were inserted on the WOW (VAJTA et al., 2000); the well of the well with the round bottom with approximately 250 µm depth. The WOWs were prepared by hand, by melting the plastic in the bottom of the well of a 4-well dish using a heated, blunted steel needle. Twelve WOWs were prepared in each well of the dish. Each EAF or COCGs was inserted into an individual WOW and was then covered by 5 µl drop of collagen gel matrix (MONTESANO et al., 1983). Collagen gel matrix was prepared by mixing on ice rat collagen gel solution prepared in our lab according to MONTESANO et al. (1983) prescription, 10 x concentrated TCM 199 Earle's salt (Sigma, M 5017), and sodium bicarbonate solution (0.5 ml 1N NaOH, 0.22 g $NaHCO_3$ and 9.5 ml H_2O) in a ratio of 7:1:2 (v:v). After 5 min of incubation at 38^oC

in CO_2 incubator culture medium was added to the well, dishes were placed in CO_2 incubator and cultured for 10 to 14 d. At the end of culture period, COCs with normal morphology, suitable for IVM, freed from follicles or follicle-like structures were placed for approximately 24 h in standard IVM culture. The morphological quality and analysis of chromatin configuration were assessed.

Experiment 2: Comparison of two systems of embedding COCGs in collagen gel. In the standard method of embedding EAF or COCGs in collagen gel as reported by other authors (TELFER, 1998; WANDJI et al., 1996; ITOH et al., 2002) a relatively large volume (200 to 400 µl) of the gel is placed on the bottom of a well or dish, then follicles in small volume of medium are placed and another volume of matrix, similar as the first one, is layered over the follicles and allowed to gel. For this experiment, we compared this standard technique to a novel, low volume method for COCGs embedding. Two layers of gel were used to embed COCGs. Two volumes were used: A) 2 x 5 µl and B) 2 x 400 µl drops of collagen gel matrix within 30 mm Petri dish. In Group A, from 6 to 12 COCGs were embedded in the gel. In Group B, from 6 to 12 individual drops were placed in the dish, and one COCGs was embedded in each gel drop. Immediately after the collagen gelled (20 min for larger, 7 min for smaller volumes at CO_2 incubator at 38ºC), culture medium was added to the dish. Growth cultures of COCGs were carried out 7 d.

Experiment 3: Comparisons of 3 systems of in vitro growth culture of intact follicles. Similarly as in the growth culture of COCGs originating from Group II follicles, we investigated the influence of the method of embedding follicles of Group I in collagen gel on the quality of recovered COCs (2 coats of 400 µl or 2 coats of 5 µl drops of gel). Moreover, an inverted culture system applied for mouse preantral follicles culture by WYCHERLEY et al. (2004) was used. Briefly, follicles were placed individually in 96-well v-bottomed culture plates in 100 µl drops of the culture medium and the plate were turned upside-down in the incubator. Since the follicle in the inverted system lies at the medium/gas interface during culture, an effect of the inverted system should be to maximize access of oxygen to the cultured follicle. The follicles were transferred every other day to newly prepared wells. Follicles growing in hanging drops of medium were turned right side up for transfer and evaluation.

Table 1
Quality and competence of COCs originating from early antral bovine follicle cultured in the WOW system for 10 to 14 d as intact follicles and isolated COCGs followed up 24 h of IVM (Qualität und Kompetenz von COCs aus frühen Antralfollikeln des Rindes, die 10 bis 14 Tage in einem WOW-System als intakte Follikel oder isolierte COCGs kultiviert und anschließend 24 h gereift wurden)

Culture system	No of cultured	No (%) of COCs with normal morphology	No (%) of matured oocytes after IVM
Intact EAF	63	3 (4.8)[a]	1 (1.6%)[a]
Isolated COCGs	55	32 (60.4)[b]	7 (12.7%)[b]

[a,b] ($P<0.01$); test χ^2

Results

Experiment 1. The effects of the WOW culture system on quality and competence of oocytes originating from cultured intact EAF (Group I) or in the isolated COCGs (Group II) for 10 to 14 d of growth and additional 24 h of IVM are shown in Table 1. A significantly higher ($P<0.01$; χ^2 test) proportion of COCs with normal morphology were obtained from cultured COCGs in comparison to intact EAF of Group I. This

system appeared to be non-useful to growth culture of intact follicles as only 4.8% of COCs suitable for IVM were recovered.

Experiment 2. There was a significant difference (P<0.01) in the proportion of COCs with normal morphology after culture between the small, individual drops and bigger volume of collagen gel (63.2% vs. 35.3%; Table 2).

Table 2
The effect of method of embedding COCGs in collagen gel on quality of COCs following 7 d of growth culture (Der Einfluss der "Einbettung" von COCGs in Kollagenauf die Qualität der COCs nach 7 Tagen Kultivierung in vitro)

Method of embedding in collagen gel	No of cultured COCGs	No (%) of COCs with normal morphology	No (%) of matured oocytes after IVM
Large volume of gel 2 coats x 400 µl	68	24 (35.3)[a]	5 (7.3%)[a]
Small drop of gel 2 coats x 5 µl	87	55 (63.2)[b]	15 (17.2%)[a]

[a,b] (P<0.01); test χ^2

Experiment 3. The proportions of COCs with normal morphology recovered after growth culture of EAF in two different volumes of collagen gel or in an inverted system are presented in Table 3. The number of COCs with normal morphology was significantly increased (P<0.01) when intact EAF were cultured in small, individual drops of collagen gel and/or in the inverted system (Fig. 1) as compared with follicles embedded in the larger volume of gel.

Table 3
The effect of culture system of intact follicles EAF on the quality of COCs following 14 d of the in vitro growth culture (Der Einfluss des Zellkultursystems von intakten Follikeln auf die Qualität der COCs nach 14 Tagen Kultivierung in vitro)

System of growth culture	No of cultured follicles	No (%) of COCs with normal morphology
Large volume of gel – 2 coats x 400 µl	117	10 (8.5)[a]
Small, individual drops of gel – 2 coats x 5 µl	100	44 (44.0)[b]
Inverted system - hanging drops of medium, without collagen gel	56	22 (39.3)[b]

[a,b] (P<0.01); test χ^2

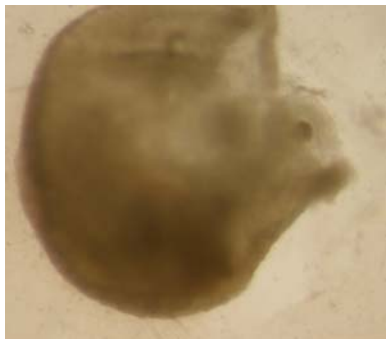

Fig. 1: Cumulus-oocyte complex freed from early antral bovine follicle following 14 d of growth culture in the inverted system (Cumulus-Oozyten-Komplex aus einem frühen Antralfollikel, der 14 Tage im hängenden Tropfen kultiviert wurde)

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Meiotic competence of oocytes from EAFs or COCGs, subjected to IVM following 14 or 7 d of growth culture, respectively, in small drops of collagen gel was assessed (Table 4; Fig. 2). A higher proportion of COCs from COCGs (63.2%) had normal morphology after growth culture and subsequently mature to M-I (30.9%; Fig. 2B), Telo I (1.8%; Fig. 2C) and M-II (18.2%; Fig. 2D) compared to intact EAF with normal morphology after growth culture (27.6%) and mature only to M-I (15.4%).

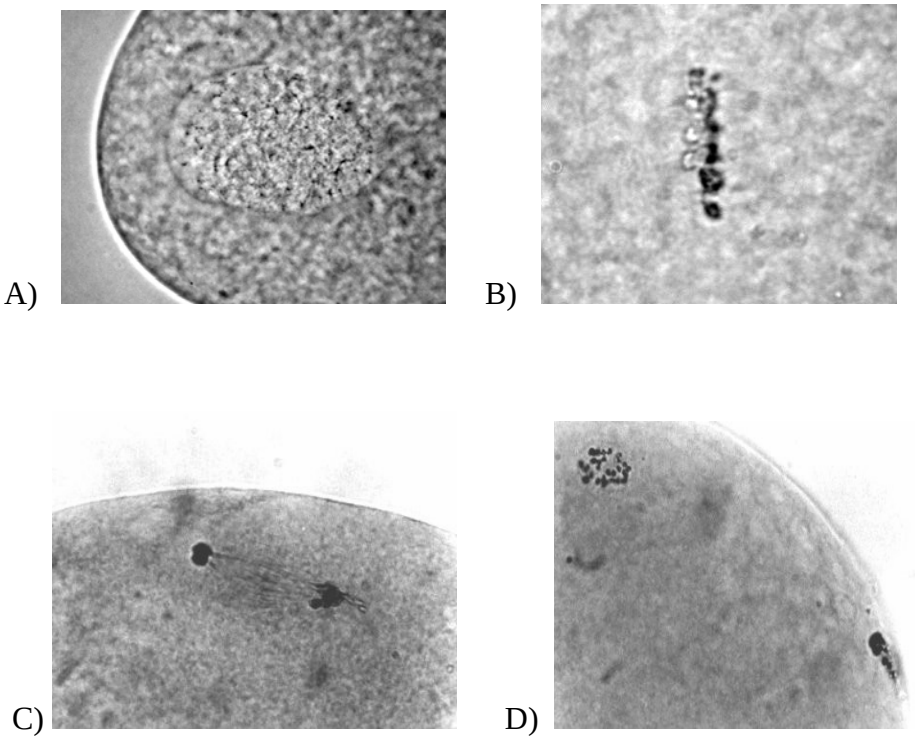

Fig. 2: Chromatin configuration in bovine oocytes after growing culture in small drops of collagen gel and subsequent IVM. (A) Germinal vesicle oocyte in Diplotene. (B) M-I stage. (C) Telo-I stage. (D) M-II with polar body. Magnification: x 1000 (Chromatinkonfiguration in Rinderoozyten, kulitiviert auf Kollagengeltropfen und anschließender IVM. (A) Oozyten im Diplotänstadium des GV. (B) Metaphase I. (C) Telophase I. (D) Metaphase II mit Polkörperchen. Vergr. x 1000)

Table 4
Meiotic competence of oocytes that grown as COCGs and isolated follicles IF embedded in small drops of collagen gel (Meiosekompetenz von Oozyten aus in vitro kultivierten COCGs eingebettet in kleine Tropfen aus Kollagengel)

Growth culture	No of cultured	No (%) of COCs for IVM	Evaluation time after	Chromatin configuration - No (%) of oocytes						
				GV	Diak[1]	Meta I[2]	Telo I[3]	Meta II[4]	Deg.[5]	Act.[6]
	46	28	growth	15	7	2	-	-	4	-
COCGs		(60.9)		(53.6)	(25.0)	(7.1)			(14.3)	
	87	55[a]	IVM	7[a]	8	17	1	10	8	4
		(63.2)		(12.7)	(14.5)	(30.9)	(1.8)	(18.2)	(14.5)	(7.3)
	53	23	growth	13	3	1	-	-	6	-
EAF		(43.4)		(56.5)	(13.0)	(4.3)			(26.1)	
	47	13[b]	IVM	8[b]	1	2	-	-	2	-
		(27.6)		(61.6)	(7.7)	(15.4)			(15.4)	

[1]Diak = diakinesis; [2]Meta I = metaphase I; [3]Telo I = telophase I; [4]Meta II- metaphase II; [5]Deg. = degenerated and undetermined oocytes; [6]Act. = activated oocytes with ♀ pronucleus; [a,b] ($P<0,01$); test χ^2.

Discussion

In vitro follicle culture conditions of are not yet optimal, as the meiotic and developmental competence of oocytes from cultured follicles is far below what one would expect from healthy in vivo grown bovine follicles. The greatest degree of success in producing live births from in vitro grown oocytes has being associated with culture of COCGs isolated from relatively large, early antral follicles (diameter approx. 0.7 mm) followed by maturation of isolated COCs and IVF (YAMAMOTO et al., 1999; HIRAO et al., 2004). However, even in the case of this class of follicles the efficiency of embryo production was rather low. A possible reason for the low efficiency of in vitro growth culture of isolated COCGs is a loss of communication between oocyte and surrounding granulosa cells as a result of migration of granulosa cells during culture and, as a consequence, disturbances in access to oocyte nutrition and signaling leading to degeneration (KĄTSKA-KSIĄŻKIEWICZ, unpublished data).

We have investigated the use of different systems for growth culture of follicles and COCGs and compared it with the system used in other experiments (TELFER, 1998; ITOH et al., 2002). In the present study, when isolated COCGs were cultured in the WOW system (VAJTA et al., 2000) they provided a similar proportion of oocytes with normal morphology as did culture in the small gel droplet, and higher than in the standard method (Experiment 2). As was expected, cultured COCGs provided a higher proportion of oocytes with normal morphology compared to that of the intact EAF. Culture of EAF in the WOW system resulted in a low proportion of oocytes with normal morphology, but 1 of the 3 cultured EAF-WOW oocytes matured to M-II, as opposed to none of 13 oocytes originating from EAF cultured in small gel drops (Table 4). The wells in WOW system may have been too small for intact follicles and, as a consequence, kept follicles from sitting inside the well. The COCGs also appeared to be slightly to large to be properly located inside the WOWs. The rate of COCs with normal morphology after growth culture in small, individual drops of collagen gel was remarkably high (Experiment 2). Most researchers use volumes between 200 to 400 µl covered with the second, similar volume of matrix for embedding follicles in collagen gel (TELFER, 1998; YAMAMOTO et al., 1999; ITOH et al., 2002). Our novel system embedding EAF or COCGs in 5 µl drops helps to prevent migration of granulosa cells out of follicle or follicle-like structure created by COCGs. At the same time, in the microdrop system, several follicles or COCGs are cultured in the same dish, which might provide the exchange of paracrine factors, oxygen and nutrient supplementations among follicles.

Since a follicle contains several thousands of cells, the question arises as to whether oxygen diffusion to the bottom of the culture well through the collagen gel coats is capable of supplying enough oxygen for follicle growth. Therefore we compared 3 methods of EAF growth culture using small and large drops of collagen gel and hanging drops of medium in the inverted system. The results did not reveal marked differences between the quality of oocytes cultured in the microdrops of gel and in the inverted system with hanging drops of medium. However, again, embedding in the large drops of collagen gel appeared to be unsuitable for follicle growth culture because significantly fewer COCs with normal morphology could be obtained. Unfortunately, even in small drops of collagen gel, meiotic competence of oocytes originated from cultured EAF was drastically reduced compared to that of those from

cultured COCGs, in spite of the fact that COCs morphology following EAF growth culture seemed to be satisfactory. These differences between oocytes from small, intact follicles and follicle-like structures formed by COCGs are perhaps not surprising because of the difference in initial diameter of the two groups of follicles. It was hypothesized that too small initial diameter of EAF and, as a consequence the immaturity of the enclosed oocytes before growth culture were associated with the low maturation rate of these oocytes after IVM culture. The good proportion of normal COC morphology after 14 d culture of EAF, and the high rate of maintanence of the GV (56-61%) in this group, indicates that these oocytes were not degenerating at the time they were removed from culture. Perhaps a longer culture period would be associated with greater meiotic competence in oocytes from early antral follicles.

Conclusions

Isolated complexes COCGs create in growth culture follicle-like structures and their oocytes achieve meiotic competence and mature to metaphase II at a 2-fold higher rate than do oocytes from smaller diameter follicles which are cultured intact. The proportion of COCs with normal morphology following growth culture significantly increased when isolated COCGs were embedded in microdrops of collagen gel rather than in large gel volumes. For the growth culture of intact follicles, the proportion of COCs with normal morphology was increased both in microdrops of gel and in culture in the inverted system compared with large gel volumes. Growth culture of isolated COCGs in the small drops of collagen gel may offer considerable advantages for future experiments in culture of oocytes from small follicles. Finally, it is worthy of comment that the determination of developmental competence following IVM/IVF of oocytes cultured in isolated COCGs and/or intact EAF will be assessed in a future experiment.

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