Interactive effects of granulosa cell apoptosis, follicle size, cumulus–oocyte complex morphology, and cumulus expansion on the developmental competence of goat oocytes: a study using the well-in-drop culture system

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Abstract

Using a well-in-drop (WID) oocyte/embryo culture system that allows identification of follicular origin, we have investigated the effects of granulosa cells (GCs) apoptosis, follicle size, cumulus–oocyte complexes (COCs) morphology, and cumulus expansion on the developmental competence of goat oocytes matured and cultured individually following parthenogenetic activation. The WID system supported oocyte maturation and embryo development to a level similar to the conventional group system. The majority of goat oocytes acquired competence for development up to the 8–16 cell stage in follicles larger than 2 mm, but did not gain the ability to form morula/blastocyst (M/Bs) until follicles larger than 3 mm in diameter. The extent of atresia affected M/Bs formation. This effect varied according to the follicle size. Cumulus expansion increased with follicle size and decreased with increasing incidence of GCs apoptosis. Oocyte developmental potential was also correlated with cumulus expansion. Regardless of the degree of follicle atresia, 73–84% of the floating cells in the follicular fluid (FF) underwent apoptosis. Correlation between floating cell density in FF and oocyte developmental potency suggests the possibility to use the floating cell density as a simple and non-invasive marker for oocyte quality. It is concluded that the developmental potential of an oocyte is determined by multifactor interactions, and multiple factors must be considered together to accurately predict the quality of an oocyte.

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Introduction

A number of studies have shown that *in vitro* development of pre-implantation stage mammalian embryos is better when cultured in groups (group culture) than cultured singly in microdrops (microdrop individual culture, MIC) (Gardner *et al.* 1994, Kato & Tsunoda 1994, Keefer *et al.* 1994, Moessner & Dodson 1995). However, apart from the large-scale production of embryos for experimental purposes, most commercial and human embryo culture systems require culturing individually or in small groups. The reason for this need is the small number of oocytes available (ovum pick-up (OPU) technology, single slaughtered valuable oocyte donors and human IVF) or the required identification after culture. Besides, sometimes zona-free eggs must be cultured in isolation from each other to prevent conglomeration. Therefore, the establishment of an individual culture system that is as efficient as group

culture has become of prime importance. Furthermore, although there are quite a few reports on individual culture of embryos, studies on individual culture of oocytes during maturation are few and with conflicting results (Carolan *et al.* 1996, Hagemann *et al.* 1998, Jewgenow *et al.* 1999, Ward *et al.* 2000).

Follicular oocytes obtained from slaughtered animals have been found highly variable in their developmental competence after *in vitro* maturation (IVM; Gordon & Lu 1990, Bilodeau-Goeseels & Panich 2002). Accurate, fast, and non-invasive markers of oocyte quality are therefore in urgent need for oocyte selection prior to IVM. Traditional methods have relied upon the measurement of follicle diameter and the morphological assessment of cumulus–oocyte complexes (COCs). Although several studies have shown that overall, oocytes from larger follicles have a greater developmental potential to the blastocyst stage *in vitro* (Pavlok *et al.* 1992, Lonergan

et al. 1994, Blondin & Sirard 1995, Blondin *et al.* 1997), diameter does not necessarily indicate whether the follicle is healthy and growing or undergoing atresia. Although COCs morphological assessment is undoubtedly popular, its accuracy as a forecaster of oocyte quality is still uncertain (Blondin & Sirard 1995, Hazeleger *et al.* 1995, Jewgenow *et al.* 1999, Ebner *et al.* 2003).

It is recognized that apoptotic cell death is an underlying mechanism of cell loss during follicular atresia (Tilly *et al.* 1991, Hsueh *et al.* 1994, Liu *et al.* 2003, Yu *et al.* 2003, 2004). While the detection of granulosa cells (GCs) apoptosis has been widely used to identify atretic follicles, very little evidence for its use as a marker of oocyte quality exists. The effect of follicular cell apoptosis on oocyte developmental competence should be best studied by individual culture that demonstrates a functional relationship between follicular origin and quality of oocytes. However, studies in this field are few and results are discrepant. With MIC systems, some studies indicated that reduced oocyte developmental capacity was related to apoptotic death of follicular cells even before morphological signs of severe atresia were detected (Jewgenow *et al.* 1999), but others suggested that even a high degree of atresia was not necessarily detrimental (Hagemann *et al.* 1999). Therefore, the effect of follicular cell apoptosis on oocyte developmental potential must be studied using an individual culture system that is as efficient and consistent as group culture. In addition, most of the above reviewed studies were conducted on cattle or other animals, not on goats.

In this study, we first established a well-in-drop (WID) system for individual oocyte/embryo culture and proved that the system was as efficient as group culture. We then studied the effects of GCs apoptosis, density of GCs in follicular fluid (FF), follicle size, COC morphology, and cumulus expansion on the developmental competence of goat oocytes using this system. We found that the developmental competence of oocytes with the same COC morphology and follicle size and the same grade of cumulus expansion may differ significantly with the level of GCs apoptosis. However, at the same level of atresia, oocyte developmental capacity may vary with follicle size, COC morphology, or grades of cumulus expansion. This suggests that the developmental potential of an oocyte is determined by multifactor interactions, and multiple factors must be considered together to accurately predict the quality of an oocyte.

Materials and Methods

Isolation of follicles and recovery of oocytes and GCs

Goat ovaries were obtained from a local abattoir and transported within 3 h to the laboratory in sterilized saline containing 100 IU/ml penicillin and 0.05 mg/ml

streptomycin and maintained at 30–35 °C. Ovaries in growth phase with neither corpus luteum nor follicles larger than 5 mm in diameter were selected. Follicles were dissected by forceps, freed of adherent stromal tissue, and processed individually in 100 µl Dulbecco's PBS (D-PBS) drops under a stereomicroscope. After their diameters were recorded to the nearest 0.1 mm with the aid of an eyepiece graticule, follicles were opened with forceps to remove COCs and GCs. The released COCs were characterized for morphological quality, picked up, and processed as described below. Follicles were then bisected and GCs on the interior wall were gently scraped into D-PBS.

The COCs recovered were divided into four grades according to the surrounding cumulus cells and quality of oocytes. Grade A, with four or more layers of cumulus cells; grade B, with one to three layers of cumulus cells; grade C, completely or partially denuded; and grade D, all others, including those with expanded cumulus and degenerated oocytes (Hashimoto *et al.* 1999). Only grades A and B oocytes from follicular growth stage ovaries were used unless otherwise specified.

Assessment of GCs apoptosis

The drop of D-PBS with GCs was collected into a 1.5 ml tube on ice. Free floating GCs were separated from FF and D-PBS by centrifugation (200 × *g*, 5 min) at 4 °C. For Hoechst 33342 staining, the GCs pellets were re-suspended in 50 µl D-PBS supplemented with 0.01 mg/ml Hoechst 33342 (Sigma) and stained in dark for 5 min. Ten microliters drop of suspension were then smeared on the slide and observed under a fluorescence microscope (1000×, under oil immersion). Cells with pycnotic nuclei (shrunken nuclei with a bright fluorescence appearance resulting from condensed chromatin) were considered as apoptotic (Fig. 1A, Shiota *et al.* 2003, Chen *et al.* 2005). Four to six fields of each slide were examined, and 200 GCs on each slide were analyzed to evaluate the degree of apoptosis of a follicle. Classes of follicles were determined as non-atretic with <5% pycnotic nuclei, early atretic with 5–30% pycnotic nuclei, mid-atretic with 30–50% pycnotic nuclei, and late atretic with >50% pycnotic nuclei.

For hematoxylin staining, the GCs pellets were re-suspended in 100 µl 4% paraformaldehyde and prefixed for 20 min at room temperature. At the end of pre-fixation, a drop of the suspension was smeared on a slide and allowed to dry. The procedures used for hematoxylin staining were those for the conventional hematoxylin-eosin (HE) staining described in the textbook (Brauer 1955) with minor modifications. The stained cells were observed under a light microscope. Cells with heavily stained, shrunken nuclei, or apoptotic bodies were considered as apoptotic (Fig. 1B, Liu *et al.* 2003).

Figure 1 Goat granulosa-cell smears stained by different methods. A is a smear stained with Hoechst 33342, in which the apoptotic cells (arrows) showed shrunken nuclei with a bright fluorescence appearance under a fluorescence microscope (magnification, $\times 1000$). B is a smear stained with hematoxylin, in which the apoptotic cells showed heavily stained, shrunken nuclei, or apoptotic bodies (arrows) under a light microscope (magnification, $\times 1000$). C is a merged picture of a smear triple-stained with Annexin V, PI, and Hoechst 33342, in which the apoptotic and necrotic cells appeared pink or white (bar = 20 μm).

For triple staining with Annexin V, PI, and Hoechst 33342, the GCs pellets were re-suspended in 100 μl binding buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2 , 10 mM Hepes, pH 7.4) and centrifuged at $200\times g$ for 5 min. Then, the cell pellets were re-suspended in 10 μl buffer, 0.5 μl Annexin V-FITC solution (Sigma), 1 μl PI solution (100 $\mu\text{g}/\text{ml}$ in PBS), and 1 μl Hoechst 33342 solution (0.1 mg/ml in PBS) and incubated for 10 min at room temperature. At the end of incubation, the cells were washed by centrifugation, smeared on a slide and observed under a Leica laser scanning confocal system (TCS SP2). Hoechst 33342 labeled nuclear chromatin was excited with the 405 nm line of a diode laser. The fluorescein isothiocyanate (FITC) and propidium iodine (PI) fluorescence was obtained by excitation with 488 nm line of an Ar/ArHr laser and the emitted light was passed through a 488 nm filter. The individual optical sections were pseudo-colored and digitally recombined into a single composite image using the Leica Confocal Software. In the merged picture, while the healthy cells looked blue, the apoptotic and necrotic cells appeared pink or white (Fig. 1C).

Assessment of GCs density in follicular fluid

After washing thrice in D-PBS, each follicle was opened under a stereomicroscope with fine forceps in a D-PBS droplet of appropriate volume, carefully avoiding mural GCs fell off because of mechanical damage. Mural GCs that fell off by any chance were carefully removed by a mouth pipette. The resulted suspension was placed on a cytometer for floating GCs density assessment.

To determine the volume of D-PBS droplets used for collection of GCs from individual follicles, we measured the volume of FF from follicles of different sizes, and found a significant positive correlation between the volume of FF and the diameter of follicles ($r=0.95$, $P<0.01$). A regression analysis established the following two equations for calculation of FF volume from follicles of different sizes:

$$v = 8.9d - 17.0 \quad (\text{when } d > 2.5 \text{ mm}), \quad v = 3.8d - 4.7 \quad (\text{when } 1.5 \text{ mm} \leq d \leq 2.5 \text{ mm}).$$

Here, v = FF volume (μl) and d = follicle diameter (mm).

Since, only follicles larger than 2.5 mm in diameter showed no mural GCs detachment and can hence be used for FF floating cell density measurement (see Results for details), and because a big deviation in FF volume was noticed among follicles in this group, the volumes of droplets for FF collection from these follicles were determined in three groups: 40 μl D-PBS was used to dilute 6–10 μl FF from 2.5 to 2.9 mm follicles, 60 μl D-PBS was used for 10–12 μl FF from 3.0 to 3.4 mm follicles, and 80 μl D-PBS for 13–18 μl FF from 3.5 to 3.9 mm follicles.

Preparation of the WID

Two kinds of WIDs were used for experiments. The WID for IVM was large with approximately 0.9 mm width and 0.5 mm depth. The WID for embryo culture was small with approximately 0.5 mm width and 0.3 mm depth. A fire-polished solid glass rod was heated on a flame for approximately 2 s, and then pressed slightly by hand to the bottom of a 35 mm polystyrene dish. The plastic melted immediately, and the rod was slightly immersed. After 15–20 s, the plastic became solidified and the rod was removed. In the WID system, 10 large wells and 20 small wells were made in each group for IVM and embryo culture, respectively. The wells were then filled with D-PBS supplemented with 0.1% of polyvinyl alcohol (PVA, Sigma Chemical Co., St Louis, MO, USA), and rigorously flushed by pipetting to remove air bubbles and possible toxic material arising from the melting process. The D-PBS was subsequently replaced with culture medium, which covered all the 10 large wells with 100 μl droplets or the 20 small wells with 50 μl droplets, covered with mineral oil and incubated overnight at 38.5 $^{\circ}\text{C}$ under 5% CO_2 in humidified air. After another rigorous flushing of the wells, the medium was replaced by a new 100 or 50 μl culture medium and used for culture. Oocytes or embryos were cultured

singly in different wells, which were numbered from 1 to 10 or 1 to 20.

In vitro maturation of oocytes

The recovered COCs were washed thrice in D-PBS and once in the culture medium before culture. Then, the COCs were put in WIDs for IVM (each per well). The culture medium was TCM-199 (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) FCS (Gibco), 1 mg/ml 17 β -estradiol, 24.2 mg/l sodium pyruvate, 0.05 IU/ml FSH, 0.05 IU/ml LH, and 10 ng/ml EGF. Maturation culture was conducted at 38.5 °C under 5% CO₂ in humidified air.

Oocyte activation

At the end of maturation culture, oocytes were examined and processed individually under a stereomicroscope. Their cumulus cells were stripped by pipetting in D-PBS containing 0.1% (w/v) hyaluronidase (Sigma). Denuded oocytes with a polar body were selected and washed in D-PBS before activation treatments. The ionomycin and 6-DMAP stocks were prepared in dimethyl sulfoxide (DMSO) and diluted to the desired concentrations in CR1aa (Rosenkrans *et al.* 1993) supplemented with 3 mg/ml BSA (Sigma) and 5% FCS before use. Oocytes were first exposed to 5 mM ionomycin for 2 min at room temperature, and then, after being extensively washed in CR1aa, they were incubated in CR1aa containing 2 mM 6-DMAP for 2 h at 38.5 °C under 5% CO₂ in humidified air (Lan *et al.* 2005).

Culture of activated oocytes.

Oocytes after activation treatment were co-cultured on cumulus cell monolayers (CCM) in CR1aa containing 3 mg/ml BSA and 5% FCS. To prepare CCM, cumulus cells were collected from *in vitro* matured COCs and cultured in DMEM/F12 (Gibco) supplemented with 10% FCS in the WID system for embryo culture. At 24 h of culture, the DMEM/F12 in WID drops with growing CCM were replaced with 50 μ l warmed CR1aa and equilibrated for 1 h prior to embryo culture. After activation treatments, oocytes were washed in CR1aa and transferred to WIDs (one oocyte per well) and incubated for 9 days at 38.5 °C under 5% CO₂ in humidified air. Half of the culture medium in each drop was renewed at 48-h intervals. At the end of co-culture, development was examined under a microscope and numbers of embryos developing to different stages were recorded.

All the procedures during maturation, activation, and embryo culture were conducted carefully to ensure developmental competence could be traced back to the oocyte's follicle of origin. To keep results constant, all the

procedures including measurement of follicle diameter, assessment of GCs apoptosis, and density and grading of COCs, and cumulus expansion were performed by one person (the first author of the paper).

Data analysis

Statistical analyses were carried out by ANOVA using Statistical Package for Social Sciences 11.5 for Windows (SPSS, Inc., Chicago, IL, USA). Where necessary, data were transformed. Differences between treatment groups were evaluated with the Duncan multiple comparison test. Data are expressed as mean $\pm$ S.E.M. and $P < 0.05$ is considered significant.

Results

Accuracy of Hoechst staining for assessment of GCs apoptosis

GCs collected from the same follicle were divided into three parts and apoptosis were assayed by Hoechst 33342 staining, hematoxylin staining, and triple staining (with Annexin V, PI, and Hoechst 33342) respectively. Among the follicles analyzed ($n = 13$), the average percentages of apoptotic granulosa cells (AGCs) revealed by triple staining, hematoxylin staining, and Hoechst staining were $38.5 \pm 3.6\%$ (ranging from 11 to 59%), $24.8 \pm 3.4\%$ (0–38%), and $26 \pm 3.4\%$ (1–38%) respectively (Fig. 2). Although the percentages of AGCs revealed by hematoxylin staining and Hoechst staining were lower than those detected by triple staining, the three sets of values were highly correlated, $r = 0.89$ and 0.97 between Hoechst and hematoxylin and between Hoechst and triple staining respectively ($P < 0.01$). Considering that triple staining and hematoxylin staining are more time-consuming and/or costly, and Hoechst staining has been used with success for apoptosis detection (Shiota *et al.* 2003, Chen *et al.* 2005), Hoechst staining was chosen for this study.

Maturation and embryo development of goat oocytes cultured under different systems

Goat oocytes from 2 to 4 mm follicles were matured, activated, and embryos cultured singly in microdrops (1 oocyte/10 μ l drop), in groups (10 oocytes/100 μ l drop for maturation, 20 oocytes/50 μ l drop for embryo culture) or in the WID system. Maturation rates did not differ ($P > 0.05$) among different culture systems (Table 1). While morula/blastocyst (M/B) rates (percent morulae/blastocysts of cleaved oocytes) did not differ between WID culture and group culture, percent cleaved oocytes decreased significantly and no embryos developed into M/Bs when cultured singly in microdrops.

Figure 2 A graph with two scatter plots showing the percentages of apoptotic granulosa cells detected by Hoechst 33342 versus hematoxylin staining and Hoechst 33342 versus triple staining respectively, among 13 different follicles. The correlation coefficients are 0.97 and 0.89 between Hoechst and hematoxylin and between Hoechst and triple staining respectively. Correlation is significant at the 0.01 level in both cases.

Effects of GCs apoptosis on oocyte developmental competence

In the 1–1.4 mm follicle group, no oocytes from non-atretic follicles matured, but some matured and cleaved after activation when percent AGCs increased to above 15% (Table 2). In the 1.5–1.9 mm follicle group, maturation rates of oocytes were significantly higher in follicles with more than 30% AGCs than in follicles with <30% AGCs. The percentage of 8–16 cell embryos was significantly higher in follicles with 30–50% AGCs than in follicles with <15% or more than 50% AGCs. In both the 2–2.9 and 3–3.9 mm follicle groups, M/B rates were significantly higher in early atretic follicles than in the non- or late atretic follicles. The overall rate of M/Bs was lower in 2–2.9 mm follicles than in 3–3.9 mm follicles

(6.3 ± 3.6 vs $15.9 \pm 5.1\%$, $P=0.05$). This indicated that (a) the majority of goat oocytes acquired competence for meiosis and development up to the 8–16 cell stage in follicles larger than 2 mm, but they did not gain the ability to form M/Bs until follicles larger than 3 mm in diameter; (b) while moderate atresia was beneficial but severe atresia was detrimental to competent oocytes, atresia of different degrees was beneficial to the incompetent oocytes.

Relationship between GCs apoptosis, cumulus expansion, and oocyte developmental competence

The COCs were matured for 24 h before cumulus expansion was assessed under a microscope. Cumulus expansion was scored in 0–4 grades. 0, no response; 1, minimum response, cells in the peripheral two layers began to expand; 2, expansion extended inwards to several layers of cumulus cells; 3, expansion of all layers of the cumulus except corona radiata cells; and 4, expansion of the whole cumulus including corona radiata cells (Sun *et al.* 2002a). Cumulus expansion capacity of goat oocytes decreased with increasing apoptosis of GCs, but increased with follicle size. Thus, few oocytes from follicles with more than 30% AGCs or from follicles smaller than 2 mm in diameter underwent a cumulus expansion beyond grade 3 (Table 3). All the data in Table 3 were recorded from the same population of oocytes as used in Table 2.

The relationship between cumulus expansion and competence of oocytes from 2 to 4 mm follicles was analyzed using the data from Table 3. Rates of M/Bs were significantly higher in oocytes with grade 3 cumulus expansion than in those showing grade 2 or 1 cumulus expansion in follicles with 5–30% AGCs (Table 4). Oocytes showing no cumulus expansion never formed M/Bs. Rates of M/Bs were significantly lower in oocytes from follicles with <5% AGCs than in oocytes from follicles with 15–30% AGCs in both grade 2 and grade 3 expansion groups. This indicates that oocyte developmental potential increases with increasing cumulus expansion, but oocytes showing the same grade of cumulus expansion may vary in their developmental competence with degrees of follicular atresia.

Table 1 Maturation and embryo development of goat oocytes from 2–4 mm follicles cultured under different systems.

Culture system	Oocytes cultured (replicates)	% Oocytes matured	% Embryos developing to		
			2-Cell	8–16 Cell	Mor/blast
MIC	33 (3)	77.4 ± 4.3^a	61.7 ± 2.5^a	26.2 ± 5.0^a	0 ^a
Group	60 (3)	86.7 ± 4.8^a	84.7 ± 4.4^b	50.6 ± 1.8^b	18.8 ± 3.2^b
WID	60 (3)	88.3 ± 3.3^a	79.1 ± 4.3^b	52.5 ± 4.9^b	14.2 ± 3.6^b

Values with common letters in their superscripts in the same column did not differ ($P>0.05$).

Table 2 Effects of granulosa cell apoptosis on development of goat oocytes from follicles of different sizes.

Follicle diameter (mm)	% AGCs	Oocytes observed	% Matured oocytes	% Embryos developing to		
				2-Cell	8–16 Cell	Mor/blast
1.0–1.4	<5	25	0 ^a			
	5–15	21	0 ^a			
	15–30	23	26.1±9.3 ^b	33.3±21.1 ^a	0 ^a	
	30–50	27	29.6±9.0 ^b	37.5±18.3 ^a	0 ^a	
	>50	21	33.3±10.5 ^b	42.9±20.2 ^a	0 ^a	
1.5–1.9	<5	43	37.2±7.5 ^a	43.8±12.8 ^a	0 ^a	
	5–15	35	40.0±8.4 ^a	50.0±13.9 ^a	14.3±14.3 ^a	0 ^a
	15–30	33	39.4±8.6 ^a	61.5±14.0 ^a	37.5±18.3 ^{a, b}	0 ^a
	30–50	36	69.4±7.8 ^b	60.0±10.0 ^a	40.0±13.1 ^b	6.7±6.7 ^a
	>50	32	65.6±8.5 ^b	57.1±11.1 ^a	16.7±11.2 ^a	0 ^a
2.0–2.9	<5	55	81.8±5.2 ^a	80.0±6.0 ^a	33.3±8.0 ^{a, b}	0 ^a
	5–15	65	83.1±4.7 ^a	83.3±5.1 ^a	44.4±7.5 ^{a, b}	8.9±4.3 ^{a, b}
	15–30	52	82.7±5.3 ^a	86.0±5.3 ^a	56.8±8.3 ^b	18.9±6.5 ^b
	30–50	49	75.5±6.2 ^a	73.0±7.4 ^{a, b}	33.3±9.2 ^{a, b}	3.7±3.7 ^a
	>50	41	73.2±7.0 ^a	56.7±9.2 ^b	23.5±10.6 ^a	0 ^a
3.0–3.9	<5	78	85.9±4.0 ^a	88.1±4.0 ^a	37.3±6.4 ^a	3.4±2.4 ^a
	5–15	71	84.5±4.3 ^a	88.3±4.2 ^a	60.4±6.8 ^b	30.2±6.4 ^b
	15–30	63	77.8±5.3 ^a	79.6±5.8 ^a	61.5±7.9 ^b	25.6±7.1 ^{b, c}
	30–50	61	73.8±5.7 ^a	75.6±6.5 ^{a, b}	38.2±8.5 ^a	11.8±5.6 ^{a, c}
	>50	53	73.6±6.1 ^a	61.5±7.9 ^b	29.2±9.5 ^a	8.3±5.8 ^a

Values with common letters in their superscripts in the same column within follicle size group did not differ ($P>0.05$).

Correlation of floating cell density in the follicular fluid with oocyte developmental capacity

When follicles of different diameters were opened under a stereomicroscope, antral layers of mural GCs detached from many (26–40%) of the follicles smaller than 2.4 mm in diameter, but this seldom occurred in the larger follicles (data not shown). Regardless of the degree of atresia, 73–84% of the floating cells in the FF of 2.5–4 mm follicles underwent apoptosis and the difference in percentages of AGCs in FF was insignificant between the non-atretic and atretic follicles (data not shown).

We then analyzed the relationship between the density of floating FF cells and the developmental competence of oocytes from 2.5 to 4 mm follicles. As shown in Table 5, the percentage of M/Bs was higher significantly ($P<0.05$) in follicles with $7.5\text{--}12.5\times 10^6$ floating FF cells than in follicles with <2.5 or $>25\times 10^6$ floating cells, and it was higher also than in follicles with 2.5–7.5 or 12.5–25 $\times 10^6$ floating cells ($P=0.052$ and 0.051 respectively). A correlation analysis on the data presented in Table 5 showed that the percentages of apoptotic GCs were highly correlated with the concentrations of floating FF cells ($r=0.7$, $P<0.01$).

Table 3 Effects of granulosa cell apoptosis on cumulus expansion of oocytes from follicles of different sizes.

Follicle diameter (mm)	% AGCs	Oocytes observed ^a	% Oocytes with different grades of cumulus expansion				
			0	1	2	3	4
1.0–1.9	<5	60	25.0±5.6 ^b	35.0±6.2 ^b	31.7±6.1 ^b	8.3±3.6 ^b	0 ^b
	5–15	52	34.6±6.7 ^{b, c}	32.7±6.6 ^b	28.8±6.3 ^b	3.8±2.7 ^b	0 ^b
	15–30	48	47.9±5.0 ^c	47.9±7.3 ^b	4.2±2.9 ^c	0 ^b	0 ^b
	30–50	38	55.3±8.2 ^c	44.7±8.2 ^b	0 ^c	0 ^b	0 ^b
	>50	24	54.2±10.4 ^c	45.8±10.4 ^b	0 ^c	0 ^b	0 ^b
2.0–2.9	<5	51	3.9±2.7 ^b	9.8±4.2 ^b	23.5±6.0 ^b	56.9±7.0 ^b	5.9±3.3 ^b
	5–15	54	9.3±4.0 ^{b, c}	16.7±5.1 ^b	25.9±6.0 ^b	48.1±6.9 ^{b, c}	0 ^b
	15–30	48	18.8±5.7 ^c	14.6±5.1 ^b	27.1±6.5 ^b	39.6±7.1 ^c	0 ^b
	30–50	41	34.1±7.4 ^d	36.6±7.5 ^{b, c}	26.8±6.9 ^b	2.4±2.4 ^d	0 ^b
	>50	35	54.3±8.5 ^e	22.9±7.2 ^c	22.9±7.2 ^b	0 ^d	0 ^b
3.0–3.9	<5	71	0 ^b	0 ^b	23.9±5.1 ^b	63.4±5.8 ^b	12.7±4.8 ^b
	5–15	65	3.1±2.2 ^{b, c}	12.3±4.1 ^{b, c}	26.2±5.5 ^b	50.8±6.2 ^{b, c}	7.7±1.0 ^{b, c}
	15–30	59	11.9±4.3 ^c	18.6±5.1 ^{c, d}	23.7±5.6 ^b	42.4±6.5 ^c	3.4±1.3 ^{b, c}
	30–50	54	31.5±6.4 ^d	27.8±6.2 ^d	29.6±6.3 ^b	11.1±4.3 ^d	0 ^c
	>50	32	46.9±9.0 ^e	31.3±8.3 ^d	21.9±7.4 ^b	0 ^d	0 ^c

Values with common letters in their superscript (b–e) in the same column within follicle size group did not differ ($P>0.05$).

^aAll the data were recorded from the same population of oocytes used in Table 2.

Table 4 Effects of cumulus expansion on maturation and development of oocytes from 2–4 mm follicles.

Cumulus expansion	% AGCs	Oocytes cultured ^a	% Oocytes matured	% Embryos developing to		
				2-Cell	8–16 Cell	Mor/blast
0	<5	0 ^b				
	5–15	7 ^b				
	15–30	16	50.0±12.9 ^c	37.5±18.3 ^c	0 ^c	
	30–50	31	48.4±9.1 ^c	33.3±12.6 ^c	0 ^c	
	>50	34	41.2±8.6 ^c	21.4±11.4 ^c	0 ^c	
1	<5	5 ^b				
	5–15	17	76.5±10.5 ^d	84.6±10.4 ^{d, e}	45.5±15.7 ^{c, d}	9.1±9.1 ^c
	15–30	18	83.3±9.0 ^d	80.0±10.7 ^{d, e}	41.7±14.7 ^{c, d}	8.3±8.3 ^c
	30–50	30	76.7±7.9 ^d	73.9±9.4 ^{d, e}	35.3±11.9 ^{c, d}	0 ^c
	>50	18	77.8±10.1 ^d	64.3±13.3 ^d	22.2±14.7 ^{c, d}	0 ^c
2	<5	29	89.7±5.8 ^d	80.8±7.9 ^{d, e}	42.9±11.7 ^{c, d}	4.8±4.8 ^c
	5–15	31	87.1±6.1 ^d	85.2±7.0 ^{d, e}	56.5±10.6 ^d	13.0±7.2 ^{c, d}
	15–30	27	88.9±6.2 ^d	83.3±7.8 ^{d, e}	55.0±11.4 ^d	20.0±9.2 ^{d, e}
	30–50	27	81.5±7.6 ^d	77.3±9.1 ^{d, e}	47.1±12.5 ^{c, d}	11.8±8.1 ^{c, d}
	>50	15	80.0±10.7 ^d	66.7±14.2 ^d	37.5±18.3 ^{c, d}	12.5±12.5 ^{c, d}
3	<5	74	87.8±3.8 ^d	93.8±3.0 ^{e, f}	45.9±6.4 ^{c, d}	1.6±1.6 ^c
	5–15	59	89.8±4.0 ^d	96.2±2.6 ^f	70.6±6.4 ^d	39.2±6.9 ^e
	15–30	44	81.8±5.9 ^d	91.7±4.7 ^{e, f}	63.6±8.5 ^d	33.3±8.3 ^e
	30–50	7 ^b				
	>50	0 ^b				

Values with common letters in their superscript (c–f) in the same column did not differ ($P>0.05$).

^aData were from the oocytes used in Table 3. ^bData were not enough to be analyzed.

Effects of GCs apoptosis on COCs morphology

Distribution of the four type COCs in 2–4 mm follicles with different percentages of AGCs are summarized in Table 6. In general, proportions of grade A COCs decreased, while those of grades B, C, and D increased with increasing apoptosis of GCs, but it was only in follicles with over 30% AGCs that the decrease in grade A and the increase in grade D COCs became significant ($P<0.05$).

Discussion

We showed in this study that MIC of goat oocytes during maturation and embryo culture did not affect maturation but impaired embryo development. In mice (Paria & Dey 1990, Canseco *et al.* 1992), sheep (Gardner *et al.* 1994), cattle (Blondin & Sirard 1995, Carolan *et al.* 1996, O'Doherty *et al.* 1997, Ward *et al.* 2000), and human (Moessner & Dodson 1995), oocytes cultured in groups have better developmental competence than oocytes

cultured singly. However, when goat oocytes were matured and embryos cultured in the WID system, rates of cleavage and M/Bs were comparable with those obtained in group culture in the same drop size and at the same embryo density. Vajta *et al.* (2000) reported similar results with culture of bovine zygotes using a similar system (the well of the well (WOW) system). This indicates that the WID system we have developed can best mimic group culture in efficiency, while allows identification of oocyte origin.

Our previous studies have shown that GCs apoptosis is an underlying mechanism of follicular atresia in the goat (Yu *et al.* 2003, 2004) as in other animals (Liu *et al.* 2003). Most of the studies on the effect of GCs apoptosis on oocyte developmental competence have so far been conducted in the bovine and using group culture systems. No such studies have been reported in the goat. Using the MIC system, while Jewgenow *et al.* (1999) concluded that reduced oocyte developmental capacity was related to apoptotic death of follicular cells even before morphological signs of severe atresia were

Table 5 Relationship between granulosa cell density in follicular fluid (FF) and oocyte developmental competence.

FF cell density ($\times 10^6/\text{ml}$)	Follicles observed	% Oocytes matured	% Embryos developing to		
			2-Cell	8–16 Cell	Mor/blast
<2.5	42	76.2±6.7 ^a	81.3±7.0 ^{a, b}	42.3±9.9 ^{a, b}	7.7±5.3 ^b
2.5–7.5	47	93.6±3.6 ^b	86.4±5.2 ^{a, b}	50.0±8.2 ^{a, b}	13.2±5.6 ^{a, b}
7.5–12.5	42	95.2±3.3 ^b	95.0±3.5 ^a	55.3±8.2 ^a	28.9±7.5 ^a
12.5–25	38	81.6±6.4 ^{a, b}	83.9±6.7 ^{a, b}	38.5±9.7 ^{a, b}	11.5±6.4 ^{a, b}
>25	36	80.6±6.7 ^{a, b}	72.4±8.4 ^b	28.6±10.1 ^b	4.8±4.8 ^b

Values with common letters in their superscripts in the same column did not differ ($P>0.05$).

Table 6 Percentages of COCs of different grades in 2–4 mm follicles at different stages of atresia.

% AGCs	Oocytes observed	% COCs of different grades			
		A	B	C	D
<5	178	89.8±2.3 ^a	7.3±2.0 ^a	2.8±1.2 ^a	0 ^a
5–15	171	77.8±3.2 ^b	11.6±2.5 ^{a, b}	7.6±2.0 ^a	2.9±1.3 ^a
15–30	183	73.8±3.3 ^b	13.1±2.5 ^{a, b}	8.2±2.0 ^{a, b}	4.9±1.6 ^a
30–50	156	40.4±3.9 ^c	16.0±3.0 ^b	14.1±2.8 ^{b, c}	29.5±2.7 ^b
>50	148	26.4±3.6 ^d	18.9±3.2 ^b	15.5±3.0 ^c	39.2±4.0 ^c

Values with common letters in their superscripts in the same column did not differ ($P>0.05$).

detected, Hagemann *et al.* (1999) suggested that even a high degree of atresia was not necessarily detrimental to bovine oocytes. Using the WID culture system, we showed that while late atresia was detrimental, early atresia was beneficial to the competent oocytes. Previous studies have demonstrated that a low level of atresia tended to improve the *in vitro* competence of bovine oocytes (Blondin & Sirard 1995, Moor *et al.* 1996, Hagemann *et al.* 1999, de Wit *et al.* 2000, Hendriksen *et al.* 2000). However, this study showed that atresia of all degrees was beneficial to the incompetent goat oocytes. The effect of atresia on incompetent oocytes has not been reported, but it is important for utilization of these oocytes.

A characteristic of cells undergoing apoptosis is that junctions with neighboring cells are disrupted (Duvall & Wyllie 1986). Therefore, GCs from interior follicle wall will enter FF after apoptosis, which may reflect the degree of follicle atresia. In this study, 73–84% of the floating cells were found apoptotic in FF of 2.5–4 mm follicles, regardless of degrees of atresia. This indicates that not the apoptotic proportion of floating FF cells, but rather their concentration in the FF might reflect the atretic status of a follicle. Our correlation analysis showed a high correlation between the percentages of AGCs and the concentrations of floating FF cells. Furthermore, we showed that the density of floating cells in FF affected oocyte maturation and development significantly. Although this suggests that the floating cell density in FF can be used as a non-invasive marker of oocyte quality, its use would likely be restricted to large follicles because antral layers of mural GCs detached from many of the small follicles in both the bovine (Irving-Rodgers *et al.* 2001) and the goat (this study).

Both the present and the previous studies in cattle (Blondin & Sirard 1995, de Wit *et al.* 2000, Zeuner *et al.* 2003) indicate a high correlation between follicle quality or GCs apoptosis and distribution of COCs with different morphology. Since, only grade A and B oocytes were used in this study, that oocytes from early atretic follicles showed a higher, while those from late atretic follicles a lower developmental potential suggested a high correlation between COCs morphology and oocyte competence. Studies on the bovine have also shown that COCs with good morphology tended to develop better *in vitro*, although some indicated that oocytes with

incomplete cumulus developed as well (Madison *et al.* 1992, Blondin & Sirard 1995, de Wit *et al.* 2000). However, both this and other studies showed that the COCs with good morphology could have come from the late atretic follicles and developed poorly (Jewgenow *et al.* 1999, de Wit *et al.* 2000). Although COCs with good morphology came mainly from the non- and early atretic follicles, COCs with poor morphology came nearly exclusively from the late atretic follicles (de Wit *et al.* 2000, the present study). In addition, although non-atretic follicles contained a high percentage of grade A COCs, oocytes from these follicles showed a lower developmental competence than those from early atretic follicles. Therefore, while COCs with advanced signs of degeneration are bound to have decreased potency to develop *in vitro*, the developmental potential of oocytes with good appearance is affected by factors other than morphology.

Goat oocytes from <0.5 mm follicles were unable to undergo GVBD when matured *in vitro*. They became GVBD-competent in 0.5 mm follicles, MI-competent in 1–1.8 mm follicles and MII-competent in 2–5 mm follicles (De Smedt *et al.* 1994, Crozet *et al.* 1995, Ma *et al.* 2003). This study showed that the majority of goat oocytes acquired competence for development up to the 8–16 cell stage in follicles larger than 2 mm, but did not gain the ability to form M/Bs until follicles larger than 3 mm in diameter. Overall, these results agree with that bovine oocytes from larger follicles tend to develop better *in vitro* (Tan & Lu 1990, Pavlok *et al.* 1992, Lonergan *et al.* 1994).

The present data indicate that both the follicle size and the level of atresia affect oocyte cumulus expansion during IVM. This has not been reported. The limited number of studies conducted so far indicates that, while not essential for nuclear maturation (Leibfried & First 1979, Sun *et al.* 2002b, Han *et al.* 2006), cumulus expansion is important for successful fertilization (Ball *et al.* 1983, Chen *et al.* 1993, Qian *et al.* 2003), because unexpanded cumulus may present a barrier to sperm penetration (Bedirian *et al.* 1975). However, both Han *et al.* (2006) and this study showed that M/B rates were highly correlated with the degree of cumulus expansion in parthenogenetic goat oocytes, suggesting that cumulus expansion contributes not only to successful fertilization but also to embryo development.

In summary, the developmental potency of goat oocytes with the same COC morphology, the same follicle size, and the same grade of cumulus expansion may differ significantly with the level of GCs apoptosis. However, at the same level of GCs apoptosis, oocyte developmental capacity may vary with follicle size, COC morphology, or grades of cumulus expansion. This suggests that the developmental potential of an oocyte is determined by multifactor interactions, and multiple factors must be considered simultaneously to accurately predict the quality of an oocyte. The correlation between floating FF cell density and oocyte development suggests the possibility to use the floating cell density in FF as a simple and non-invasive marker for oocyte quality.

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