—Research Note—

Germinal Vesicle Stages in Pig Follicular Oocytes Collected by Different Methods

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Abstract. In Exp. 1 oocytes were collected from follicles (4–6 mm) in ovaries from prepubertal gilts by aspiration of the follicles with a syringe or dissection from healthy follicles. After aspiration some oocytes with compact cumulus and granulosa cells were selected. In Exp.2 oocytes were collected from follicles (4 mm, 5–6 mm and 7–8 mm) of gilts on the 18th–19th day of the cycle. Germinal vesicle (GV) stages of oocytes were examined and categorized into GV1, GV2, GV3 and GV4. In Exp. 1 when collected by aspiration, a significantly lower percentage of oocytes (45%) was arrested at the GV1 stage than oocytes from dissected follicles (78%). However, after morphological selection of oocytes, the percentage (58%) significantly increased. In Exp 2, significantly higher percentages (86–90%) of oocytes from follicles of 5–8 mm in diameter were at the GV1 stage than oocytes from follicles in diameter of 4 mm (24%) and atretic follicles (57%). These results indicate that by aspiration some oocytes are collected from follicles that were inadequate to maintain arrest at GV1, but significantly higher percentages of oocytes at GV1 can be obtained after morphological selection of oocytes or isolation from large follicles of gilts on the 18th–19th day of the cycle.

Key words: Germinal vesicle, *In-vitro* maturation, *In-vitro* fertilization, Follicle, Gilt.

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Incomplete oocyte maturation causes failure of fertilization or anomalies such as a low rate of male pronucleus formation after IVF in pigs [1–3]. It was reported that the morphology of follicular cells attached to oocytes before culture affects *in-vitro* maturation of oocytes [4]. Therefore, for the successful maturation of oocytes, it would be important to observe details of cumulus-oocyte complexes (COC) before culture and collect morphologically intact COC. To do so Ding *et al.* [5]

and Miyano *et al.* [6] collected COC from only non-atretic intact follicles that had been dissected. However, there have been few reports on nuclear status, or germinal vesicle (GV) stages, of oocytes before culture.

The present study was undertaken firstly to compare the morphology of GV in oocytes obtained by aspiration or dissection of follicles in ovaries from prepubertal gilts. Secondly, oocytes were collected from cycling gilts on the 18th–19th day of the cycle and the morphology of their GV was observed.

Materials and Methods

Collection of oocytes from prepubertal and cycling gilts

Exp. 1: Ovaries were removed from prepubertal gilts at a local slaughterhouse and returned within 1 h of slaughter to the laboratory in saline (9 g NaCl/l, 200 i.u. penicillin GK/ml, 200 μ g streptomycin sulfate/ml) at 20–30 C. Then COC were collected from follicles 4–6 mm in diameter by two different methods. 1) COC with or without granulosa cells were collected by aspiration with a 5-ml syringe equipped with an 18-gauge needle [7] and oocytes with compact granulosa cells were selected or 2) COC with compact granulosa cells were collected from healthy antral follicles that had been dissected in PBS-PVA from ovaries following the technique described by Moor and Trounson [8].

Exp. 2: Ovaries were removed from cycling gilts on the 18th–19th day of the cycle (the onset of oestrus was checked once daily with a boar) and then COC with compact granulosa cells were collected from follicles 4–8 mm (classified into 3 groups 4 mm, 5–6 mm and 7–8 mm) in diameter by scraping the inner surface of the follicular wall with a surgical blade under the dissection microscope. When cell debris came out of follicles by scraping, the follicles were considered to be atretic.

Examination of GV stages

All the oocytes were fixed for 3–4 days in 25% acetic alcohol at room temperature, stained with 1% aceto-orcein and examined under a phase-contrast microscope. GV stages were classified as GV1, GV2, GV3 and GV4 according to the criteria used by Motlik and Fulka [9]. In brief, GV1 is the first stage of the nucleus in fully grown oocytes and characterized by a nucleolus surrounded by chromatin in a ring or horseshoe like shape. The nucleoplasm is finely granular. GV2 is the first sign of breakdown of the germinal vesicle. The chromatin forms a few small round clumps on the nuclear membrane, but the nuclear membrane, the nucleolus, and granulation of the nucleoplasm are unaffected. At the stage of GV3, the nucleoplasm loses its granulation, but the nuclear membrane still remains distinct. The clumps of chromatin on the nuclear membrane are connected with the chromatin around the nucleolus which becomes less

distinct. The network of chromatin is scattered all over the nucleus. GV4 is the last stage of GV and the nuclear membrane becomes less distinct and the nucleolus disappears completely. The chromatin is seen either as an irregular network, or else the individual filamentous bivalents are distinguishable.

The results were analyzed by the χ^2 -test.

Results

In Exp. 1, as shown in Table 1, when COC were collected from follicles of 4–6 mm in diameter in ovaries from prepubertal gilts by aspiration and fixed without selection, a significantly lower percentage of oocytes (45%) was at stage of GV1 and a significantly higher percentage of oocytes was at stage of GV3 (35%) than when collected from dissected healthy antral follicles of the same diameter (78% and 7%, respectively) and 11% of them was at stage of GV4. However, when oocytes with compact cumulus and granulosa cells were selected and fixed after aspiration, the percentage of oocytes at GV1 (58%) increased to the same value as those collected from dissected follicles and no oocytes were at the GV4 stage. In Exp 2, when COC were collected by scraping from follicles of cycling gilts on the 18th–19th day of the cycle, high percentages (86–90%) of oocytes from follicles of 5–8 mm in diameter were at the GV1 stage. All of the COC were with compact cumulus and granulosa cells. The percentage, however, significantly decreased when oocytes were collected from follicles of 4 mm in diameter (24%) and atretic follicles (57%). No oocytes from cycling gilts were at the GV4 stage.

Discussion

Motlik and Fulka [9] observed the breakdown of the pig GV of oocytes maturing *in vivo* and *in vitro*, and then divided the process into four well-defined stages, based on the chromatin changes, and on nucleolus and nuclear membrane disappearance according to time after hCG injection or *in-vitro* culture. They found that some oocytes (a total of 9%) were at the GV4 stage and/or more advanced stage, even immediately after isolation from the ovaries of prepubertal gilts, and

Table 1. Germinal vesicle (GV) stages of oocytes collected from gilts by different methods

Source of oocytes	Diameter of follicles (mm)	Collection method	Selection ^a	No. of oocytes examined	No (%) of oocytes at stage of			
					GV1	GV2	GV3	GV4
Prepubertal gilts	4–6	Aspiration ^b	–	65	29 (45) ^f	6 (9) ^f	23 (35) ^f	7 (11)
	4–6	Aspiration ^b	+	67	39 (58) ^{fg}	10 (15) ^f	18 (27) ^{fg}	0
	4–6	Dissection ^c	+	60	47 (78) ^g	9 (15) ^f	4 (7) ^g	0
Cycling gilts	7–8	Scraping ^d	–	36	31 (86) ^f	1 (3) ^f	4 (11) ^f	0
	5–6	Scraping ^d	–	29	26 (90) ^f	1 (3) ^f	2 (7) ^f	0
	4	Scraping ^d	–	25	6 (24) ^g	7 (28) ^g	12 (48) ^g	0
	Atresia	Scraping ^d	–	14	8 (57) ^h	1 (7) ^f	5 (36) ^g	0

^aOocytes with compact cumulus and granulosa cells were selected. ^bOocytes were aspirated from follicles with a 5-ml syringe equipped with an 18-gauge needle within 1 h of slaughter. ^cOocytes with compact granulosa cells were collected from healthy antral follicles that were dissected within 1 h of slaughter. ^dOocytes with granulosa cells were collected by scraping the inner surface of the follicular wall with surgical blade. ^eOvaries were removed from cycling gilts on the 18th–19th day of the cycle. ^{f,g,h}In the same source of oocytes numbers with different superscripts in the same column are significantly different ($P < 0.05$; χ^2).

that all the oocytes collected from the ovaries of gilts after PMSG and hCG injection (fixed after 4 h of hCG injection) were at the GV1 stage. In contrast, we found that many oocytes collected not only from follicles of prepubertal gilts (a total of 22%–55%) but also from follicles of 5–8 mm in diameter in ovaries of cycling gilts (a total of 10%–14%) on the 18th–19th day of the cycle were already at GV2–GV4. Compared with Motlik and Fulka's data, our results showed that more oocytes were at advanced stages of GV (GV2–GV4) before culture. Although there is no obvious reason for this difference, different ways of collection and/or selection of oocytes may be one explanation, as found in this study where selection of oocytes according to the existence of attached cumulus and granulosa cells showed significantly higher percentages of GV1 than without selection.

Ingram [10] reported that atresia of medium size and large follicles may be accompanied by nuclear maturation. Furthermore, Motlik and Fulka [9] suggested that oocytes with GV of advanced stages could be from atretic follicles. However, although significantly lower percentages of oocytes from atretic follicles were arrested at GV1, compared with those from large follicles of 5–8 mm in diameter, the lowest percentage of oocytes arrested at GV1 was from small intact follicles of 4 mm in diameter. More than half of oocytes from atretic follicles were arrested at GV1 stage in our study. Therefore, it was suggested that oocytes with GV

of advanced stages are not only from atretic follicles but also from intact follicles and that there are oocytes at GV1 even in atretic follicles. It was of interest that oocytes from small follicles showed a significantly lower percentage of GV1 than those of oocytes from large follicles in Exp. 2. In gilts on the 18th–19th day of the cycle, obviously oocytes are to be ovulated from large follicles, and oocytes in small follicles won't have a chance to do so and the follicles are likely to be atretic. Thus, it may be that at the time of selection of follicles for ovulation, an unknown mechanism selects follicles having oocytes at the GV1 stage and these continue to grow to the final stage and ovulate, or oocytes in follicles that are not selected to continue to grow and ovulate can not be arrested at the GV1 stage and the follicles become atretic.

Considering the fact that a significantly higher percentage of oocytes from large follicles, which are supposed to be ovulated, were at the GV1 stage, oocytes at the GV1 stage can be considered to be physiologically intact. However, oocytes collected by aspiration and selected for the presence of attached compact cumulus cells have been used for many studies [11–13], and although there is a possibility that oocytes with GV of advanced stages have been included in these studies, the oocytes showed high rates of maturation, fertilization and male pronucleus formation after IVF. Also in our system oocytes collected by aspiration and selected for the presence of attached compact cumulus

Fig. 1. Photomicrographs of germinal vesicle (GV) stages in pig oocytes collected from follicles (4–6 mm in diameter) in ovaries from prepubertal gilts by aspiration of the follicles with a 5-ml syringe equipped with an 18-gauge needle. GV1: An oocyte at the GV1 stage. A nucleus is surrounded by chromatin in a ring or horseshoe like shape. $\times 750$. GV2: An oocyte at the GV2 stage. The chromatin forms a few round clumps on the nuclear membrane and a few chromocenters are situated near the nuclear membrane. But the nuclear membrane and the nucleolus are unaffected. $\times 750$. GV3: An oocyte at the GV3 stage. The clumps of chromatin on the nuclear membrane are connected with the chromatin around the nucleus which becomes less distinct. The network of chromatin is scattered all over the nucleus. $\times 750$. GV4: An oocyte at the GV4 stage. The nuclear membrane becomes less distinct and the nucleolus disappears completely. The chromatin is seen as an irregular network. $\times 750$.

cells showed similar results as reported by them (data not shown). Thus, it seems that the stages of GV of oocytes before culture do not affect these events. However, low rates of *in-vitro* development of IVM-IVF embryos to the stage of blastocyst and no birth from *in-vitro* produced blastocysts in these studies might be related to the advanced stages of GV in oocytes used, because about 40% of oocytes aspirated showed GV of advanced stages even after selection, before culture.

Although the collection of oocytes by dissection of follicles is very labor intensive compared with aspiration, it ensures the collection of oocytes only from non-atretic follicles and significantly higher percentages of oocytes at the GV1 stage can be

obtained. However, selection of COC having compact granulosa cells after aspiration significantly improved the percentage of oocytes at the GV1 stage. Therefore, the selection of oocytes with compact and multiple granulosa cells seems to be essential for successful IVM-IVF in pigs.

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