

Changes in Number of Granulosa Cells, Follicular Fluid Levels and Diameter of Oocytes during Folliculogenesis in Pre-pubertal Gilts at Marketing Weight

C. M. Chiou^{1,3}, T. S. Yang², S. P. Yeh¹, M. Z. Tsai³, S. P. Cheng¹ and M. C. Huang^{1,*}

¹ Department of Animal Science, National Chung Hsing University, Taichung, Taiwan, ROC

ABSTRACT : The follicles (1.8 to 7.8 mm in diameter) were recovered from the ovaries in marketed pigs and the number of granulosa cells, the diameter of oocytes obtained from different development stages of the follicles and follicular fluid levels were determined. Correlations between size measurements and cell counts as well as the diameter of antral follicles and oocytes were also investigated. The results indicated that, while expanding in size, follicle numbers decreased with a greater atretic proportion. Granulosa cells increased in numbers continuously and remained unchanged beyond the size of 200 mm³ in non-atretic follicles, whereas a sudden drop of granulosa counts was observed in atretic follicles. Follicular fluid, on the other hand, linearly increased its volume with follicle size and differed little between those of non-atretic and atretic follicles. Diameters of oocytes in non-atretic follicles increased to its maximum when follicles expanded to 150 mm³ and maintained its size during later follicular expansion. It is concluded that, for *in vitro* culture, the optimal size of porcine follicle should be between 150 to 180 mm³ if they are collected from pre-pubertal gilts of marketing size slaughtered in an abattoir. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 12 : 1647-1651)

Key Words : Porcine, Follicle Size, Follicular Fluid, Oocyte, Granulosa Cell

INTRODUCTION

The oocytes and follicles of a porcine embryo begin to form on day 18 and the primordial germ cells are observed in genital ridges (Black and Erickson, 1968). Normally, the primordial follicles are surrounded by a single layer of cells (Oxender et al., 1979) and the primary follicle is a potential ovum surrounded by a single layer of cuboidal granulosa cells with a diameter of around 0.5 mm (Chiquoine, 1960; Guraya, 1985). In rabbits, cumulus-oocyte complexes (COCs) collected from ovarian follicles can be cultured and parthenogenetically activated *in vitro* if its diameter is above 1 mm, although maturation remained low (Ju et al., 2002). On prenatal pigs, the secondary follicle is an ovum surrounded by two or more layers of granulosa cells with a diameter of 101.7±41.8 µm and, if continue to develop, oocyte will expand its size from 45.6 to 120 µm in diameter (Black and Ericson, 1968; Motlik et al., 1984; Hyttel et al., 1997). Usually, a follicle with a diameter of up to 3 mm containing an oocyte with a diameter from 80 to 110 µm is referred to as the tertiary follicle, or is called a Graffian follicle with a fluid-filled antrum and potential ovum in gilts (Christenson et al., 1985; Hyttel et al., 1997). The granulosa cells of Graffian follicles are rich in FSH receptors and regulated by FSH to secrete estrogens (Leung

and Armstrong, 1980; Huang et al., 2002). Under the influence of FSH and estrogens, the Graffian follicles soon develop to pre-ovulate follicles with a diameter of 7 mm or more (Loeken and Channing, 1985), and contain oocytes with a diameter above 120 µm. The follicles are in a consistent state of physiological and histological changes during the maturation stages.

In porcine genetic engineering, information regarding the follicular fluid, number of granulosa cells, and diameter of oocytes is essential, but it has not been well established yet. In this present study, atretic and non-atretic follicles are collected from marketing gilts in abattoir to reveal basic data of the follicular fluid, number of granulosa cells, and diameter of oocytes. Those information is of value for *in vitro* manipulation of germ cells.

MATERIALS AND METHODS

Recovery of antral follicles

Ovaries were collected from carcass offal immediately removed from slaughtered pigs in a local abattoir and immersed into saline and carried to a laboratory at the room temperature. After arrival, single follicles were isolated from ovaries by two pliers to unrip connective tissue between follicles by mechanical force. Transparent or opaque follicles, as described by Grimes and Ireland (1986), were classified into atresia and non-atresia categories and their greatest diameter were measured by a micro ruler.

Measurement of follicular fluid and diameter of the COCs

Follicular fluid of all the isolated follicles was aspirated by inserting of by 22# needle (Hynes et al., 1996) and filled

* Corresponding Author: Mu-Chiou Huang. Tel: +886-4-22852469, Fax: +886-4-22860265, E-mail: mchuang@mail.nchu.edu.tw

² Division of Applied Biology, Animal Technology Institute Taiwan, P.O. BOX, 23 Chunan Taiwan, ROC.

³ Department of Animal Science, National Chia Yi University, Chia Yi, Taiwan, ROC.

Received July 21, 2003; Accepted May 3, 2004

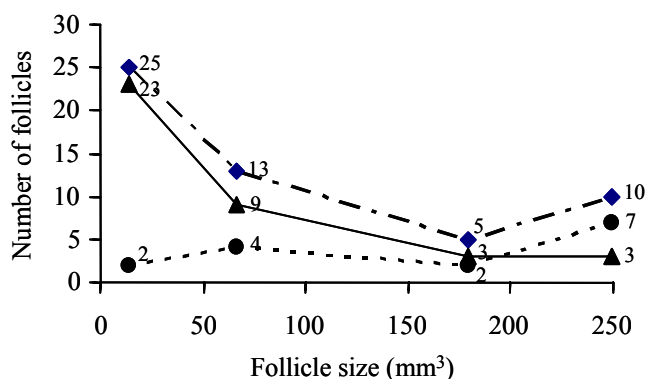


Figure 1. Number of follicles of different size obtained from slaughterhouse in pigs. Total follicle (-◆-), non-atretic follicle (-▲-), atretic follicle (-●-).

into tubes to centrifuge at 600×g for 15 min. After follicular fluid volume were measured, sediments were separated and were suspended into phosphate buffer solution, so that COCs can be separated by micro pipette, and their diameter with membrane, zona pellucida and cumulus oophorus can be measured under microscope with a micro ruler.

Recovery and count of granulosa cells

Selected follicles of different size were punched and granulosa cells were collected and suspended in 1 ml of saline with methylene blue for 10 min. An aliquot (10 μl) was placed on a hemacytometer to count granulosa cells under a reverse microscope.

Statistical analysis

Data were presented by mean and standard deviation. Significant differences between means was tested by analysis of one-way variance (ANOVA) and their correlation between variables was computed by using statistical software of SAS, 1986.

RESULTS AND DISCUSSION

Number of follicles

The follicles observed ranged from 1.9 to 7.8 mm in diameter, average weight of the collected ovaries was 2.3±0.8 g and the number of non-atretic and atretic follicles in the ovaries were showing Figure 1. It can be seen that the total number of non-atretic follicles decreased with increase in size; on the other hand, the atretic follicle was remained in low number, but slightly increased in the last stage of size increase and rendered the atretic to non-atretic ratio increased along with increasing size. Only 8% of can be classified as small follicles with volume of 60 mm³ atretic as volume of follicle was 180 mm³, an almost equal number of atretic and non-atretic follicles was found. Follicular size of 180 mm³ in pre-puberty gilts seems is a critical size in follicle growth at which decreased of granulosa cells and

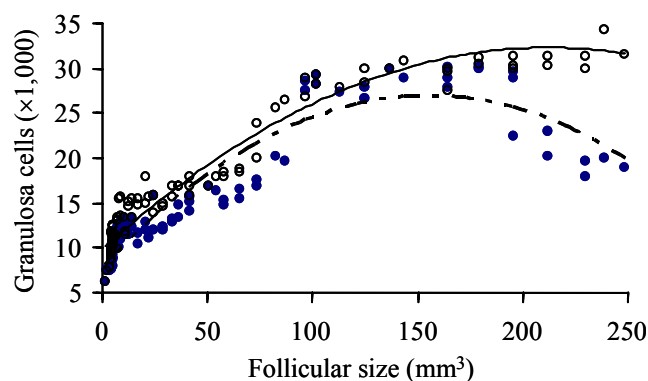


Figure 2. Numbers of granulosa cells obtained from atretic (●) ($y=-0.8x^2+248x+7,778$, $R^2=1$) and non-atretic (○) follicles ($y=-0.5x^2+212x+9,870$, $R^2=1$).

oocyte diameter occurred. This corresponded with the description by Wang and Greenwald (1991), for the ratio of healthy and atresia follicles obtained from the ovary mouse and suggesting a common mechanism may exists in mammals. In cyclic cows, the atretic follicle is always higher than non-atretic follicles. This result did not correspond with our present study, indicating once reached puberty a different mechanism controls follicular growth.

Number of granulosa cells

The correlation of the number of granulosa cells in atretic and non-atretic follicles are shown in Figure 2. The number of granulosa cells in atretic follicles was correlated with size of follicular size in a best fitting of $y=-0.8X^2+248X+7,778$ ($r=0.99$); while it was $y=-0.5X^2+212X+9,870$ ($r=0.99$) for non-atretic follicles. Two regressions illustrated a different pattern; the number of granulosa cells of the atretic follicle increased along with follicle volume reached 150 mm³, maintained as follicle volume expanded to 180 mm³, and drastically decreased thereafter. In the non-atretic follicles, granulosa cell number also increased accompanies follicle volume expansion up to 220 mm³ and maintained even reached 250 mm³ (Figure 2). The drop of cell numbers in large atretic follicles suggests a substantial apoptosis, which did not see in non-atretic follicles. In Figure 2, it is also observed that before follicle volume developed to 150 mm³, number of granulosa cells differs little between atretic and non-atretic follicles, the later drop in number of granulosa cells in atretic follicles beyond 150 mm³ suggests that a part of the granulosa cell is already in a process of apoptosis in atretic follicles during development and resulted a dramatically decrease in cell numbers once it reached to a certain stage of development.

Follicular fluid

The follicular fluid amounts of atretic and non-atretic antral follicles in different follicle size are shown in Figure 3. The increase in follicular fluid level is linearly

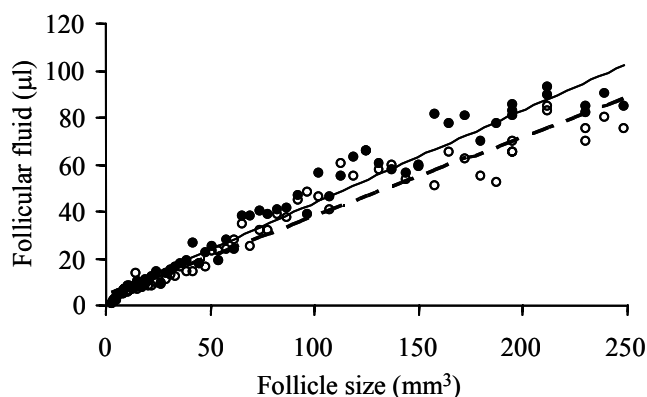


Figure 3. Contents of follicular fluid obtained from atretic (●) ($y=0.3953x+4.1058$, $R^2=0.9617$) and non-atretic (○) follicles ($y=0.3382x+4.1285$, $R^2=0.9375$).

accompanied by an increase in follicle volume in either atretic or non-atretic antral follicles and no significant difference between them. Thus, follicular fluid levels, despite atretic or non-atretic, increase in associated with the expansion of follicle diameter. However, a small but not significant gradual higher fluid level was observed from size of 115-120 mm³ onward in the atretic follicles suggesting a compensation of fluid fill in atretic when cells started to undergo apoptosis.

Oocyte diameter

The diameter of oocytes in atretic and non-atretic in the antral follicles was shown in Figure 4 and 5. The average diameter of oocytes lacking pellucida, in both atretic and non-atretic, of the antral follicles was development to 175-190 mm³ of the follicle volume. As Matas et al. (1996) described, the diameter of an oocyte in pigs can be divided into four groups: less than 105 µm, between 105 to 115 µm, between 116 to 120 µm, and above 120 µm. After being cultured *in vitro*, the penetration ability of the sperm in the larger groups was higher. However, in bovine, the diameter of the follicles is not related to the rate of sperm penetration and fertility either the male pronucle forming ability (Leibfried-Rutledge et al., 1985). These data suggest that once meiosis is initiated, the development ability *in vitro* differed little at least in bovine oocytes. On the other hand, the cleavage rates (more than 3 divisions) of the nuclear transplant rabbit embryos were greater in the small nuclear donors than in the large donors. It appeared that no difference in the development competence between large and small isolated blastomeres was observed (Ju et al., 2003).

In gilts, the average diameter of oocytes containing zona pellucida in atretic and non-atretic antral follicles is similar (Figures 4 and 5) and both were maximum when developed up 20-160 mm³ of the follicle volume. These data clearly indicated that oocytes increase its size with the expansion in

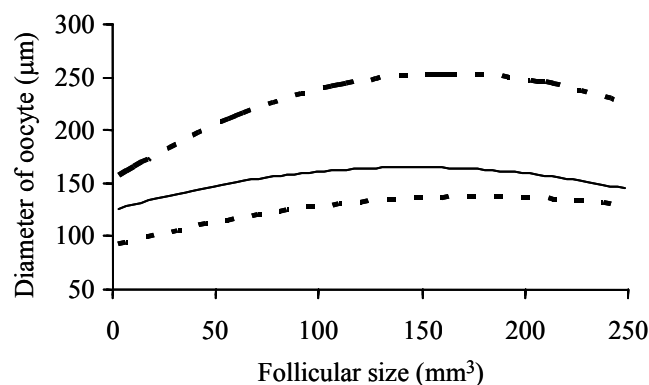


Figure 4. Diameters of oocyte obtained from non-atretic follicles. Content cumulus (---) ($y=-0.0038x^2+1.2336x+152.49$, $R^2=0.6039$); content zona pellucida (—) ($y=-0.0019x^2+0.5544x+124.65$, $R^2=0.377$) and non-zona pellucida (.....) ($y=-0.0015x^2+0.5256x+90.237$, $R^2=0.4766$).

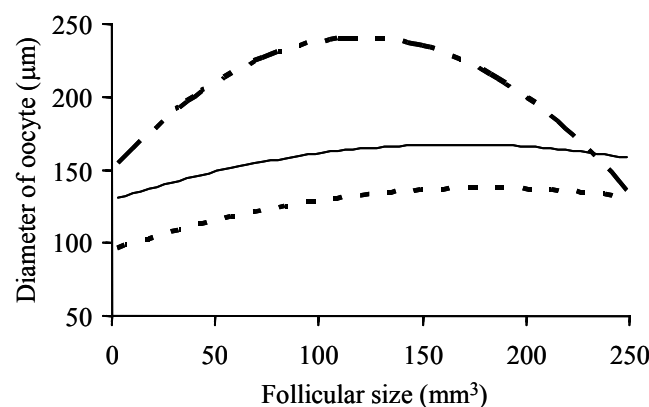


Figure 5. Diameters of oocyte obtained from atretic follicles. Content cumulus (---) ($y=-0.0063x^2+1.4984x+150.37$, $R^2=0.6614$); content zona pellucida (—) ($y=-0.0014x^2+0.4559x+129.41$, $R^2=0.4492$) and non-zona pellucida (.....) ($y=-0.0013x^2+0.4652x+94.98$, $R^2=0.4653$).

follicle diameter. Also, in those atretic oocytes became slightly larger than in non-atretic presumably some in the atretic follicle were in their degeneration process showing less incompleteness physicality. This result observed from porcine was similar to those described by Arlotto et al. (1996) and Fair et al. (1995) in bovines that the expansion in diameter of the oocytes was accompanied by an increase in follicle diameter and when antral follicle diameter reached beyond 4 mm there was no more further changes in diameters of the oocytes. Suzuki et al. (1994), on the other hand, reported that the diameter of oocytes from immature bovines was larger than those from matured ones after insemination. The causes were not clear. The diameter of the oocytes increases rapidly before the follicle forms a small antral and the rapid synthesis of protein or DNA by the oocytes took place while the diameter was less than 110 µm. After that, the synthesis of protein or DNA nearly stopped (Fair et al., 1995, 1996). In gilts, Christmann et al.

(1994) showed that an oocyte with a diameter of less than 90 μm was not capable of resuming meiosis. In bovine, Blondin and Sirard (1995) indicated that oocytes obtained from follicles with a diameter of 3 mm or smaller were not capable of developing to more than the sixteen-cell stage *in vitro*. Those data all suggested that oocytes need to reach an optimal size to function properly.

The average diameter of oocytes containing cumulus oophorus in atretic and non-atretic antral follicles, and in small or middle antral follicles, was similar. However, the oocytes containing cumulus oophorus in large non-atretic follicles was larger than those of atretic follicles (Figures 4 and 5). Oocytes containing cumulus oophorus in non-atretic in the antral follicles could obtain a maximum of 160 mm^3 of the follicle volume, but in atretic follicles, once above 130 mm^3 it regressed and caused a significantly difference in size between atretic and non-atretic group. In addition, those COCs obtained from middle size follicles had compacted cumulus oophorus, and make it difficult to peel during the isolation process. This is also observed in bovines, Lonergan et al. (1994) described COCs obtained from follicles greater than 6 mm in diameter, although surrounded by more layers of cumulus oophorus than those smaller, they were easier to peel into a piece of cumulus oophorus. Also in bovine, denuded oocytes obtained from larger follicles were 30% more than those obtained from small follicles (Fukui and Sakuma, 1980), while beyond 5.1 mm in diameter (about 150 mm^3 in volume), follicles were nearly as complex in structure as the matured and rendered cumulus oophorus surrounding the COCs looser and easier to peel during the isolation process.

The average diameter of oocytes containing cumulus oophorus in atretic follicles decreased in larger follicles (about 150 mm^3 in volume) together the looser COCs obtained from atretic follicles indicating a part of the cells were already in apoptosis during the isolation process, as suggested by Guthrie et al. (1995) and Jolly et al. (1994). Therefore, oocytes obtained from atretic follicles had a higher proportion of denuded oocyte (Fukui and Sakuma, 1980). In cyclic cows, the proportion of atretic follicles in small or middle size was higher than the larger follicles (Carolan et al., 1996). Whether it is also true in gilts after reaching puberty is not known and worthwhile to reveal for the further investigation of control mechanism in follicular growth.

REFERENCES

- Arlotto, T., J-L. Schwartz, N. L. First and M. L. Leibfried-Rutledge. 1996. Aspects of follicle and oocyte stage that affect *in vitro* maturation and development of bovine oocytes. *Theriogenology* 45:943-956.
- Black, J. L. and B. H. Erickson. 1968. Oogenesis and ovarian development in the prenatal pig. *Anat. Rec.* 161:45-56.
- Blondin, P. and M. A. Sirard. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol. Reprod. Dev.* 41:54-62.
- Carolan, C., P. Lonergan, P. Monget, D. Monniaux and P. Mermillod. 1996. Effect of follicle size and quality on the ability of follicular fluid to support cytoplasmic maturation of bovine oocyte. *Mol. Reprod. Dev.* 43:477-483.
- Chiquoine, A. D. 1960. There development of the zona pellucida of the mammalian ovum. *Am. J. Anat.* 106:149-170.
- Christenson, R. K., J. J. Ford and D. A. Redmer. 1985. Maturation of ovarian follicles in the prepubertal gilt. *J. Reprod. Fert. Suppl.* 33:21-36.
- Christmann, L., T. Jung and R. M. Moor. 1994. MPF components and competence in growing pig oocytes. *Mol. Reprod. Dev.* 38:85-90.
- Fair, T., P. Hyttel and T. Greve. 1995. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Mol. Reprod. Dev.* 42:437-442.
- Fair, T., P. Hyttel, T. Greve and M. Boland. 1996. Nucleus structure and transcriptional activity in relation to oocyte diameter in cattle. *Mol. Reprod. Dev.* 43:503-512.
- Fukui, Y. and Y. Sakuma. 1980. Maturation of bovine oocytes cultured *in vitro*: relation to ovarian activity, follicular size and the presence or absence of cumulus cells. *Biol. Reprod.* 22:669-673.
- Grimes, R. W. and J. J. Ireland. 1986. Relationship of macroscopic appearance of the surface of bovine ovarian follicles, concentrations of steroids in follicular fluid, and maturation of oocytes *in vitro*. *Biol. Reprod.* 35:725-732.
- Curaya, S. S. 1985. *Biology of Ovarian Follicles in Mammals*, pp. 3-14. Springer-Verlag, Berlin.
- Guthrie, H. D., B. S. Cooper, G. R. Welch, A. D. Zakaria and L. A. Johnson. 1995. Atresia in follicles growth after ovulation in the pig: Measurement of increased apoptosis in granulosa cells and reduced follicular fluid estradiol-17 β . *Biol. Reprod.* 52:920-927.
- Huang, W. T., S. H. Lu, P. C. Tang, S. C. Wu, S. P. Cheng and J. C. Ju. 2002. Biochemical compositions of follicular fluid and the effects of culture conditions on the *in vitro* development of pig oocytes. *Asian-Aust. J. Anim. Sci.* 15:1403-1411.
- Hynes, A. C., M. T. Kane and J. M. Sreenan. 1996. Partial purification from bovine fluid of a factor of low molecular mass with inhibitory effects on the proliferation of bovine granulosa cells *in vitro* and on rat follicular development *in vivo*. *J. Reprod. Fert.* 108:185-191.
- Hyttel, P., T. Fair, H. Callesen and T. Greve. 1997. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology* 47:23-32.
- Jolly, P. D., D. J. Tisdall, D. A. Heath, S. Lun and K. P. McNatty. 1994. Apoptosis in bovine granulosa cells in relation to steroid synthesis, cycle adenosine 3',5'-monophosphate response to follicle-stimulating hormone and luteinizing hormone, and follicular atresia. *Biol. Reprod.* 51:934-944.
- Ju, J. C., T. H. Chen, J. K. Tseng, C. Tsey, S. P. Yeh, P. C. Chou, C. H. Chen and C. T. Liu. 2002. Cytoskeletal patterns, *in vitro* maturation and parthenogenetic development of rabbit GV oocytes. *Asian-Aust. J. Anim. Sci.* 15:1695-1701.
- Ju, J. C., J. S. Yang, C. T. Liu, C. H. Chen, J. K. Tseng, P. C. Chou

- and S. P. Cheng. 2003. Differential influences in sizes and cell cycle stages of donor blastomeres on the development rabbit embryos. *Asian-Aust. J. Anim. Sci.* 16:15-22.
- Leibfried-Rutledge, M. L., E. S. Critser and N. L. First. 1985. Fertilization potential of follicular oocytes classified by stage of cycle and size of follicle. *Theriogenology* 23:753-759.
- Leung, P. C. K. and D. T. Armstrong. 1980. Interactions of steroids and gonadotropins in the control of steroidogenesis in the ovarian follicle. *Ann. Rev. Physiol.* 42:71-82.
- Lonergan, P., P. Monaghan, D. Rizos, M. P. Boland and I. Gordon. 1994. Efficiency of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture *in vitro*. *Mol. Reprod. Dev.* 37:48-53.
- Loeken, M. R. and C. P. Channing. 1985. Direct evidence for de-novo synthesis of LH receptors in cultured pig granulosa cells in response to FSH. *J. Reprod. Fert.* 73:343-351.
- Matas, C., E. Martinez, J. M. Vazquez, J. Roca and J. Gadea. 1996. *In vitro* penetration assay of boar sperm fertility: Effect of various factors on the penetrability of immature pig oocytes. *Theriogenology* 46:503-513.
- Motlik, J., N. Crozet and J. Fulka. 1984. Meiotic competence *in vitro* of pig oocytes isolated from early antral follicles. *J. Reprod. Fert.* 72: 323-328.
- Oxender, W. D., B. Colenbrander, D. F. M. Van De Wiel and C. J. G. Wensing. 1979. Ovarian development in fetal and prepubertal pigs. *Biol. Reprod.* 26:715-721.
- SAS. 1986. SAS Users Guide, SAS Institute Inc., Cary, NC.
- Suzuki, H., X. Yang and R. H. Foote. 1994. Surface characteristics and size changes of immature, *in vitro* matured and *in vitro* fertilized bovine oocytes. *Theriogenology* 41:307.
- Wang, X., S. K. Roy and G. S. Greenwald. 1991. *In vitro* DNA synthesis by isolated preantral to preovulatory follicles from the cyclic mouse. *Biol. Reprod.* 44:857-863.