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Morphological and Cellular Criteria of Ovaries, Follicles and Oocytes for *In Vitro* Maturation in the Pig

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체외배양을 위한 돼지 난소 및 난포란의 형태학적 조건

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초 록

돼지난포란의 안정된 체외배양체계를 위하여 도살장에서 채취된 난소로부터 난포 및 난자-난구세포체의 형태적인 선정기준을 설정하고 이의 이론적 배경을 확립하였다.

난소를 난포상태 및 분포나 황체 존재 여부에 따라 A, B 및 C 의 세 가지 형태로 분류하고 각 type의 난소에서 직경 3~5mm 인 난포로부터 난포란을 회수하였다. 회수된 난포란을 난구세포 부착상태에 따라 Good, Fair 및 Poor 의 세 가지 형태로 분류하여 각각을 호르몬이 첨가된 M16+FCS 배양액으로 35 시간 동안 체외배양하여 성숙율을 비교 검토하였다. 난소의 형태에 따라 회수된 난포란 중 Good 또는 Fair 형태는 Type A 및 C 난소에서 85%를 차지한 반면, Type B 난소에서는 53%에 불과하였다. 또한 이들 난포란을 체외배양한 결과 Type A 및 C 에서 회수된 난포란은 90 및 85%의 높은 성숙율을 보인 반면, Type C 의 난포란은 33%의 저조한 성숙을 나타냈다. 한편 핵형 분석 및 조직학적 분석에서도 Type C 난소의 경우 난포란의 핵형이 대부분 GVBD 및 퇴화형태를 보였으며, 폐쇄난포의 비율도 Type A 난소의 53%에 비해 월등히 높은 85% 를 나타내어 성숙율 비교실험의 결과와 일치되는 경향을 나타냈다.

따라서 본 실험의 선별기준에 의한 돼지 난소 및 난포란의 형태적 분류 작업에 의해 난포란의 체외배양 성적 향상 및 안정된 배양체계의 확립이 가능하였다.

INTRODUCTION

Mammalian oocyte has unique properties which are not found in somatic cells. It has a large amount of materials to be used during early development of embryo upon fertilization without genomic activation. This fact makes mammalian oocyte be one of the favorite experimental materials not only in basic researches such as cellular, molecular and developmental biology, but in the applied researches of reproductive biology such as *in vitro* fertilization (Mattioli *et al.*, 1989), embryo transfer (Lu *et al.*, 1990), cloning of embryos (Bondioli *et al.*, 1989) and production of transgenic animals (Hammer *et al.*, 1985). Therefore, it is of importance to maintain the supply of good quality of materials at a consistent basis to do such works. Especially, porcine oocyte may be a good model for many domestic animals, and suitable for research purpose due to availability of the large number of the oocytes from an ovary. The ovaries can also be obtained very easily from lo-

cal slaughter houses in large number. Therefore, these ovaries obtained from slaugter house may be practical source to ensure the supply of the oocytes.

There have been many studies on the maturation of follicular oocytes which were recovered from pigs either with controlled reproduction or with unknown state of reproductive cycle(Chung *et al.*, 1991; Byun *et al.*, 1989; Eng *et al.*, 1986; Gerard *et al.*, 1979; Richter and McGaughey, 1979; Tsafriri and Channing, 1975). However, the results were often variable and not reproducible. Although some morphological details of oocyte maturation have been established(Fukui and Sakuma, 1980), it is not always clear to select follicles and the oocytes on the basis of known description of published works. There should be some other selecting criteria for oocyte maturation *in vitro* to supply good oocytes which then will give substantial information along with some of established morphological assessment (Homa *et al.*, 1988).

This study made efforts for the selection of the ovaries, follicles and oocytes for culture by morphological and cytological analysis to provide a reliable culture system for the production of embryo *in vitro*.

MATERIALS AND METHODS

1. Chemicals

All of the chemicals used for preparation of culture media were purchased from BDH Chmical Co.(Poole, U. K.).

2. Selection of pig ovaries and follicular oocytes

Ovaries were collected in warm 0.85% NaCl solution from a local slaughter house. They were brought to laboratory in a Dewar flask within an hour. Ovaries were trimmed of other connective tissues prior to washing three times in physiological saline at 37℃ to remove blood and dirts. They were kept in physiological saline in a beaker on warmer plate during whole process. Since it was difficult to classify ovaries definitely, ovaries were arbitrarily divided into three groups, A, B and C types, depending on the distribution of follicular size on the surface of ovary and the presence or absence of corpus luteum(CL). The proportion of each types was determined in 144 ovaries for this study. The number of visible and collectable follicles, distribution of follicular size and number of corpus lutea were also examined in 3 different types of ovaries. The size of follicles was classified into 3 groups, $>$ 5, 3~5 and $<$ 3mm in diameter by experience.

3. Oocyte recovery from the classified ovaries

Ovaries were blotted on sterile gauze to remove saline and blood by applying slight pressure. Ovaries were cut into 3 or 4 pieces with a blade knife(No. 10, Paragon, Paragon Razor Co., Sheffield, U. K.), and then were blotted thoroughly with a sterile gauze to remove blood. They were transferred into 3ml of M2 + 4% (V/V) FCS medium in a watchglass at 37℃. Only the follicles with 3~5mm in diameter were punctured with a pair of watchmaker's forceps. The released oocyte-cumulus complexes(OCCs) were recovered from the watchglass prior to washing three times in the medium with a finely pulled Pasteur pipette under a dissecting microscope.

4. Classification of the recovered OCCs

The washed OCCs were again divided into 3 groups depending on the appearance of ooplasm and attachment of cumulus cell layers (McGaughey *et al.*, 1979). Briefly, the OCCs with several layers of cumulus cells and uniform ooplasm were designated 'good' type. 'Fair' type has 4~5 cell layers with uniform ooplasm, and

'poor' type has one cell layer with the exposed zona pellucida.

5. Nuclear analysis of the oocytes

Only two types of the OCCs, good and fair, recovered from the follicles were stripped of cumulus cells by the treatment of 300 IU/ml hyaluronidase(Type IV-s, Sigma) with repeated pipetting. Immediately after the collection of the OCCs, the chromatins of the oocyte were analysed by rapid staining method(Byun *et al.*, 1991) and fluorescent staining method using Hoechst 33258(Lee and Byun, 1989). Chromosomes of the oocyte were classified as normal dispersed germinal vesicle, abnormal condensed GV and GVBD according to McGaughey (1978). For more accurate analysis of GV chromosomes, 100mg/ml dibutyryl cyclic adenosine monophosphate (Sigma) were contained in M2+FCS to prevent GVBD which may occur during the collection of the oocytes. The oocytes matured *in vitro* were also processed for nuclear analysis at the end of culture.

6. *In vitro* maturation of follicular oocyte

After washing three times in M2+FCS, the OCCs were further washed three times in 2 ml of M16+15%(V/V)FCS+10 IU PMS+10 IU hCG(M16+FCS+Gn) in a watchglass prior to culture. The OCCs were cultured in 0.2 to 0.3ml droplets of the medium under liquid paraffin oil (BDH Co.) in a sterile, disposable plastic dish (35×10mm, Falcon plastics, Becton Dickinson Co., N. J., U.S.A.). The medium and oil had been equilibrated in an incubator for a minimum of 24h. About 30 OCCs were deposited in a droplet of the medium. The OCCs were cultured for 35h in an atmosphere of 5% CO_2 in air with 100% humidity at 39℃.

7. Histological analysis of ovaries

Three types of ovaries were cut into pieces, and the tissues were fixed in 10% formalin in phosphate buffered saline for 3 days at room temperature. They were washed, dehydrated in ascending alcohols and cleared in xylene prior to embedding into paraffin(Paraplast, Monoject Scientific Co., St. Louis, MO, U.S.A.). The paraffin blocks were sectioned into 2mm in thickness, and processed according to standard haematoxylin and eosin staining. From the sections normal or atretic follicles were analysed according to the criteria reported(Centola, 1982) in the three different types of ovaries. Photographs were taken under a light microscope using a camera and PAN F film(ASA 50, Ilford Co., U.K.) with appropriate exposures.

RESULTS AND DISCUSSIONS

1. The proportion of three different types of ovaries

Total 144 ovaries were classified into 3 groups according to distribution of developed follicles. Ovaries in type A contain relatively uniform size of follicles with 3~5mm in diameter(medium size) and a few corpus lutea and white bodies on the surface(Fig. 1a). The proportion of type A was 26% in 3 collections(Table 1). This type of ovaries was considered as preferable samples for most of experiments. Type B ovaries were covered with small follicles with < 3mm in diameter, and CL and white bodies were distributed over the surface of the ovaries(Fig. 1b). The proportion of type B was the highest, being 44% among the collected ovaries(Table 1). If differently sized follicles were present on the surface of the ovaries, they were grouped into type C(Fig. 1c) and 30% of the ovaries belonged to this group. Since the morphological appearance of the ovaries obtained from slaughter house were so variable, the classification may provide certain standard for the selection of follicles.

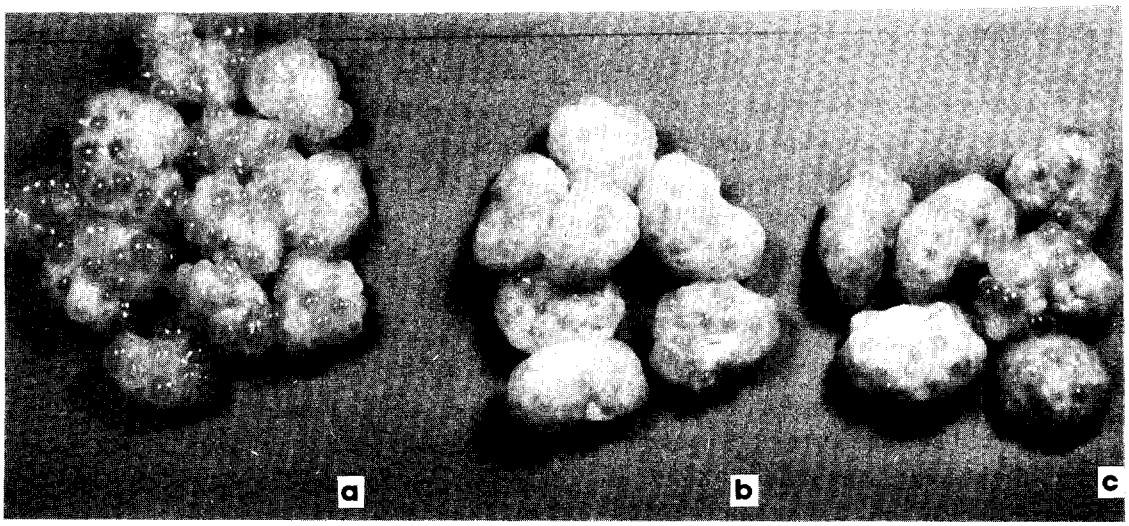

Fig. 1. The appearance of ovaries classified into 3 groups depending on the distribution of properly sized follicles, corpus lutea and white bodies. Representative ovaries in Type A(a) covered with properly sized follicles, Type B(b) with small sized follicles and Type C(c) with various sizes of follicles and white bodies are shown.

Table 1. Classification of ovaries obtained from a slaughter house by the follicular distribution[1]

Type of ovary	No. of ovaries(%)	Appearance and characteristics
Type A	38(26)	Follicles sized 3~5mm in diameter. Regular distribution. A few corpus lutea.
Type B	63(44)	Follicles sized < 3mm in diameter. Many corpus lutea.
Type C	43(30)	Various size of follicles. Irregular distribution. A few corpus lutea.
Total	144(100)	

1. The results were obtained from 3 experiments.

2. Distribution of different size of follicles in the ovary

The numbers of differently sized follicles and CL were examined in the classified ovaries to clarify whether the classification by the appearance was appropriate in terms of distribution of follicular size, CL and white bodies. The total numbers of visible follicles on the surface of the ovaries varied ranging from 30 to 50 follicles in 3 types of ovaries(Table 2). However, type B contained more follicles although 63.8% of follicles were small(< 3mm in diameter). The follicles sized 3 to 5mm in diameter were distributed at the highest rate in type A, being 42%. Type C ovaries have large proportion of smaller follicles, however, various size of follicles were found. This quantitative analysis demonstrated that the selection of the ovaries was appropriate for obtaining enough number of follicles properly sized. However, the number of CL was higher in type B than that of type C,

Table 2. Distribution of different sizes of follicles in the classified ovaries[1]

Types of ovary	Total No. of follicles[2]	% of follicular size at (in diameter)			% of CL[3]
		>5mm	3~5mm	<3mm	
Type A	29.9[b]	22.4	42.0[a]	19.7[b]	16.0[b]
Type B	47.9[a]		11.1[b]	63.8[a]	25.1[b]
Type C	34.0[b]	12.6	18.5[b]	31.3[b]	37.6[a]

1. Results from the analysis of 144 ovaries.
2. The values with different superscripts in the same column significantly differ ($p<0.01$).
3. Abbreviation is CL, corpus luteum.

thus suggesting that the presence of CL is not related to the number of proper follicles for oocyte maturation. The success of oocyte maturation *in vitro* often depends entirely on the sources of follicular oocytes once the other variables are established. It has been reported that follicular size from which oocytes are recovered affects the maturity of the oocytes in various species(Tsuji *et al.*, 1985; Leibfried and First, 1979; Smith *et al.*, 1978; Tsafriri and Channing, 1975).

3. Classification of the OCCs

The recovered OCCs from each types of ovaries were also examined to elucidate whether the quality of the OCCs is related to each classified ovaries. Although properly sized follicles were selected for this experiment, the recovered OCCs were various. Three different groups of the OCCs were found in the medium sized follicles of A, B and C types of ovaries when the OCCs were classified according to the tightness and thickness of cumulus cells(Fig. 2).

The distribution of 'good' and 'fair' OCCs used for most of culture studies, however, was highest in A type of ovaries(85% of follicles,

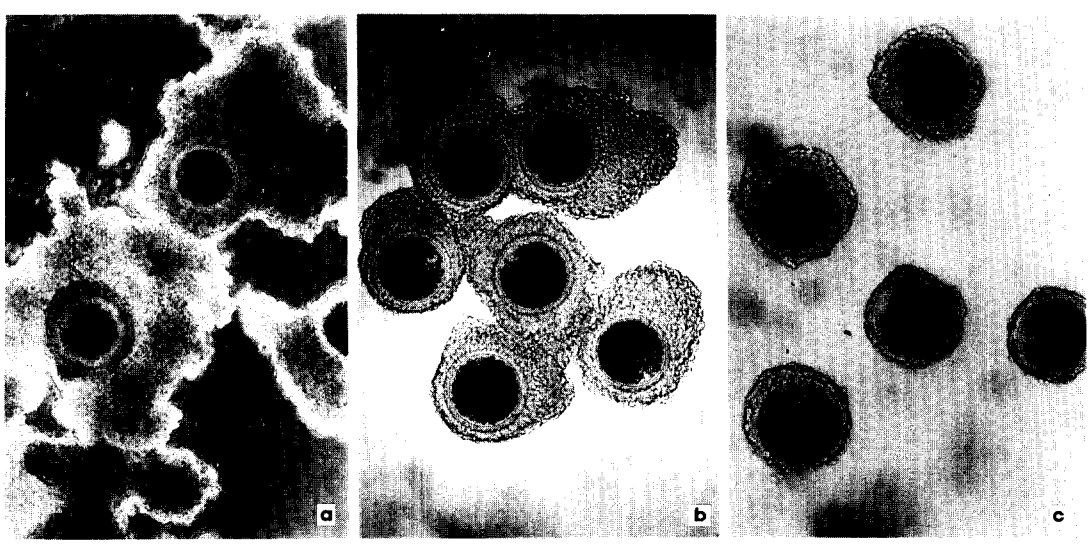

Fig. 2. Representative OCCs released from medium-sized follicles showing 3 different groups: 'good'(a), 'fair'(b) and 'poor'(c). The differences in the tightness and thickness of cumulus cell layers are clearly seen.

Fig. 3). Most of the OCCs from type B ovaries were grouped as 'poor' with a few layers of cumulus cells. In contrast, 'good' or 'fair' groups of the OCCs were sufficiently found in type C ovaries. The average proportion of 'good' OCCs from 3 types of ovaries was 28%, which was similar to 25% of good oocytes recovered from small or medium sized follicles in works of McGaughey(1978). The selection method used in this experiment would be very useful for obtaining constant pool of good oocytes under similar endocrinological control and developmental stage. Practically A and C types of ovaries may be used for the recovery of the oocytes for culture. From the data presented in Fig. 3, about 33~35% of follicular oocytes may be grouped as 'good' OCCs. The higher proportion of 'good' OCCs may attribute to the procedure method described in this study may provide a practice for the stable supply of 'good' OCCs from porcine ovaries.

4. *In vitro* oocyte maturation in different OCCs

The morphology of ovaries and the content of follicles were consistently related as shown in previous sections. However, it is critical whether the morphological criteria work properly in a biological system. The maturation rate of the recovered OCCs from each types of ovaries demonstrated that the morphological

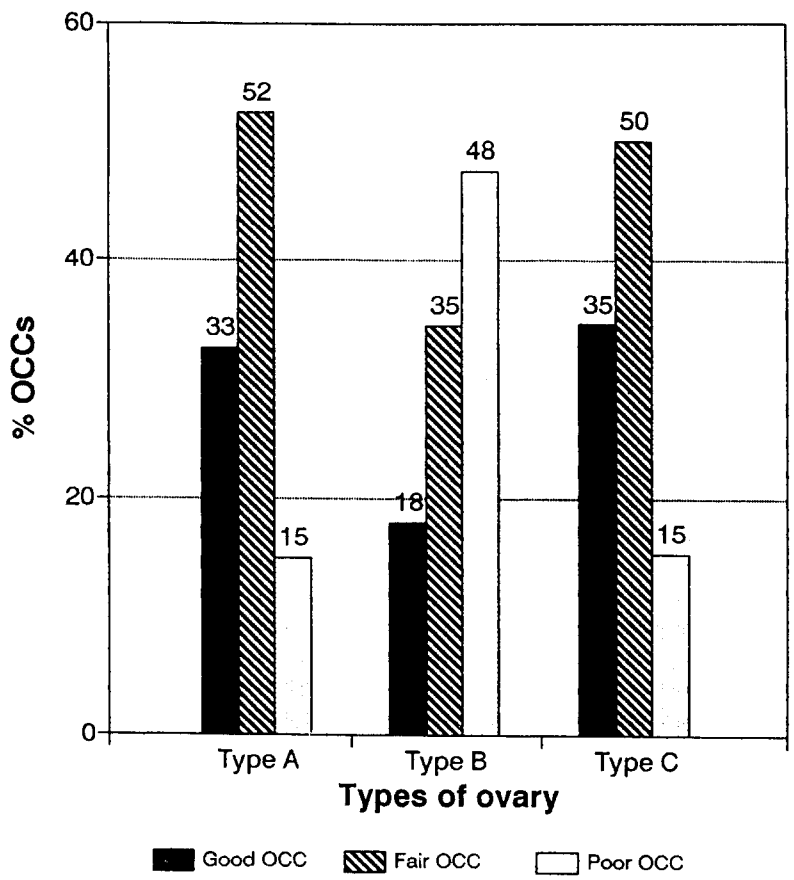

Fig. 3. Proportions of 'good', 'fair' and 'poor' OCCs collected from follicles in 3 different types of ovaries.

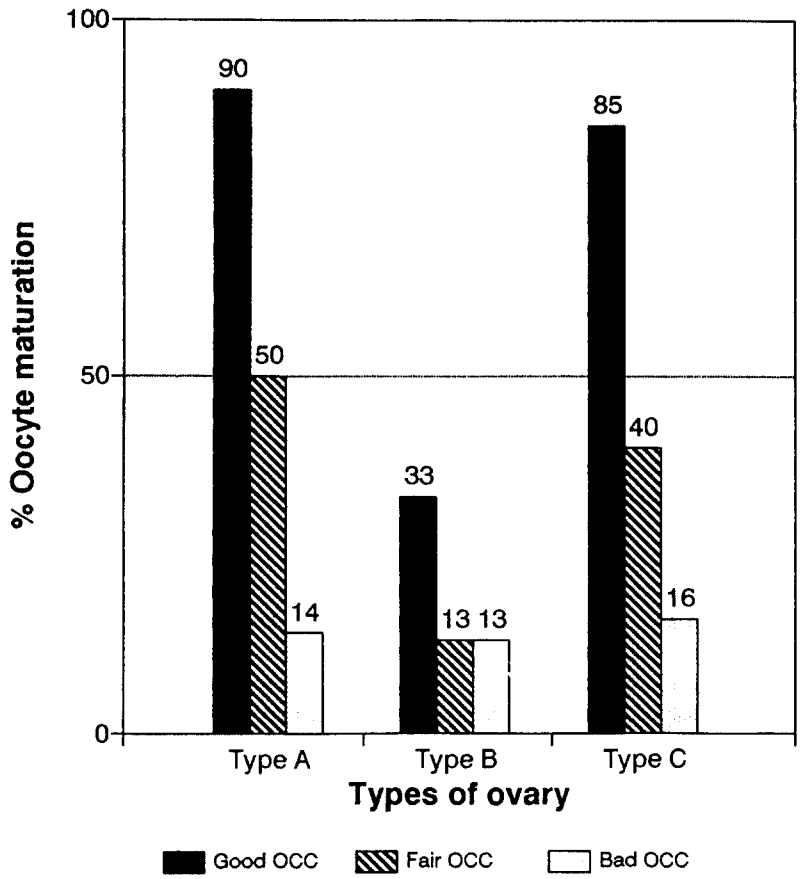

Fig. 4. The rates of oocyte maturation *in vitro* in 3 different groups of the OCCs collected from 3 types of ovaries.

selection was in good agreement with biological properties. The oocytes with the first polar body were considered as maturation in this analysis after 35h of culture in M16+FCS+Gn. 'Good' OCCs from type A and C ovaries showed 90 and 85% of maturation, respectively (Fig. 4). The results were higher than those of other reports. Even 'fair' OCCs matured upto 50 and 40% in type A and type C ovaries, respectively. In contrast, 'good' and 'fair' OCCs from type B ovaries showed 33 and 13% of maturation, respectively.

The results clearly demonstrated that selection of ovaries by the appearance was appropriate in terms of number of properly sized follicles and good oocytes resulting higher maturation *in vitro*. It also suggests that although properly sized follicles of B type ovaries have 'good' OCCs, they are not necessarily good oocytes since the rate of oocyte maturation was very low. Many of the oocytes contained highly disorganized cytoplasm when examined immediately after collection from the B ovaries. Therefore, it would be useful to follow the sequential procedure as shown in this experiment. The procedure described in detail will provide first substantial information for all studies using porcine ovaries from slaughter house.

5. Nuclear analysis of the oocytes recovered

From above experiment it was not clear why the oocyte from certain type of ovaries showed low rate of maturation after 35 h of culture. The possible factors for low maturation rate may be either cytoplasmic or nuclear or even endocrinological one. The visible factor easily detectable is nuclear status of the oocytes with similar morphology. Therefore, the oocytes were analysed immediately after selection and recovery. Type A and C ovaries showed normally dispersed chromatin in 85 and 68% of analysed oocytes, respectively(Fig. 5).

Type B ovaries showed about 35% of similar GV oocytes. Thus the nuclear chromatins may be one of the reasons for the low maturation rate in the oocytes of type-B ovaries. The maturation rates are in good agreement with this proportions of normal GV in 3 different ovaries. Along with normal GV, abnormally dispersed chromatins were frequently found in the oocytes of type C ovaries, and 25% of GVBD chromatin were also found in this type, consistent with the morphological criteria of follicles(Fig. 6). McGaughey (1978) postulated that the incidence of oocytes with fibrous GV chromatin was highest in the 'good' category, whereas 'poor' category contained high incidences of the oocytes in the diffuse and degenerate configuration. In human oocytes about 2.0~3.6% of the collected immature oocytes contained GVBD

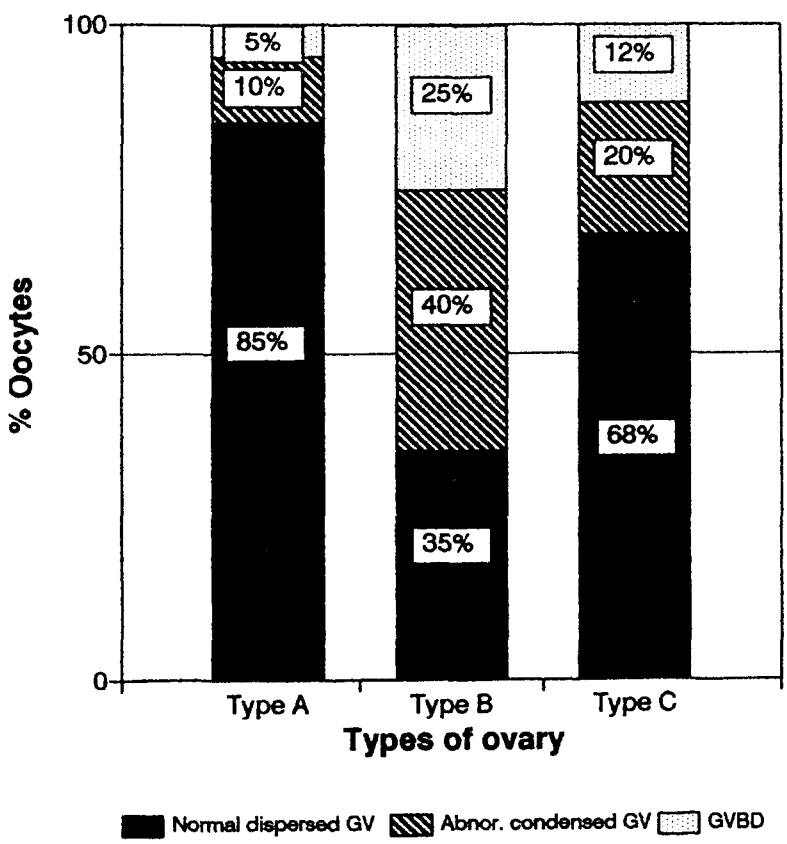

Fig. 5. Nuclear analysis of recovered oocytes from the medium-sized follicles of each types of ovaries.

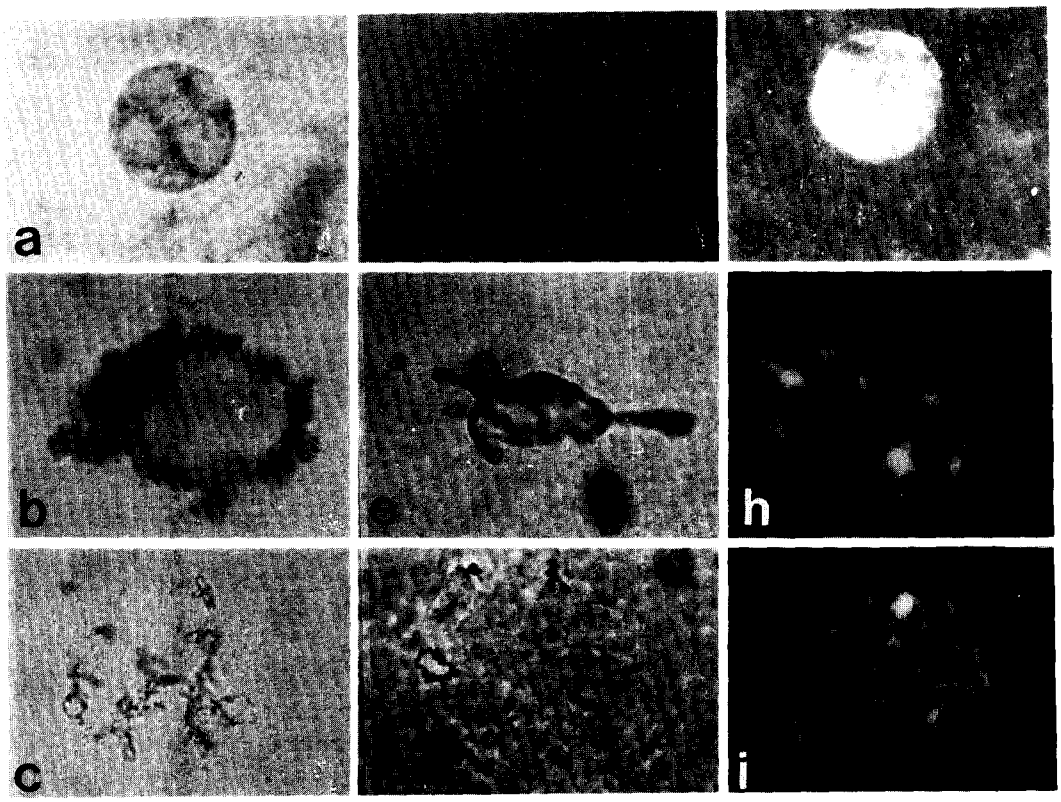

Fig. 6. Nuclear configurations of 'good' and 'fair' oocytes collected from follicles sized 3~5mm in diameter in 3 different types of ovaries. The chromatins of normally dispersed GV(a, d and g), abnormally condensed GV(b, e and h) and GVBD(c, f and i) were visualized by the rapid staining or Hoechst 33258(×500).

chromatins(McNatty *et al.*, 1979; Shea *et al.*, 1975). It has been also revealed that the rate of the oocyte with GVBD chromatins was about 7% in human ovaries(Gougeon and Testart, 1986). Therefore, it appears to be general that the immature, follicular oocytes contain various status of nuclei at any specific times.

The results presented in this experiment provided the establishment of the selection of ovaries, follicular and the OCCs and theoretical basis for those selection procedure by demonstrating morphological, nuclear and biological properties in classified samples.

6. Distribution of atretic or normal follicle in different types of ovaries

It seems that the oocytes with normal morphology and abnormal chromatins may differ in the follicle before the recovery of the oocytes. Therefore, this possibility was examined in the classified ovaries. The histological examination demonstrated that disorganization in the association between the oocyte and follicular cells and the piknotic nuclei of follicular cells were predominant in the follicles of types B and C ovaries (Fig. 7). The proportion of such follicles was 53% in type A ovaries (Fig. 8). This results

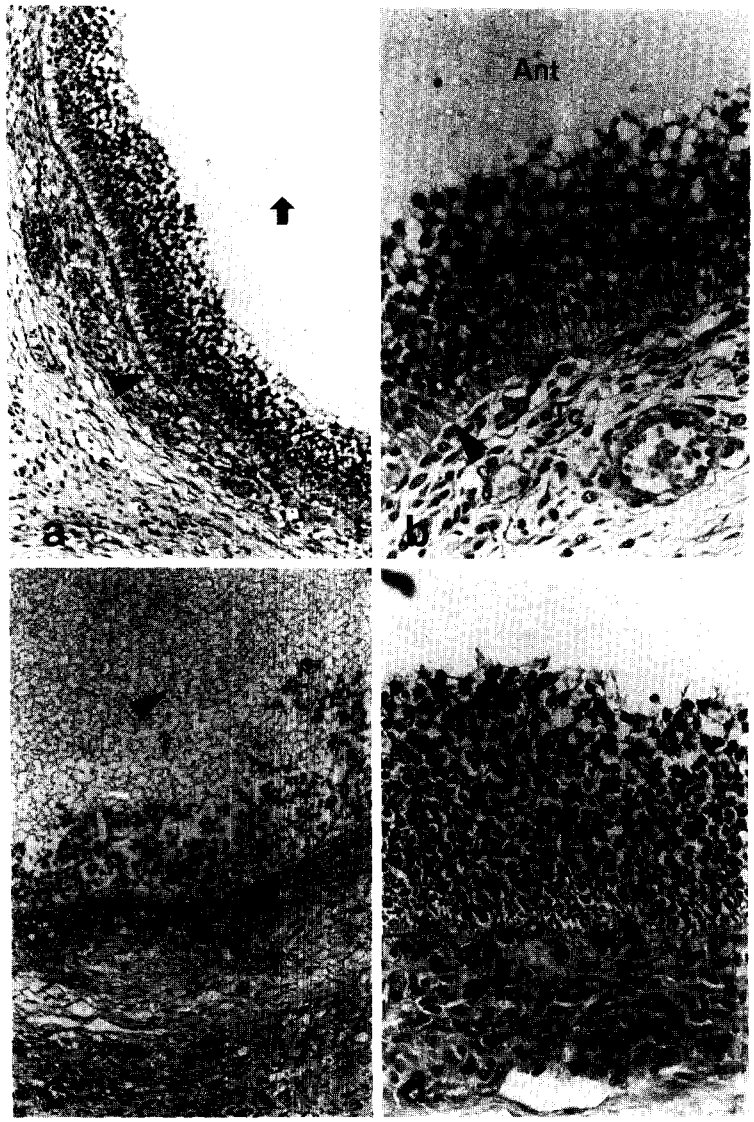

Fig. 7. Follicular organization in normal and atretic follicles. A very clear limiting structure of the follicle(an arrowhead), and clear follicular cavity(an arrow), are seen in the normal follicle(a). The folliclular cells(Fc) tightly connected to the basement membrane(an arrowhead) and theca cells(Tc) in another normal follicle(b). No precipitation was found in the antrum(Ant) of normal follicle. The precipitation was very extensive in the antrum of atretic follicle(an arrowhead). Disorganization of cellular association and piknotic nuclei of follicle cells(an arrow) were found in an atretic follicle (c). Different degrees of atresia were found in the follicle cells with piknotic nuclei (an arrow) in another atretic follicle(d).

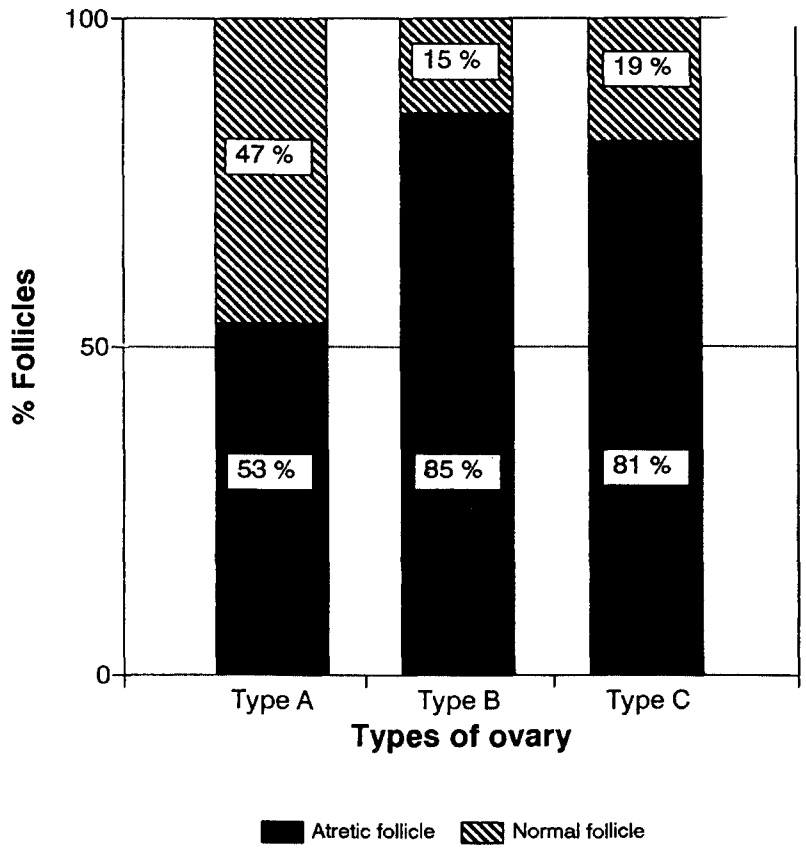

Fig. 8. The proportion of normal and atretic follicles distributed in 3 different types of ovaries.

suggest that even type A ovaries contain a large proportion of atretic follicles within the tissue. The rate of atretic follicle in type A was similar to that of preantral follicles reported by Centola(1982). The report suggested that the atretic follicles accounted for 75, 73 and 84% of all the oocytes examined in small-, medium- and large-sized antral follicles, respectively, and approximately 56% of all preantral follicles were atretic. Although the rate of atretic follicle was determined in this study, no information is available about the exact interrelationship between the morphology of follicles and atresia by the histological analysis. The data only demonstrated that appropriate selection procedure could provide consistent supply of good oocytes rather than random selection after collection of the OCCs from available ovaries.

The histological criteria for the identification of atretic follicles in the mammals ovary are well established (Hunter *et al.*, 1989; Ryan, 1981; Byskov, 1978). Generally, it is known that many oocytes in mammalian ovaries are atretic (McGaughey *et al.*, 1979; Himelstein-Braw *et al.*, 1976), and most of non-atretic oocytes are small and incapable of maturation in culture(Channing *et al.*, 1980; Tsafriri and Channing, 1975). Also, the oocytes from atretic follicles underwent GVBD at very low level and 20% of the oocytes were necrotic in human study(Gougeon and Testart, 1986). But once a follicle begins to degenerate *in vivo*, it will probably not return to

the ovulatory pathway(Hirshfield, 1989). The size of follicles and atresia are reported as important factors for normal maturation *in vitro* (Centola, 1982; Channing *et al.*, 1980). Therefore, the selection of ovaries, and follicles may avoid this atretic follicles as few as possible, thus providing a good system for basic and applied researches.

SUMMARY

The theoretical basis for the selection of the ovaries, follicles and oocyte−cumulus complexes(OCCs) for *in vitro* culture by morphological and cytological analysis to provide a reliable culture system was established. Ovaries were arbitrarily divided into three groups, A, B and C types, depending on the distribution of follicular size. The recovered OCCs were again divided into 3 groups, such as 'good', 'fair' and 'poor', depending on the appearance of ooplasm and attachment of cumulus cell layers. The OCCs were cultured in M16+FCS+Gn for 35h in an atmosphere of 5% CO_2 in air with 100% humidity at 39℃. The distribution of 'good' and 'fair' OCCs was highest in type A of ovaries (85% of follicles). Most of the OCCs from type B of ovaries were grouped as 'poor'. 'Good' OCCs from type A and C ovaries showed 90 and 85% of maturation, respectively. In contrast, 'good' and 'fair' OCCs from type B ovaries showed 33 and 13% of maturation, respectively. Type A and C ovaries showed normally dispersed chromatin in 85 and 68% of analysed oocytes, respectively, type B ovaries showed about 35% of similar GV oocytes. The histological examination demonstrated that atretic follicles were predominant in the follicles of type B and C ovaries. The proportion of such follicles was 53% in type A ovary.

The results clearly demonstrated that selection of ovaries by the appearance was appropriate in terms of number of properly sized follicles and good oocytes resulting higher maturation *in vitro*.

REFERENCES

Bondioli KR, Ellis SB, Prior JH, Williams MW and Harpold MM. 1989. The use of male-specific chromosomal DNA fragments to determine the sex of bovine preimplantation embryos. Theriogenology, 31: 95-104.

Byskov AG. 1978. Follicular atresia. In: The vertebrate ovary, Jones RE (ed.), Plenum Press, New York, NY, U.S.A., pp. 533-562.

Byun TH, Lee SH and Song HB. 1991. Development of a rapid staining method for nucleus of the oocyte from domestic animals. Korean J. Anim. Sci., 33:25-31.

Centola GM. 1982. Light microscopic observations of alterations in staining of the zona pellucida of porcine follicular oocyte: possible early indication of atresia. Gamete Res., 6:293-304.

Channing CP, Schaerf FW, Anerson LD and Tsafriri A. 1980. Ovarian follicular and luteal physiology. Int. Rev. Physiol., 22: 117-201.

Eng LA, Kornegay ET, Huntington J and Wellman T. 1986. Effects of incubation temperature and bicarbonate on maturation of pig oocytes *in vitro*. J. Reprod. Fert., 76: 657-662.

Fukui Y and Sakuma Y. 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.

Gerard Y, Menezo Y, Rombauts P, Szollosi D and Thibault C. 1979. *In vitro* studies of oocyte maturation and follicular metabolism in the pig. Annls. Biol. Anim. Biochim.

Biophys., 19: 1521-1535.

Gougeon A and Testart J. 1986. Germinal vesicle breakdown in oocytes of human atretic follicles during the menstrual cycle. J. Reprod. Fert., 78: 389-401.

Hammer RE, Pursel VG, Rexroad CE, Jr., Wall RJ, Bolt DJ, Ebert KM, Palmiter RD and Brinster RL. 1985. Production of transgenic rabbits, sheep and pigs by microinjection. Nature, 315: 680-683.

Hirshfield AN. 1989. Rescue of atretic follicles *in vitro* and *in vivo*. Biol. Reprod., 40: 181-190.

Homa ST, McGaughey RW and Racowsky C. 1988. Isolated subpopulations of mass-harvested pig oocytes and their characterization by size, incidence of atresia and competence to mature in culture. J. Reprod. Fert., 82:81-86.

Hunter MG, Grant SA and Foxcroft GR. 1989. Histological evidence for hetero-geneity in the development of preovulatory pig follicles. J. Reprod. Fert., 86: 165-170.

Lee SH and Byun TH. 1989. Analysis of mammalian oocytes and embryos using DNA-specific dyes. J. Inst. Biotechnol., Korea Univ., 1: 43-46.

Leibfried L and First NL. 1979. Characterization of bovine follicular oocytes and their ability to mature *in vitro*. J. Anim. Sci., 48:76-86.

Lu KH, Gordon I, Gallagher M and McGovern H. 1990. Pregnancy established in cattle by transfer of fresh and frozen embryos derived from *in vitro* maturation, fertilization of oocytes and their subsequent culture *in vitro*. Theriogenology, 33:278.

Mattioli M, Bacci ML, Galeati G and Seren E. 1989. Developmental competence of pig oocytes matured and fertilized *in vitro*. Theriogenology, 31: 1201-1207.

McGaughey RW. 1978. *In vitro* oocyte maturation. In: Method in mammalian reproduction, Daniel JC, Jr. (ed.), Academic Press, New York, NY, U.S.A., pp. 1-19.

McGaughey RW, Montgomery DH and Richter JD. 1979. Germinal vesicle configurations and patterns of polypeptide synthesis of porcine oocytes from antral follicles of different size, as related to their competency for spontaneous maturation. J. Exp. Zool., 209:239-254.

McNatty KP, Smith DM, Makris A, Osathanond R and Ryan KH. 1979. The micro- environment of the human antral follicle: interrelationships among the steroid levels in antral fluid, the population of granulosa cells, and the status of the oocyte *in vivo* and *in vitro*. J. Clin. Endocr. Metab., 49: 851-860.

Richter JD and McGaughey RW. 1979. Specificity of inhibition by steroids of porcine oocyte maturation *in vitro*. J. Exp. Zool., 209: 81-90.

Ryan RJ. 1981. Follicular atresia: some speculations of biochemical markers and mechanisms. In: Dynamics of ovarian function, Schwartz NB and Hunzicker-Dunn M (eds.), Raven Press, New York, NY, U.S. A., pp. 1-11.

Shea BF, Baker RD and Latour JPA. 1975. Human follicular oocytes and their maturation *in vitro*. Fert. Steril., 26: 1075-1082.

Smith DM, Tyler JPP and Erickson GF. 1978. Effects of medium composition and progesterone on maturation *in vitro* of rabbit oocytes from Graafian follicles of different sizes. J. Reprod. Fert., 54: 393-400.

Tsafriri A and Channing CP. 1975. Influence of follicular maturation and culture conditions on the meiosis of pig oocytes *in vitro*. J. Reprod. Fert., 43:149-152.

Tsuji K, Sowa M and Nakano R. 1985. Relationship between human oocyte maturation and different follicular sizes. Biol. Reprod., 32:

413-417.

변태호, 심금섭, 송해범. 1989. 배양액의 종류가 돼지 난포난의 체외성숙에 미치는 영향. 한국축산학회지, 31:363-372.

정형민, 박세필, 오종훈, 이훈택, 정길생. 1991. 가토 복수가 돼지 난포난의 체외성숙에 미치는 효과. 한국가축번식학회지, 15:221-224.