Oestrous cycle stage influences the morphology and maturation of porcine oocytes *in vitro*

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ABSTRACT: The objective of the study was to characterize the effect of the oestrous cycle stage on the yield, morphology and meiotic competence of porcine oocytes. A total of 46 cycling gilts, at 8.5–9 months of age, were used as oocyte donors. Their oestrous cycle was synchronized by Regumate and the onset of oestrus was checked (Day 0). The gilts were slaughtered at the early (Days 1–5), middle (6–10) and late (11–14) luteal or early (Days 15–16), middle (17–19) and late (20–21) follicular phase. Oocytes were isolated separately from medium (5–9 mm) and small (≤ 4 mm) follicles. Cumulus-oocyte complexes with dark, evenly granulated cytoplasm and at least two compact layers of cumulus cells were selected as useful for maturation. They were matured by a standard protocol, denuded from cumulus cells, fixed in glutaraldehyde, stained with 33258-Hoechst and examined by epifluorescence. The oocytes collected from small and medium follicles differed in their yield, morphology and meiotic competence regardless of the phase. The mean number (± S.E.M.) of oocytes isolated per donor was higher (187.7 ± 48.4 vs. 16.9 ± 6.1) but the mean percentage (± S.E.M.) of useful oocytes was lower (22.4 ± 7.5% vs. 80.2 ± 6.8%; $P < 0.01$) for small than for medium follicles. The mean number (± S.E.M.) of useful oocytes per donor was significantly ($P < 0.01$) higher (42.1 ± 16.8 vs. 11.9 ± 4.3) but the mean percentage of matured oocytes was significantly ($P < 0.01$) lower (48.4 ± 17.8% vs. 79.9 ± 7.9%) for small than for medium follicles. The oocyte population collected from small follicles varied during the oestrous cycle. The mean number (± S.E.M.) of oocytes isolated per donor from small follicles increased during the luteal and decreased during the follicular phase, except for the late follicular phase when it increased again. The mean percentage (± S.E.M.) of useful oocytes did not differ too much during this period, except for the late follicular phase when it decreased significantly ($P < 0.01$). The mean number (± S.E.M.) of useful oocytes per donor increased during the luteal and decreased during the follicular phase, but the differences were not significant except for the late follicular phase ($P < 0.01$). Similarly, the mean percentage (± S.E.M.) of matured oocytes increased during the luteal and decreased during the follicular phase, and the differences were significant. Compared with the oocyte population from small follicles, the oocyte population from medium follicles was less variable in the period from the middle luteal to middle follicular phase, when these follicles were present on the ovaries. It can be concluded that the porcine oocyte population changes in terms of quantity, morphological quality and meiotic competence according to the stage of follicular development. The late luteal and early follicular phases appeared to be most productive for oocyte recovery, because more morphologically normal oocytes with greater meiotic competence were collected, as compared with the other stages of the oestrous cycle.

Keywords: gilts; follicular development; oocytes; morphology; meiotic competence

The quality of oocytes affects the efficiency of early embryo development and pregnancy establishment after embryo transfer. The meiotic and developmental competence of oocytes is acquired gradually with follicle growth, and they are completed during maturation. The relationship between the follicular status, morphology of bovine oocytes and their *in vitro* developmental competence was described (de Wit et al., 2000; Vassena et al., 2003).

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In cattle the morphology, meiotic and developmental competence of oocytes change during the growth, static, dominant and regression phases of each of two or three follicular waves that emerge during the oestrous cycle (Salamone et al., 1999). In contrast, in pigs no two-wave hypothesis has been suggested; folliculogenesis has been described as continuous growth and atresia of ovarian follicles independent of changes in plasma FSH and steroid concentrations, without evidence of cohort or dominant follicles in the luteal phase (Guthrie and Cooper, 1996; Kanitz et al., 2001). It seems that the process of selection and growth of dominant follicles, which leads later to atresia of subordinate follicles, takes a long period of the oestrous cycle, especially at the late luteal and early follicular phase (Schnurrbusch et al., 1990).

This study was designed to characterize the effect of early, middle and late stages of the luteal and follicular phases of the oestrous cycle on the yield, morphology and meiotic competence of oocytes in cycling pubertal gilts.

MATERIAL AND METHODS

Oocyte donors. A total of 46 Landrace × Czech Large White crossbred cycling gilts (age, 8.5 to 9 months; weight, 130–150 kg) were used as oocyte donors. Their oestrous cycle was synchronized by Regumate (Intervet, France) over a 16-day period (daily 20 mg altrenogest per gilt). Four to five days after the treatment, the oestrus onset was checked (Day 0). Gilts were slaughtered at the early (Days 1–5), middle (6–10) and late (11–14) stage of the luteal phase or at the early (Days 15–16), middle (17–19) and late (20–21) stage of the follicular phase at an experimental abattoir. Ovaries of each donor were evaluated for morphology and those with adequate follicular status were used. They were transported within 20 min at 39°C to the laboratory.

Oocyte collection. Oocytes were isolated from all subordinate follicles located on the ovaries, separately from medium (5–9 mm) and small (2–4 mm) follicles by aspiration and cutting, respectively. Large dominant follicles (≥ 10 mm) were not included in this study. Macroscopically healthy cumulus-oocyte complexes with a dark, evenly granulated cytoplasm and at least two compact layers of cumulus cells were selected as useful for maturation.

Oocyte maturation. Oocytes were matured in 500 µl of TCM-199 medium (Earle's salt), with the addition of 0.20mM sodium pyruvate, 0.57mM cysteamin, 50 IU/ml penicillin, 50 µg/ml streptomycin (Sigma Chemicals Co., Prague, Czech Republic), 10% BFS (bovine foetal serum, Sigma Chemicals Co., Prague, Czech Republic) and gonadotropins (P.G.600 15 IU/ml, Intervet, Holland) in Nunc 4-well multi-dish (Nunc, Intermed, Denmark) for 47 hours at 39°C in a humidified atmosphere of 5% CO_2 in air.

Oocyte examination. After culture the oocytes were denuded from cumulus cells by vortexing, fixed in 2.5% aqueous glutaraldehyde solution (v/v), and stained with bisbenzimide-33258 Hoechst in citrate buffer (10 µl/ml in 0.154M sodium chloride and 0.015M trisodium citrate) for 10 min at room temperature. Wet mounts were prepared in 5 µl glycerine buffer (0.1M CH_3COOH 22.2 ml, 0.2M Na_2HPO_4 27.8 ml and glycerine 50 ml) after the oocytes were rinsed three times in PBS-Dulbecco. They were examined by epifluorescence at a 400× magnification.

Evaluated parameters. We assessed the number of isolated and useful oocytes per donor; the quality and meiotic competence of oocytes were evaluated as a ratio of useful to isolated oocytes and that of matured to useful oocytes. The oocytes at metaphase II with an extruded polar body after culture were considered as matured ones.

Statistical analysis. The data were expressed as mean percentages ± S.E.M. and analyzed by the ANOVA procedure, Chi-square test, Version 6.1 for Windows software (SPSS, Inc.).

RESULTS

Oocytes from small and medium follicles regardless of the phase

Oocytes collected from small and medium follicles differed in their yield, morphology and competence for maturation regardless of the phase. Evidently, a higher number of oocytes per donor was isolated from small than from medium follicles. On the other hand, the percentage of useful (± S.E.M.) oocytes was significantly lower ($P < 0.01$) for small than for medium follicles. The mean number (± S.E.M.) of useful oocytes collected per donor from small follicles was significantly higher ($P < 0.01$) than that of useful oocytes collected per donor from medium follicles. However, the percentage of matured oocytes (± S.E.M.) was signifi-

Table 1. Characteristics of oocytes from small and medium follicles regardless of the phase (mean ± S.E.M.)

Follicle size	Oocytes			
	isolated/donor (*n*)	useful/isolated (%)	useful/donor (*n*)	matured/useful (%)
Small	187.7 ± (48.4)	22.4 ± (7.5)[a]	42.1 ± (16.8)[a]	48.4 ± (17.8)[a]
Medium	16.9 ± (6.1)	80.2 ± (6.8)[c]	11.9 ± (4.3)[c]	79.9 ± (7.9)[c]

Values with different superscripts within the same column are significantly different (a–c, $P < 0.01$)

cantly lower ($P < 0.01$) for small than for medium follicles (Table 1).

Oocytes from small and medium follicles as related to the phase

The population of oocytes collected from small follicles varied in the parameters evaluated during the whole oestrus cycle, from the early luteal to late follicular phase.

The mean (± S.E.M.) number of oocytes isolated per donor from small follicles increased during the luteal phase, reached maximum at the early follicular phase, decreased during the middle follicular phase and increased again at the late follicular phase. The mean percentage (± S.E.M.) of useful oocytes did not differ throughout either the luteal or follicular phase, except for the late follicular phase when it decreased significantly ($P < 0.01$).

The mean number (± S.E.M.) of useful oocytes collected per donor from small follicles increased during the luteal phase, reached the peak at the early follicular phase and decreased thereafter, but the differences between the stages of each phase were not significant, except for the values related to the late follicular phase. Similarly, the mean percentage (± S.E.M.) of matured oocytes increased during the luteal phase and decreased during the follicular phase, with the differences between the three stages of the same phase being significant (Table 2). In comparison with the population of oocytes collected from small follicles, the population of oocytes collected from medium follicles was less variable in terms of the evaluated parameters in the period from the middle luteal to the middle follicular phase when these follicles were present on the ovaries. The mean number (± S.E.M.) of oocytes isolated per donor from medium follicles remained similar for the whole time. The mean percentage (± S.E.M.) of useful oocytes did not differ during this period, except for the middle follicular phase when it decreased significantly ($P < 0.05$). Similarly, the mean number (± S.E.M.) of useful oocytes collected per donor from medium follicles did not change significantly at any stage of the two phases. As in the oocytes from small follicles, the mean percentage (± S.E.M.) of matured oocytes increased from the middle to late luteal phase and decreased from the early to middle follicular phase, but no significant difference between stages was found (Table 3).

Table 2. Characteristics of oocytes from small follicles in the luteal or follicular phase (mean ± S.E.M.)

Phase	Stage	Oocytes			
		isolated/donor (*n*)	useful/isolated (%)	useful/donor (*n*)	matured/useful (%)
Luteal	early	144.8 ± (34.4)	21.2 ± (5.1)[a]	30.7 ± (14.5)[a]	30.4 ± (17.7)[a]
	middle	183.0 ± (36.3)	20.3 ± (13.7)[a]	37.1 ± (22.2)[a]	55.1 ± (11.9)[b]
	late	207.7 ± (53.1)	21.3 ± (5.0)[a]	44.3 ± (3.6)[a]	73.9 ± (4.6)[c]
Follicular	early	267.2 ± (89.8)	26.4 ± (5.7)[a]	70.6 ± (18.7)[a]	60.3 ± (6.4)[a]
	middle	174.8 ± (11.0)	26.3 ± (4.0)[a]	45.9 ± (7.3)[a]	46.0 ± (10.6)[b]
	late	244.5 ± (41.5)	11.7 ± (4.8)[b]	28.5 ± (6.5)[b]	20.8 ± (1.7)[c]

Values with different superscripts within the same column and inside the same phase are significantly different (a–b, a–c, b–c, $P < 0.01$)

Table 3. Characteristics of oocytes from medium follicles in the luteal or follicular phase (mean ± S.E.M.)

Phase	Stage	Oocytes			
		isolated/donor (*n*)	useful/isolated (%)	useful/donor (*n*)	matured/useful (%)
Luteal	early	none*			
	middle	12.8 ± (4.1)	84.3 ± (4.4)[a]	10.8 ± (3.6)[a]	73.0 ± (8.6)[a]
	late	26.5 ± (2.5)	77.4 ± (2.2)[a]	20.5 ± (2.5)[a]	87.2 ± (3.6)[a]
Follicular	early	15.5 ± (1.5)	93.5 ± (0.6)[a]	14.5 ± (1.5)[a]	86.2 ± (1.5)[a]
	middle	17.0 ± (6.3)	75.5 ± (6.5)[b]	12.8 ± (1.5)[a]	76.8 ± (7.9)[a]
	late	none*			

Values with different superscripts within the same column and inside the same phase are significantly different (a–b, $P < 0.05$)

*no medium follicles on ovaries

Comparisons of the oocyte populations, as related to follicle size and stages of the oestrous cycle, are shown in Figure 1 and 2.

DISCUSSION

A sufficient number of mature and fertilizable oocytes with high developmental competence is a prerequisite for *in vitro* embryo production in farm animals; in addition, these oocytes are also essential for further advancements in the field of biotechnology and biomedicine dependent on both the quantity and the quality of oocytes.

In pigs, the ovaries of prepubertal and early pubertal gilts are the main sources of oocytes for the production of genetically valuable embryos or embryos used for experimental purposes. It has been reported that oocytes of prepubertal gilts appeared less meiotically and developmentally competent than those of their more adult counterparts. A lower reproductive potential was observed also in primiparous sows, therefore, the use of pubertal but cycling gilts was recommended for IVF and breeding programs (Marchal et al., 2001; Brussow et al., 2002; Ratky et al., 2005). Higher meiotic and developmental competence observed in oocytes after the onset of puberty is acquired gradually during multiple oestrous cycles (Bagg et al., 2004). In cycling gilts, morphological quality and meiotic competence of oocytes vary during the oestrous cycle, because the follicular population is a mixture of both healthy and atretic follicles. Oocytes are usually harvested regardless of the oestrous cycle stage

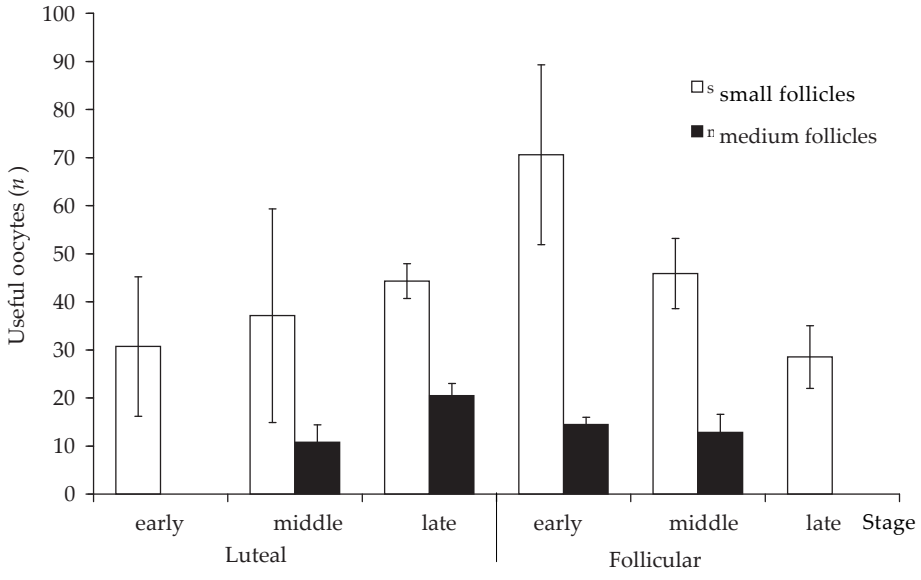

Figure 1. Mean number ± S.E.M. of useful oocytes per donor related to follicle size and phase

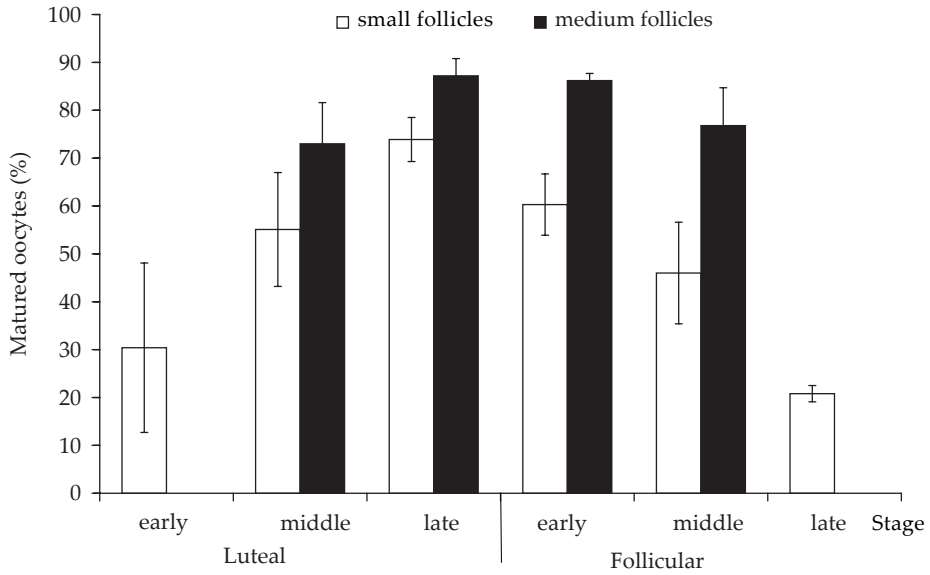

Figure 2. Mean percentage ± S.E.M. of mature oocytes related to follicle size and phase

of donors and they originate mostly from small and medium follicles, because a higher number of these follicles is present on ovaries.

In this study, we collected oocytes from cycling pubertal gilts and evaluated the qualitative and quantitative parameters of oocyte populations from small and medium follicles during the whole oestrous cycle, i.e. early, middle and late stages of both the luteal and the follicular phase.

It has been documented that the size of follicles from which oocytes are derived has a significant effect on the quality of oocytes, their maturation, fertilization and development both in pigs (Bolamba and Sirard, 2000; Marchal et al., 2002; Krisher, 2004;) and in cattle (Raghu et al., 2002). Factors within follicles, at later stages of their development, play an important role during oocyte maturation (Algriany et al., 2004).

Full competence for maturation is achieved by both porcine and bovine oocytes recovered from follicles larger than 3 mm in diameter. Porcine oocytes from follicles smaller than 3 mm are not fully meotically competent, because they are cytoplasmically deficient (Sun et al., 2001; Marchal et al., 2002). They are also smaller in size and show a lower glutathione content before and after maturation (Liu et al., 2002). Despite this, a relatively high proportion of oocytes derived from small follicles is able to resume meiosis, synthesize cyclin B, phosphorylate MAP kinase and translocate cortical granules; however, their ability to complete maturation, be fertilized and start embryo development is limited (Sun et al., 2001).

In our study, a very high proportion of oocytes, over 90%, was harvested from small follicles (≤ 4 mm), regardless of the oestrous cycle phase. Morphological quality as well as meiotic competence of these oocytes were found to be very low, which is in agreement with the results of Marchal et al. (2002) and Liu et al. (2002). On the other hand, the oocytes isolated from medium follicles (4–9 mm), though lower in numbers, were better in their morphological quality and were more capable of completing nuclear maturation. Good morphological quality and better developmental competence in porcine oocytes collected from larger follicles were reported by Yoon et al. (2000). These authors described that larger follicles, as compared with small ones, yielded more morphologically normal and more meiotically competent oocytes (22% vs. 14% and 91% vs. 58%). In our experiments, these values for oocytes from medium follicles, as compared with those from small follicles, were approximately 80% vs. 22% and 80% vs. 48%, respectively.

The size of the follicle affects the quality of immature oocytes, potentially implicating messenger RNA or protein stores as factors involved in oocyte developmental competence. Metabolism may play a critical role in oocyte quality, because the glycolytic activity of matured oocytes is correlated with increased embryonic development (Krisher, 2004). In porcine oocytes from larger follicles, competence to undergo nuclear and cytoplasmic maturation is improved due to their ability to accumulate intracellular glutathione and

extracellular steroid hormones during maturation (Liu et al., 2002).

There is a general agreement that, in pigs as well as in cattle, the size of the follicle is changing during the oestrous cycle due to its growth and atresia. It is known that slightly atretic follicles produce morphologically good-quality oocytes but follicles with higher degree of atresia produce morphologically poor-quality oocytes with low developmental ability (Jewgenow et al., 1999). A high incidence of atresia among follicles grown at the early and middle luteal phase was described by Guthrie et al. (1995a,b). In our study, the population of oocytes from small and medium follicles increased in the number of morphologically normal and meiotically competent oocytes between the early and medium luteal phase, with the maximum being reached in the late luteal and early follicular phases; thereafter, the numbers of those oocytes began to decrease. Similarly to our results, a higher oocyte recovery rate at the late luteal and early follicular phase, as compared with the late follicular phase, was observed by Schnurrbusch et al. (1990). At the late luteal phase, selection for growth and maturation of presumptive preovulatory follicles begins from medium follicles, while subordinate, smaller follicles become atretic (Ratky et al., 1995). Knox (2005) also reported that peak numbers of small and medium follicles without any larger follicles were observed on gilt ovaries at the late luteal and early follicular phase. During the early follicular phase, numbers of small and medium follicles rapidly decline, while a pool of medium follicles is selected for the ovulation (Knox, 2005). Also in our study, a decreasing number of morphologically normal oocytes with lesser meiotic competence was found during the follicular phase, with the minimum at its late stage. This can be explained by preovulatory follicle dominance when the size of subordinate follicles decreases during the middle and late follicular phases (Ratky et al., 1995). Only fifteen percent of follicles survived to ovulate at the late follicular phase (Guthrie and Garrett, 2000, 2001).

It can be concluded that in pigs the population of oocytes changes in terms of quantity, morphological quality and meiotic competence according to the stage of follicular development. The late luteal and early follicular phases appear to be the most productive for oocyte recovery, because more morphologically normal oocytes with greater meiotic competence are collected, as compared with the other stages of the oestrous cycle.

REFERENCES

Algriany O., Bevers M., Schoevers E., Colenbrander B., Dieleman S. (2004): Follicle size-dependent effects of sow follicular fluid on *in vitro* cumulus expansion, nuclear maturation and blastocyst formation of sow cumulus oocytes complexes. Theriogenology, 62, 1483–1497.

Bagg M.A., Vassena R., Papasso-Brambilla E., Grupen C.G., Armstrong D.T., Gandolfi F. (2004): Changes in ovarian, follicular, and oocyte morphology immediately after the onset of puberty are not accompanied by an increase in oocyte developmental competence in the pig. Theriogenology, 62, 1003–1011.

Bolamba D., Sirard M.A. (2000): Ovulation and follicular growth in gonadotropin-treated gilts followed by *in vitro* fertilization and development of their oocytes. Theriogenology, 53, 1421–1437.

Brussow K.P., Ratky J., Torner H., Egerszegi I., Schneider F., Solti L., Tuchscherer A. (2002): Follicular and oocyte development in gilts of different age. Acta Veterinaria Hungarica, 50, 101–110.

de Wit A.A., Wurth Y.A., Kruip T.A. (2000): Effect of ovarian phase and follicle quality on morphology and developmental capacity of the bovine cumulus-oocyte complex. Journal of Animal Science, 78, 1277–1283.

Guthrie H.D., Cooper B.S. (1996): Follicular atresia, follicular fluid hormones, and circulating hormones during the midluteal phase of the estrous cycle in pigs. Biology of Reproduction, 7, 543–547.

Guthrie H.D., Garrett W.M. (2000): Changes in porcine oocyte germinal vesicle development as follicles approach preovulatory maturity. Theriogenology, 54, 389–399.

Guthrie H.D., Garrett W.M. (2001): Apoptosis during folliculogenesis in pigs. Reproduction, 58, 17–29.

Guthrie H.D., Grimes R.W., Cooper B.S., Hammond J.M. (1995a): Follicular atresia in pigs: measurement and physiology. Journal of Animal Science, 73, 2834–2844.

Guthrie H.D., Cooper B.S., Welch G.R., Zakaria A.D., Johnson L.A. (1995b): Atresia in follicles grown after ovulation in the pig: measurement of increased apoptosis in granulosa cells and reduced follicular fluid estradiol-17 beta. Biology of Reproduction, 52, 920–927.

Jewgenow K., Heerdegen B., Muller K. (1999): *In vitro* development of individually matured bovine oocytes in relation to follicular wall atresia. Theriogenology, 51, 745–756.

Kanitz W., Brussow K., Becker F., Torner H., Schneider F., Kubelka M., Tomek W. (2001): Comparative aspects of follicular development, follicular and oocyte matu-

ration and ovulation in cattle and pigs. Archiv fur Tierzucht – Archives of Animal Breeding, 44, 9–23.

Knox R. (2005): Recruitment and selection of ovarian follicles for determination of ovulation rate in the pig. Domestic Animal Endocrinology, 29, 385–397.

Krisher R.L. (2004): The effect of oocyte quality on development. Journal of Animal Science, 82, 14–23.

Liu R.H., Li Y.H., Jiao L.H., Wang X.N., Wang H., Wang W.H. (2002): Extracellular factors affecting nuclear and cytoplasmic maturation of porcine oocytes collected from different sizes of follicles. Zygote, 10, 253–260.

Marchal R., Feugang J.M., Perreau C., Venturi E., Terqui M., Mermillod P. (2001): Meiotic and developmental competence of prepubertal and adult swine oocytes. Theriogenology, 56, 17–29.

Marchal R., Vigneron C., Perreau C., Bali-Papp A., Mermillod P. (2002): Effect of follicular size on meiotic and developmental competence of porcine oocytes. Theriogenology, 57, 1523–1532.

Raghu H.M., Nandi S., Reddy S.M. (2002): Follicle size and oocyte diameter in relation to developmental competence of buffalo oocytes *in vitro*. Reproduction, Fertility and Development, 14, 55–61.

Ratky J., Brussow K.P., Hunter M.G. (1995): Endoscopic studies of ovarian follicle development during the estrus cycle in Hungarian large white gilts. Archiv fur Tierzucht – Archives of Animal Breeding, 38, 427–435.

Ratky J., Torner H., Egerszegi I., Schneider F., Sarlos P., Manabe N., Brussow K.P. (2005): Ovarian activity and oocyte development during follicular development in pigs at different reproductive phases estimated by the repeated endoscopic method. The Journal of Reproduction and Development, 51, 109–115.

Salamone D.F., Adama G.P., Mapletoft R.J. (1999): Changes in the cumulus-oocyte complex of subordinate follicles relative to follicular wave status in cattle. Theriogenology, 52, 549–561.

Schnurrbusch U., Schmette C., Elze K. (1990): Recovery and light microscopic evaluation of follicular oocytes of swine and relationship between the degeneration rate of oocytes and the estrus phase. Deutsche Tierarztliche Wochenschrift, 97, 390–395.

Sun Q.Y., Lai L., Bonk A., Prather R.S., Schatten H. (2001): Cytoplasmatic changes in relation to nuclear maturation and early embryo developmental potential of porcine oocytes: effects of gonadotropins, cumulus cells, follicular size, and protein synthesis inhibition. Molecular Reproduction and Development, 59, 192–198.

Vassena R., Mapletoft R.J., Alloodi S., Singh J., Adams G.P. (2003): Morphology and developmental competence of bovine oocytes relative to follicular status. Theriogenology, 60, 923–932.

Yoon K.W., Shin T.Y., Park J.I., Roh S., Lim J.M., Lee B.C., Hwang W.S., Lee E.S. (2000): Development of porcine oocytes from preovulatory follicles of different sizes after maturation in media supplemented with follicular fluids. Reproduction, Fertility and Development, 12, 133–139.

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