Jpn. J. Vet. Res., **38**, 127–137 (1991)

TIMING OF SEQUENTIAL CHANGES IN CHROMOSOME CONFIGURATIONS DURING THE 1st MEIOTIC DIVISION OF PIG OOCYTES CULTURED *IN VITRO*

Marlon B. OCAMPO, Lerma T. C. OCAMPO* and Hiroshi KANAGAWA

(Accepted for publication: November 6, 1990)

This study examines the timing of changes in chromosome configurations of pig oocytes derived from small antral follicles of follicular and/or inactive stage donors using modified 199 medium supplemented with gonadotropins (Follicle Stimulating Hormone (FSH), 10 IU / ml; Human Chorionic Gonadotropin (hCG), 10 IU / ml) and Glucosamine (0.539 mg / ml). Oocytes (n=1,215) were fixed at the end of 3 hourly intervals from 0–48 hr of culture. Results were expressed as the percentage of oocytes at each stage of maturation for each time point. The germinal vesicle (GV) stage was observed for the first 17.6 hr; germinal vesicle breakdown (GVBD) stage between 17.6−26.4 hr; metaphase I (M-I) from 26.4−30.9 hr; anaphase I (A-I) ranged from 30.9−33.4 hr; telophase I (T-I) at 33.4−34.4 hr; and metaphase II (M-II) at 34.4−48 hr.

Key words: 1st meiosis, chromosome, pig oocytes, in vitro

INTRODUCTION

The meiotic competence of mammalian oocytes is associated with the ability to synthesize and activate maturation promoting factor (MPF) molecules. In the pig, the MPF increases gradually during preincubation and by 8–12 hr has reached a level sufficient to overcome maturation inhibiting activity of the growing ooplasm (14). In vivo, a latent period of 4–8 hr is required for the induction of the oocyte maturation process after hCG injection (10). In in vitro, resumption of meiosis as previously reported ranged from 12–20 hr (4, 13, 14).

The timing for completion of the first meiotic division in vitro by pig oocytes as noted by several authors, varies between 26–48 hr (2, 5, 10, 15, 16). However, in vivo, the timing of nuclear maturation to metaphase II appears to be 28–32 hr after the arrival of the maturation signal (10) or 36 hr after hCG injection (7). In this study, we examined the timing of sequential changes in the appearance of the

Department of Theriogenology, Faculty of Veterinary Medicine and * Department of Animal Science, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan

chromatin material during nuclear progression of pig oocytes through meiosis I using maturation conditions known to support normal development with modification (15). Oocytes from the small antral follices of follicular and/or inactive stage donors were selected for this purpose.

MATERIALS AND METHODS

Oocyte preparation and culture conditions. Ovaries from slaughtered gilts or sows were collected and transported to the laboratory (1 hr) in a thermos containing saline solution (0.9 % NaCl, 100 IU / ml penicillin, 100 ug / ml streptomycin) at 35–37°C. The ovaries were rinsed 2–3 times in fresh transport medium prior to oocyte collection. Oocytes were obtained from 2–5 mm follicles using an 18 gauge sterile needle and 10 ml syringe. The follicular fluid aspirates were pooled in a sterile culture dish and allowed to sediment for 10–20 min. The cumulus oocyte complexes (COCs) were recovered and selected according to McGaughey et al., 1979 (9), then washed 3–4 times before culture in 0.2 ml of maturation medium droplets (10–15 COCs) under paraffin oil at 39°C and 5 % CO_2 : 95 % air atmosphere. The maturation medium was M199 (1) supplemented with 10 % fetal bovine serum (FBS ; Sigma, Cell Culture Lab., USA), gonadotropins (FSH, 10 IU / ml : Antrin, Denkaseiyaku Co. Ltd., Japan ; hCG, 10 IU / ml : Gonatropin, Teikokuzoki, Japan) and glucosamine (0.539 mg / ml : Sigma, Cell Culture Lab., USA). The pH of the medium after equilibration with 5 % CO_2 in air was 7.4. The time that elapsed between aspiration and culture was approximately 1 hr.

Experimental design. The COCs were cultured for 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45 and/or 48 hr. At the end of culture for each time point, the adhering cumulus cells were removed mechanically by pipetting into and out of a finely drawn hematocrit tube with an inner diameter similar to that of a nude oocyte. The naked oocytes were then put on slides and covered with a coverslip supported by a vaseline/paraffin mixture. Slides were then fixed with methanol : acetic acid solution (3 : 1) for at least 24 hr, stained with 1 % aceto-orcein solution and examined by phase-contrast and Nomarski's differential interference microscopy. Analysis of their nuclear status was ascribed to one of the following ; GV, containing a single large nucleus with fine filaments of chromatin before gradually condensing to form a ring of condensed chromatin around the compact nucleolus ; GVBD, distinguished by disappearance of compact nucleolus, nuclear membrane and gradual condensation of chromatin material ; metaphase I (M–I), the formation of individual bivalents is completed ; anaphase I (A–I), characterized by the elongation and separation of two chromosome sets towards opposite poles ; telophase I (T–I), separation of the two chromosome sets is completed ; and metaphase II (M–II), emission of first polar body. The results were expressed as the percentage of oocytes at each stage of maturation for each time point.

RESULTS

Table 1 presents the total number of COCs (ranging from 50–102 oocytes) cultured and fixed at each time point from 5 replicates and the corresponding changes in the chromosome configurations throughout maturation. The percentages of oocytes at each time point is represented by the areas under the curves of Fig. 1. The total area, consisting of 6 curves, represents 48 hr and was computed to facilitate the monitoring of changes in the chromosome configurations as shown at the bottom of the

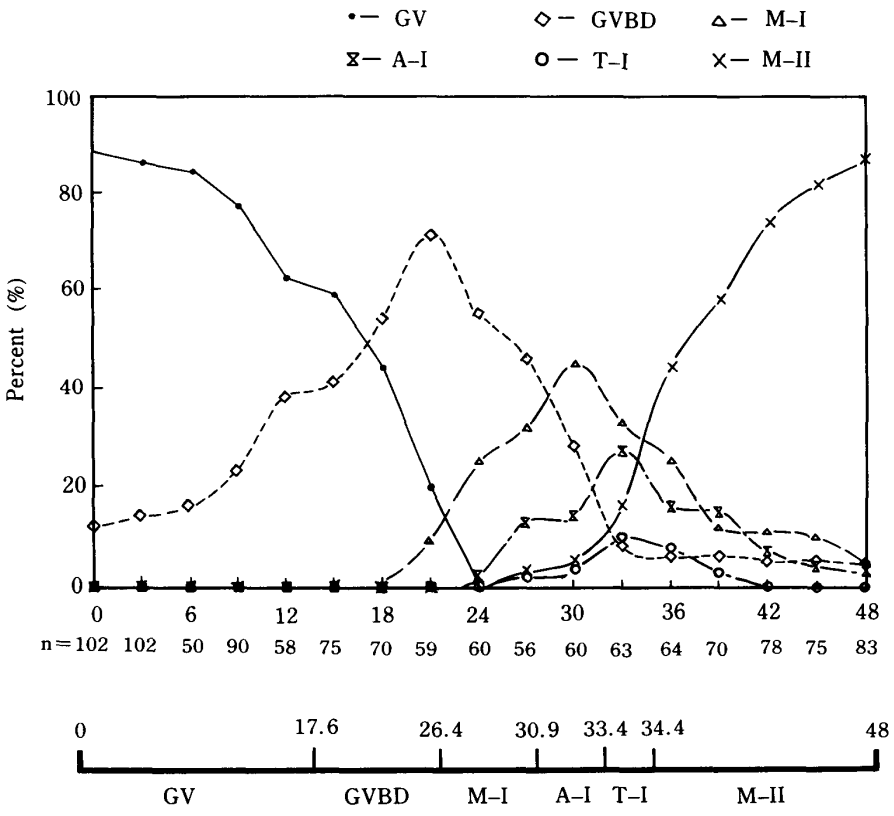


Fig. 1 Changes in the chromosome configurations of pig oocytes under the maturation conditions employed in this study. Six stages were chosen to facilitate the analysis of nuclear status namely; GV, GVBD, M-I, A-I, T-I, and M-II. The total number of samples from 5 replicates fixed at each time point ranged from 50~102 oocytes. The time sequence of various chromosome configurations during the 1st meiotic division is shown at the bottom of the graph. Each stage represents a fraction of 48 hr.

Table 1 Nuclear status of pig follicular oocytes cultured at different times during meiotic maturation

Nuclear status	Time (hr) of culture																
	0	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
GV	90	88	42	69	36	44	31	12	10	—	—	—	—	—	—	—	—
GVBD	12	14	8	21	22	31	38	42	33	26	16	—	—	—	—	—	—
M-I	—	—	—	—	—	—	—	5	15	18	27	21	16	8	11	10	4
A-I	—	—	—	—	—	—	—	—	—	7	6	17	10	10	3	5	2
T-I	—	—	—	—	—	—	—	—	—	1	2	6	5	2	—	—	—
M-II	—	—	—	—	—	—	—	—	—	—	3	10	28	40	54	50	72
Degene-rated	—	—	—	—	—	—	1	—	2	4	6	9	5	10	10	10	5
Total	102	102	50	90	58	75	70	59	60	56	60	63	64	70	78	75	83

(—) ··· No oocytes at this stage was observed at this particular time

graph. The area under each curve was expressed as a fraction of 48 hr allowing an estimate of the mean time that oocytes spent at each nuclear status. This varies between individual oocytes including those that did not reach M–II during the 48 hr period of incubation (3.6 %). The oocytes found degenerated were excluded from the results and represents only a small fraction at each time point (5.0 %).

In some instances, the nuclear membrane cannot be seen, so the assignment of the chromosome configurations to a specific stage was based on the appearance of the chromatin material. The control consisted of 102 oocytes fixed and stained immediately after recovery. In 88 % of these oocytes, the normal configurations of GV stage were noted for the first 17.6 hr (Fig. 3A, B), whereas GVBD combined with chromosome condensation stage (Fig. 3C) were observed between 17.6–26.4 hr. This represents the transition period between dictyate and diakinesis. M–I (Fig. 3D) occurred between 26.4–30.9 hr. A–I (Fig. 3E) and T–I (Fig. 3F) were noted only at intervals between 30.9–33.4 hr and 33.4–34.4 hr, respectively, indicating a short period of these stages. M–II (Fig. 3G) was the predominant stage between 34.4–48 hr.

Discussion

The present results demonstrate the effectiveness of choosing compact COCs with evenly granulated ooplasm for in vitro maturation studies as evidenced by the absence of any later stages in the control group (0 hr) fixed soon after collection. The maturation conditions employed here also proved to support resumption of meiosis, i. e., undergoing GVBD and progresses to M–II. The use of more time points at 3 hr intervals during the complete period of pig meiotic maturation allowed a more precise estimate of changes in chromosome configurations and the extent of time spent at each stage. Further, it should be emphasized that only the timing of changes in the chromosome configurations up to emission of the first polar body under the maturation conditions employed here were studied.

Earlier reports on the time sequence of GVBD in the pig revealed that oocytes cultured for 20 hr in medium with 10 ug/ml cycloheximide remained in GV stage though with a highly condensed bivalents in nucleoplasm (6). In this study, GV stage was observed for the first 17.6 hr of culture before meiotic resumption, resulting in dissolution of compact nucleolus and nuclear membrane (GVBD) lasting up to 26.4 hr. The time at which this stage occurred complements those of Motlik and Fulka, 1976 (16–20 hr) (13) and Motlik et al., 1984 (24 hr) (12) when pig oocytes approach their full size in antral follicles (>0.8 mm) and before they are capable of completing it (i. e. reaching M–II). GVBD in pig coincides with the appearance of active MFP molecules in the ooplasm. Fusion studies have demonstrated that MPF increases gradually during preincubation and reached a level enough to overcome maturation inhibiting activity of growing ooplasm by 8–12 hr (14). Data by Fulka et al., 1986 (4)

on the sensitive period of pig oocytes to cycloheximide support this claim. They concluded that proteins important for GVBD of pig oocytes were present in sufficient amounts at 12 hr of culture, a difference of about 5–14 hr compared to our observation. This difference has been correlated to the size of antral follicles used (FULKA et al., 1986; 5 mm vs OCAMPO et al., 1990; 2–5 mm). Furthermore, oocytes from follicles >1 mm in diameter acquired the competence to resume meiotic maturation in vitro but only oocytes from follicles of about 2 mm in diameter completed the 1st meiotic division in vitro. This was synchronously associated with a decrease in the nucleolar transcriptional activity of the oocytes (12). This suggests that the ability to undergo GVBD and continue to M–I is acquired earlier during folliculogenesis than the ability to reach M–II (18).

Within the follicle, granulosa cells are responsible for the maintenance of the oocytes in the dictyate stage (18), whereas continuous protein synthesis is necessary to maintain M–I and prematurely condensed chromosomes in a typical configurations in vitro. The timing of the existence of this particular stage however, has not been clarified. In the current study, the M–I stage was observed between 26.4–30.9 hr. Subsequently, A–I and T–I were seen occasionally indicating that the separation of two chromosome sets, polar body extrusion and reorganization of the remaining chromosomes for the second meiotic division are quickly accomplished. Similar findings on the brief completion of A–I to T–I stage were reported in bovine oocytes (17).

In vivo, the completion of nuclear maturation is achieved 36 hr after hCG injection (7) or 28–32 hr after the onset of the maturation signal (10). In vitro, subject to different culture conditions, the completion of the 1st meiotic division varies greatly (3, 8, 11, 19). Relative to our previous findings on pig oocytes maturation in vitro (15), the emission of the first polar body is attained between 34.4–48 hr of culture closely resembling the in vivo condition after hCG injection. Unpublished results on in vitro fertilization (IVF) of in vitro matured pig oocytes, under similar conditions and using ejaculated spermatozoa, showed that cytoplasmic maturation is acquired from 36 hr of culture as evidenced by male pronucleus formation. Thus full cytoplasmic maturation is tantamount to successful fertilization and embryonic development.

Collectively, these findings clarified the mean time spent by each phase of 1st meiotic division of pig oocytes in vitro. Furthermore, our data show the possibilities of resolving the factors influencing meiotic competence, molecular and cellular changes which are associated with maturation of pig oocytes in vitro. The effect of individual factors (FSH, hCG, glucosamine) on IVF studies of pig oocytes matured in vitro is currently being investigated.

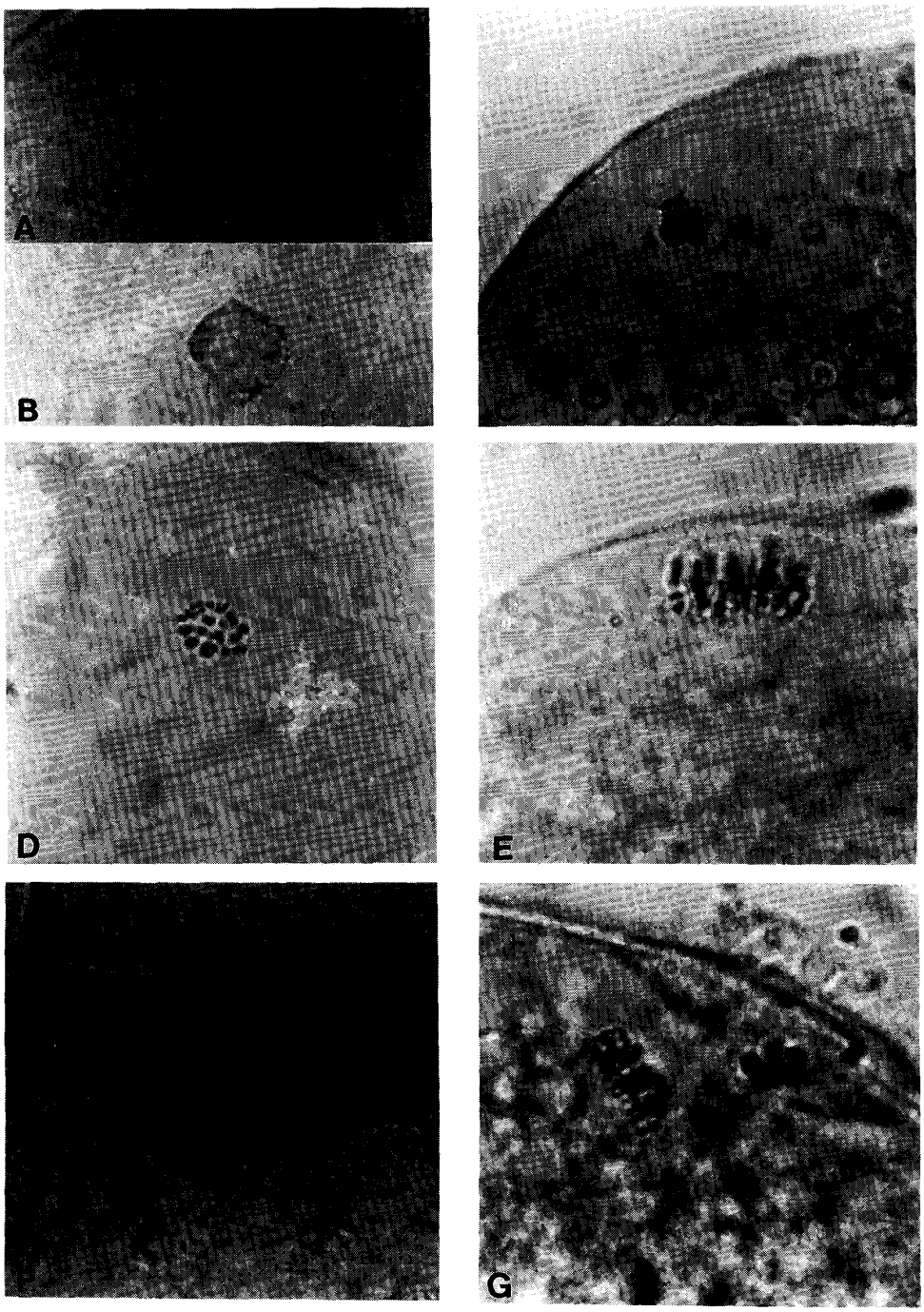
REFERENCES

1) CHENG, W. T. K. (1985): In vitro fertilization of farm animal oocytes. Ph. D. Thesis. Council for Nation Academic Awards, England
2) EDWARDS, R. G. (1965): Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. Nature., **208**, 349–351
3) FOOTE, W. D. & THIBAULT, C. (1969): Recherches experimentales sur la maturation in vitro des ovocytes de truie et veau. Ann. Biol. Anim. Bioch. Biophys., **9**, 329–349
4) FULKA, J. Jr., MOTLIK, J., FULKA, J. & JILEK, F. (1986): Effect of cycloheximide on nuclear maturation of pig and mouse oocytes. J. Reprod. Fert., **77**, 281–285
5) KAMEYAMA, Y. & ISHIJIMA, Y. (1989): In vitro maturation of follicular oocytes obtained from pig ovaries at a slaughterhouse. Jpn. J. Fert. Steril., **34**, 751–756
6) KUBELKA, M., MOTLIK, J., FULKA, J. Jr., PROCHAZKA, R., RIMKEVICOVA, Z. & FULKA, J. (1988): Time sequence of germinal vesicle breakdown in pig oocytes after cycloheximide and P-Aminobenzamidine block. Gamete Res., **19**, 423–431
7) HUNTER, R. K. & POLGE, C. (1966): Maturation of follicular oocytes in the pig after injection of hCG. J. Reprod. Fert., **12**, 525–531
8) McGAUGHEY, R. W. (1977): The culture of pig oocytes in minimal media and the influence of progesterone and estradiol–17B on meiotic maturation. Endocrinology, **100**, 39–45
9) McGAUGHEY, R. W., MONTGOMERY, D. H. & RICHTER, J. D. (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
10) MEINECKE, B. & MEINECKE-TILLMAN, S. (1979): Effects of gonadotropins on oocyte maturation and progesterone production by porcine ovarian follicles cultured in vitro. Theriogenology, **11**, 351–365
11) MINATO, Y. & TOYODA, Y. (1982): Induction of cumulus expansion and maturation division of porcine oocytes cumulus complexes in vitro. Jpn. J. Zootech. Sci., **53**, 480–487
12) MOTLIK, J., CROZET, J. (1984): Meiotic competence in vitro of pig oocytes isolated from early antral follicles. J. Reprod. Fert., **72**, 323–328
13) MOTLIK, J. & FULKA, J. (1976): Breakdown of GV in pig oocytes in vivo and in vitro. J. Exp. Zool., **198**, 155–162
14) MOTLIK, J. & FULKA, J. (1986): Factors affecting meiotic competence in pig oocytes. Theriogenology, **25**, 87–96
15) OCAMPO, M. B., OCAMPO, L. T. C., HISHINUMA, M., TAKAHASHI, Y. & KANAGAWA, H. (1990): in prep.
16) SATO, E., IRITANI, A. & NISHIKAWA, Y. (1978): Rate of maturation division of pig follicular oocytes cultured in vitro. Jpn. J. Zootech. Sci., **49**, 400–405
17) SUSS, U., WUTHRICH, K. & STRANZINGER, G. (1988): Chromosome configurations and time sequence of the 1st meiotic division in bovine oocytes matured in vitro. Biol.

Reprod., **38**, 871–880

18) TSAFRIRI, A. & CHANNING, C. P. (1975) : Influence of follicular maturation and culture conditions on the meiosis of pig oocytes in vitro. J. Reprod. Fert., **43**, 149–152

19) YOSHIDA, M., BAMBA, K. & KOJIMA, Y. (1989) : Effects of gonadotropins and estradiol 17–B on the timing of nuclear maturation and CME in pig oocytes cultured in vitro. Jpn. J. Anim. Reprod., **35**, 86–91


A
B
D
E
G

EXPLANATION OF PLATES

Fig. 2 Chromosome configurations of pig oocytes 1st meiotic division cultured in vitro in the presence of FSH (10 IU / ml), hCG (10 IU / ml) and glucosamine (0.539 mg / ml).

A) GV with very fine filaments of chromatin fixed soon after collection. The nucleolus is out of focus. 200X

B) GV of an oocyte cultured for 12 hr. The compact nucleolus is surrounded by a ring of heterochromatin. 200×

C) Oocyte at early dictyate stage fixed after 24 hr of culture with a visible condensed bivalents. 200×

D) Oocyte at M–I stage in which the formation of condensed bivalents is completed. Polar view with individual bivalents seen clearly in the plane of focus. 200×

E) Anaphase I is distinguished by the separation of two homologous chromosome sets. 500×

F) Telophase I, separation of two chromosome sets is completed. 200×

G) Metaphase II, with emission of the 1st polar body. 500×