CHAPTER 4

Effect of follicle-stimulating hormone on nuclear and cytoplasmic maturation of sow oocytes in vitro

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Abstract

A series of experiments were conducted to evaluate the effects of FSH supplementation during IVM on porcine oocyte nuclear maturation, and subsequent fertilization, cleavage and embryo development. Cumulus-oocyte-complexes (COCs) were cultured 40 h without FSH (control), 40 h with FSH (FSH 0-40 h), or 20 h with FSH followed by a 20 h culture period without FSH (FSH 0- 20 h). Nuclear stage of oocytes was assessed at intervals from 12 to 40 h of IVM. Furthermore, oocytes were in vitro fertilized, fixed and stained to determine normally fertilized and polyspermic oocytes. Additionally, COCs were matured with FSH, fertilized and zygotes cultured in NCSU-23. The percentage of cleaved embryos and blastocysts were determined and the number of nuclei was counted. The presence of FSH during the first 20 h of IVM retarded germinal vesicle breakdown. After 40 h of culture 84, 67 and 58% MII oocytes were observed in the FSH 0-20 h, FSH 0-40 h and control groups, respectively. After IVF, penetration rates were similar at 27, 26 and 29%, while the proportion of polyspermic oocytes was 7, 19 and 11% of penetrated oocytes for control, FSH 0-40 and FSH 0-20 h groups, respectively. Cleavage and blastocyst rates differed among treatments (21, 29 and 38%, and 7, 15 and 20% for control, FSH 0-40 and FSH 0-20 h groups, respectively). No differences in blastocyst cell number were found among groups. Blastocyst rates, based on number of cleaved embryos, were 51 and 52% for the FSH 0-40 and FSH 0-20 h groups, which differed significantly from the control group (31%). The results indicate that FSH has a stimulatory effect on nuclear and cytoplasmic maturation of sow oocytes. Addition of FSH for the first 20 h of culture was most beneficial, based on cleavage and blastocyst development rates.

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Introduction

Oocytes are arrested in the diplotene stage of the first meiotic division and resume meiosis immediately before ovulation. During this period of maturation, the oocyte changes from a developmentally incompetent cell to one with the capacity to direct and support the events of fertilization and early embryonic development. In the same period, cumulus cells lose contact with the oocyte and intercellular communication between these cells undergoes a progressive reduction. When oocytes from small and medium sized follicles are removed from their follicular environment and matured in vitro, spontaneous nuclear maturation does occur; only a low proportion of oocytes develop to blastocysts following IVF and early culture in vitro. These deficiencies may be attributed to abnormalities in cytoplasmic maturation, even though apparently normal nuclear maturation is observed. To increase the developmental ability of in vitro matured oocytes, gonadotropic hormones are added to the maturation medium. Although most in vitro maturation protocols currently utilize LH, FSH, or a combination of both, the effect of gonadotrophins on IVM and subsequent fertilization and early embryo development is still controversial [1].

Exposure of porcine cumulus-oocyte-complexes (COCs) to a combination of eCG, hCG and porcine follicular fluid (pFF) [2] or to eCG and pFF [3] during the first half of the maturation period increases male pronuclear formation following IVF. Addition of FSH during in vitro maturation increases the proportion of metaphase II (MII) pig oocytes [4-7]. Rath et al. [8] observed an increase of MII-stage oocytes only when FSH together with pFF was added to the maturation medium, while Bing et al. [7] reported that FSH affects nuclear progression only when cysteamine is added to the maturation medium. Maturation in the presence of FSH has no effect on male pronuclei formation following fertilization of in vitro matured pig oocytes [4,8,9]. However, the positive effect of pFF in the maturation medium on male pronuclei formation [9] or cleavage rate [8] is further enhanced in the presence of FSH. Singh et al. [5] observed a significant increase in the proportion of polyspermic oocytes when maturation medium was supplemented with FSH. It remains to be investigated whether FSH is required for only the first half or the whole maturation period. Moreover, results from the studies mentioned above [4-8] were obtained by using an IVM system where fetal calf serum (FCS) was added to the maturation medium. Naito et al. [9] reported that FCS at concentrations higher than 1% interferes with the effect of FSH with the progression of meiosis. Hence, the present study was undertaken to investigate the effect of duration of a physiological concentration of recombinant FSH, supplemented to a chemically defined maturation medium, both on the progression of meiosis of porcine oocytes and on the developmental competence of the matured oocytes following IVF.

Material and methods

Culture media

All chemicals for the preparation of culture media were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. The basic medium for IVM (OMM) was M199 (Gibco BRL, Paisley, UK) supplemented with 2.2 mg/ml $NaHCO_3$, 0.1% polyvinyl alcohol (PVA) and 100 µM cysteamine [10,11]. Depending on the treatment, recombinant human FSH (hFSH)-Org 32489 (Organon, Oss, The Netherlands) was added

to a final concentration of 0.05 IU/ml. The IVF medium was modified Tris-buffered medium [12] containing 1 mM caffeine and 0.1% (w/v) BSA (Fraction V, fatty acid free). For washing of the presumptive zygotes, IVC-wash medium, NCSU-23 [13] supplemented with 0.4% BSA and 20 mM HEPES, was used. The IVC medium was NCSU-23 supplemented with 0.4% BSA. All media were pregassed in a CO_2 incubator for at least 2 h before use.

Selection and culture of COCs

Ovaries were collected from adult sows at a local slaughterhouse, and were transported to the laboratory within 2 h in a thermoflask. After dissection of the surrounding tissue, the ovaries were flushed under running tapwater and kept at 30°C in saline supplemented with penicillin and streptomycin. COCs were aspirated from 2 to 6 mm follicles using an 18 g winged infusion set needle attached to a 50 ml polystyrene conical tube by means of a suction pump under pressure. The follicular contents were allowed to sediment and washed three times with a Tyrode's lactate-HEPES medium [14] supplemented with 0.1% (w/v) PVA. Oocytes surrounded by a compact cumulus cell mass were collected in HEPES-buffered M199 (Gibco BRL, Paisley, UK) supplemented with penicillin and streptomycin and washed three times in OMM.

IVM, IVF and IVC

In vitro maturation, IVF and IVC took place at 38.5°C in a humidified atmosphere of 5% CO_2 in air. After selection, 35-50 COCs were transferred into a four-well culture dish (Falcon, Becton Dickinson, UK) containing 500 µl of OMM, which had been previously covered with warm mineral oil and equilibrated. During IVM, COCs were cultured for a 40 h period in OMM with or without hFSH. After culture, cumulus cells were removed by repeated pipetting, and the denuded oocytes were washed twice in IVF medium. Thirty-five to 50 oocytes were placed in 50 µl drops of IVF medium previously covered with mineral oil and equilibrated. Fresh extended (BTS extender) semen from two boars, irrespective of breed, was obtained from the local AI station. Five-hundred microliters of semen from each boar was pooled together in a 15 ml polystyrene conical tube (Greiner, Frickenhausen, Germany) and 2 ml of equilibrated IVF-medium was added. After centrifuging at 700 x *g* for 5 min at 25°C, the supernatant was removed and the sperm pellet was resuspended in 1 ml IVF-medium. This procedure was then repeated. The concentration was adjusted to 10^6 motile sperm/ml, and the sperm suspension warmed to 39°C before being adding to the IVF drops. For fertilization, 50 µl of the diluted sperm suspension were added to each 50 µl drop containing the oocytes, giving a final sperm concentration of $5x10^5$ sperm/ml. The sperm and oocytes were co-incubated for 24 h. The presumptive zygotes were then washed in IVC-wash medium, transferred to 500 µl IVC medium covered with mineral oil, and cultured in groups of 35-50 for 6 days.

Experiments

Four experiments were performed to investigate the effect of FSH during IVM on nuclear progression, fertilization, and embryo development.

Experiment 1

During IVM, COCs were cultured for 40 h in OMM without hFSH (control group), for 40 h in OMM with hFSH (FSH 0-40 h group), or for 20 h in OMM with hFSH, followed by 20 h in OMM without hFSH (FSH 0-20 h group). Oocytes from all treatment groups were washed three times after the first 20 h maturation period, with medium used in the second maturation period. At termination of IVM, the percentage of oocytes at germinal vesicle (GV), metaphase I (MI), and MII stage was assessed, and the expansion of the cumulus cells was observed. The experiment consisted of eight replicates.

Experiment 2

COCs were cultured for 20, 27, 33 and 40 h using the same treatments as described in Experiment 1. At each time point, the percentage of oocytes at GV, MI, and MII stages was determined to assess nuclear progression. The experiment consisted of four replicates. Additionally, an experiment was performed in which COCs were cultured under the same conditions for 0, 12, 16 and 20 h with or without FSH. This additional experiment consisted of three replicates.

Experiment 3

COCs were cultured for 40 h using the same hFSH treatments as described for Experiment 1, then fertilized in vitro. Twenty-four hours after the onset of IVF, the nuclear stage of oocytes was assessed. The experiment consisted of four replicates.

Experiment 4

COCs were cultured for 40 h with the same hFSH treatments as described for Experiment 1, then fertilized in vitro, and placed into culture for 6 days in IVC medium. Two and 7 days after onset of IVF the presumptive zygotes were examined for cleavage and blastocyst formation, respectively. At the end of the culture period embryos were fixed, stained with 4,6-diamino-2-phenyl-indole (DAPI), and the number of nuclei in each blastocyst was counted. The experiment consisted of six replicates.

Assessment of nuclear maturation and fertilization

The nuclear status of the oocytes was determined by DAPI staining. The oocytes were fixed in phosphate buffered saline (PBS) with 2% (w/v) paraformaldehyde, washed with PBS, stained with 2.5% (w/v) DAPI, and mounted on slides [15]. The nuclear state of the stained oocytes was assessed under a fluorescence microscope. Oocytes in which diffuse or slightly condensed chromatin could be identified were classified as being in the GV stage. Oocytes that possessed clumped or strongly condensed chromatin which formed an irregular network of individual bivalents (prometaphase) or a metaphase plate but no polar body were classified as being in the MI stage. Oocytes with either a polar body or two bright chromatin spots were classified as being in the MII stage. With respect to fertilization, oocytes with two pronuclei or with one pronucleus together with one decondensed sperm head, or cleaved embryos with two to four normal blastomeres were considered normally fertilized. Oocytes with more than two pronuclei or more than one

penetrated sperm head were considered as polyspermic, and oocytes without penetrated sperm heads were considered as unfertilized.

Statistical analysis

Statistical analysis was conducted with SPSS software (SPSS, IL, USA) using an analysis of logistic regression [16] following a binomial distribution. The data from Experiments 1, 3 and 4 were analyzed by the model: $\ln \pi/(1-\pi) = \alpha$ + treatment, and data from Experiment 2 were analyzed following the model: $\ln \pi/(1-\pi) = \alpha$ + treatment + time, where π = frequency of positive outcome, and α the intercept. Treatment and time are independent categorical variables in these models. Data from blastocyst cell numbers were analyzed by Student's t-test.

Table 1. Effect of duration of hFSH exposure on nuclear maturation rates of porcine oocytes after 40 h of culture (Experiment 1)

Treatment	Total oocytes N[1]	Stage of oocytes % (mean ± S.E.M.)			
		GV	MI	MII	Degenerated
Control (no FSH)	640	18 ± 3^{a}	12 ± 2^{a}	58 ± 5^{a}	$11 \pm 2^{a,b}$
FSH 0-40 h	642	12 ± 3^{b}	$9 \pm 1^{a,b}$	67 ± 4^{b}	12 ± 2^{a}
FSH 0-20 h	611	3 ± 2^{c}	5 ± 2^{b}	84 ± 4^{c}	8 ± 2^{b}

Within columns, values with different superscripts (a, b, c) are significantly different ($P<0.05$); [1]The experiment was replicated eight times.

Results

Effect of FSH on nuclear maturation and cumulus cell expansion (Experiment 1).

The effect of FSH supplementation on nuclear progression of porcine oocytes after 40 h of IVM, is shown in Table 1. Both FSH (0-20 and 0-40 h) treatments decreased the percentage of GV-stage oocytes ($P=0.0016$ and <0.0001, respectively) and increased the percentage of MII-stage oocytes ($P=0.0039$ and <0.0001, respectively), when compared to the control group. Exposure of COCs to FSH for only the first 20 h of IVM resulted in more MII-stage oocytes at the end of culture, than exposure to FSH for the entire 40-h culture period ($P< 0:0001$), and resulted in fewer GV-stage oocytes than either of the other treatments. Images of COCs at the start of culture, and after 40 h of culture with or without FSH are shown in Figure 1. After IVM, COCs in the control group were clumped together, and cumulus cells appeared black and shrunken (Fig. 1b), while COCs in both FSH groups showed expanded cumulus (Fig. 1c).

Effect of FSH on the kinetics of nuclear maturation (Experiment 2)

At the start of culture, oocytes in both groups were in the GV stage. The effect of FSH on the kinetics of nuclear progression from 12 to 20 h of culture is presented in Table 2. Over time, a decrease in the percentage of GV-stage oocytes occurred in the control

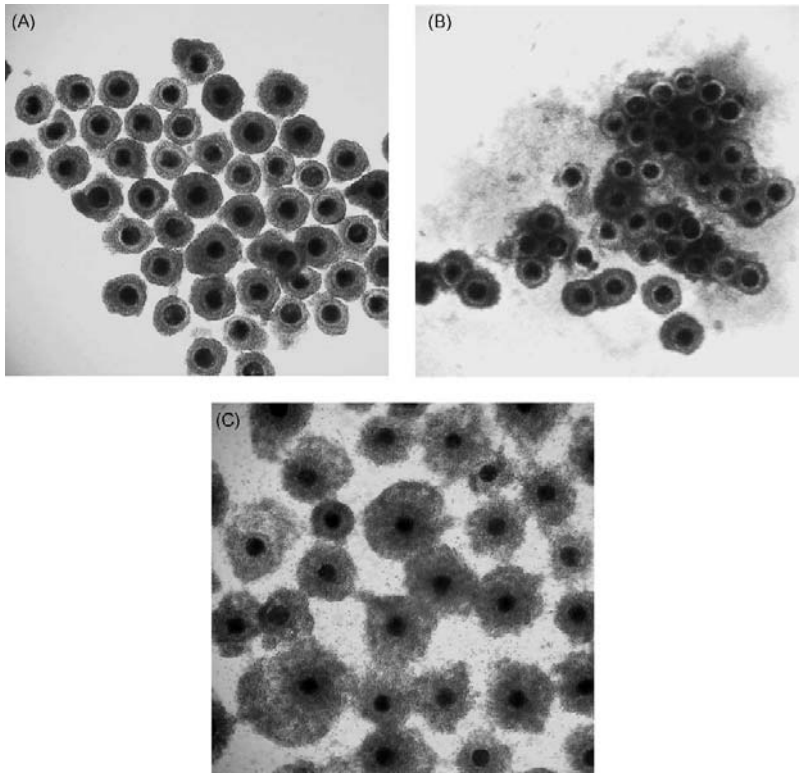

Figure 1. Cultured porcine COCs at the onset of culture (A), after 40 h of culture in maturation medium without FSH (B) and with 0.05 IU FSH/ml (C) (magnification: 30x).

Table 2. Effect of hFSH on kinetics of nuclear progression of porcine oocytes after 12, 16 and 20 h of culture (Experiment 2)

Treatment	Time of culture (h)	Total oocytes N[1]	Stage of oocytes % (mean ± S.E.M.)			
			GV	MI	MII	Degenerated
Control (no FSH)	12	158	75 ± 3^{a}	20 ± 4^{a}	1 ± 1^{a}	4 ± 2^{a}
	16	146	61 ± 3^{b}	29 ± 1^{a}	6 ± 4^{a}	5 ± 3^{a}
	20	131	49 ± 5^{c}	34 ± 7^{b}	12 ± 4^{b}	5 ± 1^{a}
FSH	12	134	85 ± 3^{a}	7 ± 1^{a}	3 ± 2^{a}	5 ± 3^{a}
	16	143	80 ± 5^{a}	10 ± 1^{a}	3 ± 2^{a}	7 ± 2^{a}
	20	129	75 ± 2^{a}	12 ± 4^{a}	2 ± 2^{a}	10 ± 2^{a}

Within the same column, values with different superscripts (a, b, c) are significantly different ($P<0.05$).
[1]The experiment was replicated three times.

group (P< 0.0001), while no significant decrease of GV-stage oocytes was observed in the group exposed to FSH (P=0.1157). The effect of FSH on the kinetics of nuclear progression stages during 20-40 h of culture is depicted in Fig. 2. At 20 h of culture fewer GV-stage oocytes (P=0.0022) were seen in the control group than in either of the FSH groups. In the culture period from 20 to 27 h, the percentage of GV-stage oocytes in the FSH 0-20 h group decreased more rapidly (P=0.0005) than was observed in the control or the FSH 0-40 h groups. The decrease of GV-stage oocytes in the control group was similar to that in the FSH 0-40 h group. No differences were observed among the treatment groups in formation of MII-stage oocytes until 33 h of culture. At the end of culture, more (P=0.0001) MII-stage oocytes were present in the FSH 0-20 h group than the control or FSH 0-40 h groups. The percentages of MII-stage oocytes were 60±8%, 66±5% and 85±3% in the control, FSH 0-40 and FSH 0-20 h groups, respectively.

Effect FSH during maturation on fertilization (Experiment 3)

The effect of FSH during maturation on IVF is presented in Table 3. The penetration rates in all treatment groups were similar, with the proportion of fertilized oocytes ranging from 26 to 29%. More polyspermic oocytes were found in the FSH 0-40 h group than in the control group (P=0.0137). The number of degenerated oocytes in the control group was higher (P=0.0044) than in the FSH 0-40 h group, but similar to the FSH 0-20 h group.

Effect of FSH during maturation on embryo development (Experiment 4)

The effects of FSH during maturation on subsequent cleavage and embryo development rates are presented in Table 4. Cleavage rates were higher for the FSH 0-20 and FSH 0-40 h groups than the controls (P=0.0001 and 0.0042, respectively). Also, cleavage rate was higher for the FSH 0-20 h group than the FSH 0-40 h group (P=0.0002). At 2 days after the onset of fertilization, the majority of cleaved embryos in all groups were at the four-to eight-cell stage and granulation in the cytoplasm appeared in the center of the blastomeres. Both FSH groups had a higher number of blastocysts than the control group (P<0.0001), while more blastocysts were found in the FSH 0-20 h group than in the FSH 0-40 h group (P=0.0066). Across treatment groups, the mean number of cells per blastocyst was similar and ranged from 20 to 70 cells per embryo.

Table 3. Effect of presence of hFSH during in vitro maturation of porcine oocytes on in vitro fertilization as assessed 24 h after the onset of IVF (Experiment 3)

Treatment	Oocytes in IVF N[1]	Fertilized oocytes % (mean ± S.E.M.)			Unfertilized oocytes % (mean ± S.E.M.)			
		Total	Normally fertilized[2]	Poly-spermic[2]	GV	MI	MII	Degenerated
Control (no FSH)	433	29±8[a]	93±2[a]	7±2[a]	14±4[a]	4±1[a]	42±0[a]	12±3[a]
FSH 0-40 h	502	26±10[a]	82±5[b]	19±5[b]	0±0[b]	1±1[b]	66±11[b]	6±2[b]
FSH 0-20 h	489	27±8[a]	89±4[a,b]	11±4[a,b]	2±0[b]	2±1[b]	61±9[b]	8±2[a,b]

Within columns, values with different superscripts (a, b) are significantly different (P<0.05). [1]The experiment was replicated four times. [2]Percentage of oocytes penetrated.

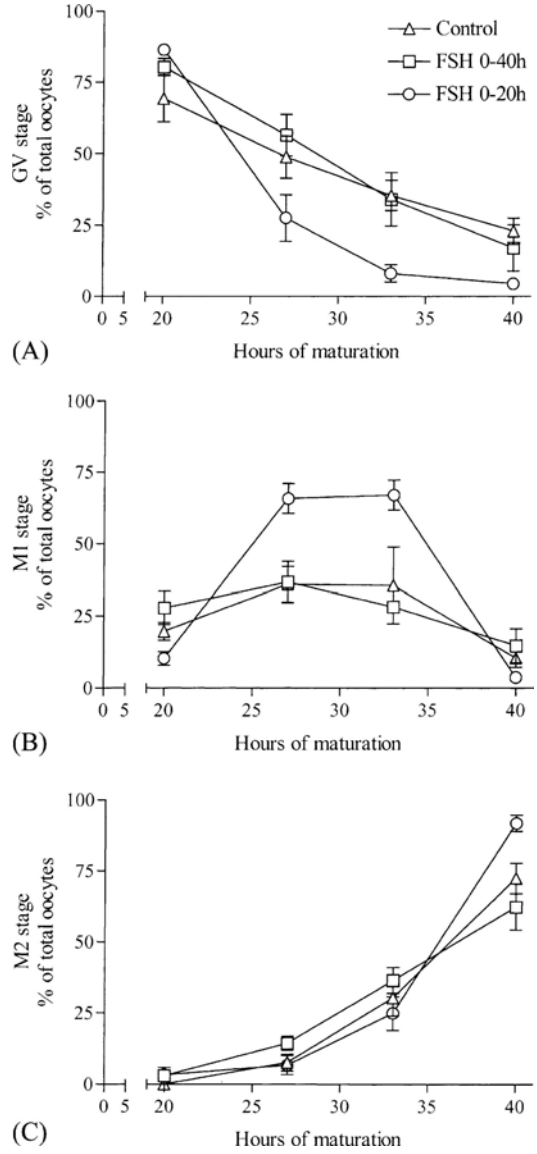

Figure 2. Kinetics of meiotic progression in porcine oocytes from 20 to 40 h of culture (Experiment 2). Nuclear stages of oocytes were scored as GV(A), MI (B), or MII (C). Sow COCs were cultured for 40 h in maturation medium without FSH (control; triangles) or with FSH (0.05 IU/ml) (FSH 0-40 h; squares), or cultured for 20 h in maturation medium with FSH (0.05 IU/ml) followed by culture in maturation medium without FSH (FSH 0-20 h; circles).

Table 4. Effect of the presence of FSH during IVM of porcine oocytes on the cleavage rate and blastocyst formation following IVF (Experiment 4)

Group	Oocytes N[1]	Cleaved embryos at 2 days % (mean ± S.E.M.)	Blastocysts at 7 days %(mean ± S.E.M.)	Cells per blastocyst (mean ± S.E.M.)
Control (no FSH)	815	21 ± 3[a]	7 ± 2[a]	37 ± 2[a]
FSH 0-40 h	711	29 ± 5[b]	15 ± 3[b]	38 ± 1[a]
FSH 0-20 h	727	38 ± 5[c]	20 ± 4[c]	38 ± 1[a]

Within columns, values with different superscripts (a, b, c) are significantly different (P < 0:05). [1]The experiment was replicated six times.

Discussion

The data presented clearly demonstrate that IVM of sow oocytes in the presence of FSH not only enhances nuclear maturation but also improves the developmental capacity of the oocytes as reflected by the enhanced rate of blastocyst formation. The presence of FSH during the first half or the entire culture period enhanced MII formation and induced cumulus cell expansion. Similar effects on nuclear progression [4-7] and on cumulus cell expansion [6,17-19] after exposure of pig COCs to FSH during the whole maturation period have been reported. Generally, we observed a higher proportion of oocytes that progressed to the MII stage in the absence of FSH as compared to the aforementioned studies. This might be due to the use of sow oocytes instead of oocytes from prepubertal gilts. Recently, it has been reported that oocytes from sows have higher maturation potency than oocytes from prepubertal gilts [20]. Our data on the kinetics of meiosis showed that addition of FSH retards GVBD, with the most apparent effect occurring during the first 20 h of maturation.

Thereafter, retardation by FSH has almost disappeared. Remarkably, omission of FSH after 20 h resulted in an acceleration of the progression of meiosis, resulting in more oocytes that reached the MII stage by 40 h. Presence of eCG and hCG during the first 20 h, or during the entire maturation period of 40 h for COCs from prepubertal gilts resulted in the same proportion of MII oocytes [2]. Retardation of nuclear maturation by FSH is in agreement with previous studies in bovine [21,22] and rodent [23-25] oocytes showing transient inhibition of maturation by FSH. The mechanism by which FSH induces oocyte maturation is not completely known. However, there are many reports indicating that cumulus cells play a mediating role in the FSH action on oocyte maturation. Recently, it has been shown that bovine oocytes obtained from small and medium sized follicles do not contain mRNA of FSH receptors [26]. In addition, culture of murine denuded oocytes in the presence of FSH [27] did not affect oocyte maturation, indicating that the effect of FSH on meiosis is exerted through cumulus cells. In contrast, Mattioli et al. [4] observed acceleration of nuclear progression when porcine oocytes were cultured in maturation medium supplemented with FCS and FSH.

The proportion of penetrated oocytes and those showing polyspermy were low compared with other studies [3-5,7,8]. It has been reported that oocytes from adult animals showed lower penetration rates, but also lower rates of polyspermy than oocytes from prepubertal gilts [20]. Although polyspermy rates were low, FSH treatment resulted in a higher level of polyspermic oocytes. It is not likely that this is due to an abnormal distribution of cortical granules, as MII-stage oocytes from both control and FSH treated groups showed

a cortical granule distribution nicely underlying the plasma membrane (Schoevers, personal observation).

FSH treatment during half or all of IVM of sow COCs resulted in enhanced cleavage following fertilization, whereas the presence of FSH during the first half of the culture period resulted in the highest yield of blastocysts. The enhanced blastocyst formation may be due to the higher percentage of MII oocytes at the end of culture, although it appeared that only a small percentage of MII oocytes were penetrated following in vitro fertilization. Expression of the blastocyst rate as the ratio of blastocyst and cleaved embryos revealed approximately 30% for the control and approximately 50% for both FSH treatments. Apparently, addition of FSH during the maturation of sow oocytes increases the competence of cleaved embryos to develop to the blastocyst stage, indicating that FSH not only enhances nuclear maturation but also promotes cytoplasmic maturation of sow oocytes. In vitro maturation of cow oocytes in the presence of FSH does not affect the proportion of MII oocytes, but cleavage and blastocyst formation is increased [22]. As the blastocyst to cleaved embryo ratio was similar for both FSH treatments, we may state that exposure of sow COCs to FSH is only necessary for the first 20 h of culture. Further exposure to FSH may have a negative influence on the cytoplasmic maturation of sow oocytes as determined by cleavage and development rates. Using pronucleus formation as parameter for cytoplasmic maturation, Funahashi and Day [2] found higher proportions of oocytes with male and female pronuclei after 20 h of culture with gonadotrophic hormones as compared with culture with hormones throughout the maturation period. Rath et al. [8] did not observe an effect on the blastocyst formation as expressed by the ratio of blastocysts to cleaved embryos following the in vitro maturation of prepubertal gilt oocytes in the presence of FSH. The relative high percentage of blastocysts obtained in our study, as compared to the study of Rath et al. [8], is probably due to the use of sow oocytes. In bovine species it has been shown that oocytes from prepubertal calves have less developmental competence [28,29].

In conclusion, our results indicate that FSH initially retards GV breakdown, but overall has a stimulatory effect on the nuclear and cytoplasmic maturation of sow oocytes.

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