BIOLOGY OF REPRODUCTION **72,** 399–406 (2005)
Published online before print 29 September 2004.
DOI 10.1095/biolreprod.104.034553

Dynamic Changes in Meiotic Progression and Improvement of Developmental Competence of Pig Oocytes in Vitro by Follicle-Stimulating Hormone and Cycloheximide[1]

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ABSTRACT

The effects of FSH, LH, and epidermal growth factor (EGF) on the dynamics of nuclear maturation and subsequent embryo development were examined in pig oocytes cultured either conventionally or after preincubation with cycloheximide (CHX). In conventional culture, FSH or EGF significantly increased the rate of attainment of metaphase II (MII) for both gilt (50.0% ± 4.2% and 54.8% ± 4.3%, respectively; control, 5.8% ± 1.8%; $P < 0.001$) and sow (87.6% ± 3.4% and 78.8% ± 3.9%, respectively; control, 7.8% ± 2.5%; $P < 0.001$) oocytes. Gilt oocytes treated with both FSH and EGF showed an additive response (93.7% ± 2.1%). Treatment with LH had no effect. Preincubation with CHX caused the majority (84–100%) of both gilt and sow oocytes to undergo germinal vesicle breakdown. Compared to those treated with LH and/or EGF (both >80%), fewer FSH-treated oocytes reached metaphase I (43.8% ± 5.3%, $P < 0.001$) by 14 h and MII (48.4% ± 5.9%, $P < 0.001$) by 24 h, although the majority (71%) did mature to MII by 36 h after removal of CHX. After in vitro fertilization, higher proportions of both CHX-pretreated and untreated, FSH-exposed oocytes cleaved (71.3% ± 2.9% and 75.3% ± 3.1%, respectively) compared with those not treated with FSH (37.7% ± 3.0% and 43.0% ± 2.9%, respectively; $P < 0.001$). Pretreatment with CHX significantly increased blastocyst yield for both FSH-treated (32.8% ± 2.0% and 10.3% ± 1.5%, respectively; $P < 0.001$) and untreated (16.7% ± 1.5% and 9.4% ± 1.2%, respectively; $P < 0.001$) oocytes. Polyspermy rates were unaffected. In conclusion, pig oocytes meiotically arrested by CHX before maturation retain and improve their developmental competence. FSH stimulates nuclear maturation but slows meiotic progression.

follicle-stimulating hormone, in vitro fertilization, luteinizing hormone, meiosis, oocyte development

INTRODUCTION

Pig oocytes have been successfully matured and fertilized in vitro (IVF) [1], and in vitro culture (IVC) conditions that support development to the blastocyst stage have been established [2]. Recent work has focused on improving the culture conditions for in vitro maturation (IVM) to improve the efficiency of in vitro production (IVP) of embryos. In early studies of pig IVM/IVF, nuclear maturation was achieved, but problems existed with poor male pronuclear (MPN) formation and polyspermic fertilization. This showed that cytoplasmic maturation as well as nuclear maturation was necessary for the IVP of viable embryos. Poor MPN formation is avoided by supplementing the maturation medium with cysteine to increase the concentration of glutathione in matured oocytes, but the problem of polyspermy remains [3, 4]. Further modification of the maturation medium is required to improve oocyte developmental competence and blastocyst yield. Despite numerous attempts, few laboratories achieve rates of blastocyst development greater than 30% [5–7].

A better understanding of the inter- and intracellular processes underlying oocyte maturation is essential to improve pig IVP. Many factors are beneficial for oocyte maturation and subsequent embryo development [8, 9], but their mechanisms of action are poorly understood. In particular, it is not known precisely how FSH and/or LH influence oocyte maturation. These hormones commonly are added to maturation medium, because in vivo, the gonadotropin surge induces oocyte maturation in preovulatory follicles [10]. However, oocytes removed from follicles will mature spontaneously in the absence of FSH and LH [11]. Epidermal growth factor (EGF) has an effect on oocyte IVM similar to that of gonadotropins and may be used in the medium [12], but it may have differential effects on nuclear and cytoplasmic maturation [13]. Previous studies concerning the influence of these treatments on nuclear maturation investigated only the accumulated rate of attainment of metaphase II (MII), ignoring the dynamics of meiotic progression and its relationship to cytoplasmic maturation and developmental competence [14, 15]. In addition, most previous studies used complex maturation media containing follicular cell constituents, follicular fluid or serum, in which unspecified hormones and growth factors may have been present.

It is possible that aspirated oocytes may have already acquired developmental competence, with only limited potential for change during conventional culture. Oocytes from larger follicles usually are more competent than those from smaller ones [16–18], and those from sows develop better than those from prepubertal gilts [19–21]. The heterogeneity of oocytes from different sources leads to asynchronous meiotic progression during IVM, especially because pig oocytes need a longer culture period than those of other species [11, 22–25]. Although oocytes usually are ''fully grown'' in middle- or large-sized follicles, they are less developmentally competent when matured in vitro than when they are matured in vivo [26]. This may be caused by removal of a final growth or maturation phase. Reducing nuclear morphological variation (meiotic synchronization) before maturation, by preincubation without gonadotropins

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Received: 21 July 2004.
First decision: 24 August 2004.
Accepted: 16 September 2004.

ISSN: 0006-3363. http://www.biolreprod.org

[23] or with dibutyryl cAMP (dbcAMP) [22, 27], appears to enhance pig oocyte developmental potential. However, the latter treatment does not improve the development of embryos reconstructed by somatic nuclear transfer [14].

In vivo administration of FSH (''coasting'' for 2 days) and LH before oocyte aspiration by ovum pickup results in approximately 80% of bovine oocytes developing to blastocysts following IVPs [28]. To allow a similar treatment in vitro, meiosis needs to be reversibly arrested [29, 30]. This can be achieved physiologically by coculture with different follicle components, such as theca cells [31], but the identity of the meiosis inhibitor is unknown. Recently, butyrolactone I and roscovitine, which are specific inhibitors of Cdc2 (a universal G_2/M-phase regulator in eukaryotic cells) [32], have been found to arrest meiosis in vitro [7, 29, 33–35]. These drugs reversibly block meiotic resumption and may be used to synchronize subsequent nuclear maturation [36]. However, there is little evidence to suggest any significant improvement in oocyte developmental competence, and to our knowledge, no proof of full-term development in any species has been reported [34, 37].

Protein synthesis is essential for meiotic resumption of oocytes in vitro in the pig, as in some other mammals [38, 39]. Cycloheximide (CHX), a nonspecific protein-synthesis inhibitor, can reversibly block meiotic resumption in porcine and bovine oocytes [38–40]. The CHX-pretreated and matured bovine oocytes can be successfully fertilized [41], and they develop to the blastocyst stage at a rate similar to that of untreated ones [42–44]. The birth of live calves from CHX-pretreated oocytes has confirmed that the effect of CHX truly is reversible [43, 45].

A reliable culture system in a relatively simple, defined maturation medium, together with a highly predictable meiotic progression, is necessary to study the effects of any given factor on oocyte maturation and to assess developmental capacity. We have shown that nuclear maturation of pig oocytes can be efficiently synchronized at the germinal vesicle (GV) stage by pretreatment with CHX [25, 46]. Le Beux et al. [47] have reported that CHX is more effective than butylactone or roscovitine for achieving reversible meiotic arrest of porcine oocytes. However, it remains unknown whether CHX treatment can affect fertilization or subsequent embryo development. The present study examined the effects of FSH, LH, and EGF on the nuclear dynamics of pig oocyte maturation and evaluated the feasibility of producing pig embryos from CHX-pretreated oocytes.

MATERIALS AND METHODS

Culture Media

All chemicals and reagents were from Sigma-Aldrich (Poole, U.K.) unless otherwise stated. The basic defined maturation medium (medium B) was Medium 199 containing Earle salts, 25 mM Hepes and sodium bicarbonate, 3 mM L-glutamine, 0.1% (w/v) BSA, 0.57 mM cysteine, 100 IU/ml of penicillin, and 0.1 mg/ml of streptomycin. The basal medium was supplemented with FSH (porcine, 50 ng/ml; National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK]), LH (porcine, 0.2 μg/ml; NIDDK), or EGF (human recombinant, 10 ng/ml) to formulate media F, L, and E, respectively, or supplemented in combinations to produce media LE, FL, FE, and FLE. The IVF medium was modified Tris-buffered medium (pH 9.9 at 4°C, balanced to pH 7.2 at 39°C in 5% CO_2 for 12–24 h, containing 113 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris [crystallized free base], 11 mM glucose, and 5 mM sodium pyruvate) supplemented with 0.1% (w/v) BSA, 20 μM adenosine (freshly prepared), 0.2 mM reduced glutathione (freshly prepared), and no antibiotics. The basic embryo IVC medium was NCSU 23 (pH 8.3 at 4°C, balanced to pH 7.4 at 39°C in 5% CO_2 for 12–24 h, containing 108.7 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25.1 mM $NaHCO_3$, 5.6 mM glucose, 1.0 mM glutamine, 7.0 mM taurine [freshly prepared], 5.0 mM hypotaurine [freshly prepared], and 0.4% [w/v] BSA) supplemented with 0.2 mM reduced glutathione (freshly prepared), 50 IU/ml of penicillin, and 50 μg/ml of streptomycin. During the first 48 h of culture, glucose in the basic IVC medium was replaced with 4.5 mM sodium lactate (DL-lactic acid) and 0.3 mM sodium pyruvate [4].

IVM of Oocytes and Assessment of Meiotic Stage

Pig ovaries were collected from a local commercial abattoir and transported to the laboratory in a warm flask in PBS (30–35°C) within 3 h of slaughter. Selected ovaries were washed three times in sterile PBS. Follicles (diameter, 3–5 mm) with a translucent appearance and extensive vascularization were aspirated using a 21-gauge needle attached to a 5/10-ml syringe primed with 0.5 ml of Dulbecco PBS (DPBS; Ca^{2+}-free). The fluid was expelled into sterile Petri dishes (diameter, 5 cm) and held at 39°C while being inspected for oocytes. Cumulus-oocyte complexes (COCs), with more than three intact and compact cumulus layers were selected for culture after washing in DPBS supplemented with 0.1% polyvinyl alcohol (PVA) and in maturation media depending on the experiment. Groups of 25–50 COCs were cultured in single dishes (diameter, 35 mm; Nunc, Roskilde, Denmark) at a ratio of one oocyte per 10 μl of maturation medium. To synchronize meiotic maturation, COCs first were preincubated in maturation medium supplemented with 5 μg/ml of CHX for 12 h [25]. The COCs then were thoroughly washed (three times in DPBS with 0.1% PVA and three times in maturation medium without CHX) and further cultured without CHX for various periods. All culture drops containing oocytes were covered with a thin layer of mineral oil pre-equilibrated with medium B and incubated in 5% CO_2 in humidified air at 39°C.

To assess meiotic progression, single dishes of COCs were withdrawn from the incubator and the cumulus cells removed by vortexing for 2–3 min in medium containing 0.1% (w/v) hyaluronidase (type IV), 2 mM EDTA, 125 mM NaCl, 3 mM sodium citrate, and 10 mM Na_2HPO_4. Oocytes were mounted under coverslips on slides and fixed for 48 h with acetic acid:ethanol (1:3 v/v). After staining with 1% lacmoid in 45% acetic acid and destaining with acetoglycerol (20% acetic acid and 20% glycerol), the oocytes were examined under a phase-contrast microscope at 400× magnification. Meiotic stages were classified as GV, GV breakdown (GVBD; diakinesis and prometaphase I), metaphase I (MI), anaphase I/telophase I (AI/TI), and MII as described [25].

IVF, Assessment of Fertilization Parameters, and Embryo Culture

For sperm preparation, fresh extended pig semen (stored for up to 5 days at 21°C; PIC 225 semen, Pig Improvement Company, Oxford, U.K.) was washed twice by centrifugation (5 min, 500 × *g*) in DPBS (Ca^{2+}- and Mg^{2+}-free) supplemented with 0.1% BSA, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. The sperm pellet was then resuspended in IVF medium and the sperm concentration determined by a hemocytometer after dilution in 18% NaCl saline. The sperm suspension (1×10^6 sperm cells/ml) was preincubated for a short period (10 min) at 39°C before coincubation with oocytes. At the end of maturation culture, dishes of oocytes were denuded of cumulus cells by brief vortexing in warm maturation medium (1–2 min), washed in the same medium, and transferred to IVF medium droplets, which were covered with mineral oil and preincubated for 2 h. Before insemination, oocytes were set in IVF-medium droplets (5–10 oocytes in 30 μl of IVF medium; 4–7 drops per dish [diameter, 5 cm]) in the incubator for 20–30 min. An equal volume of sperm suspension (30 μl) was introduced into each droplet, producing a final sperm concentration of 5×10^5 cells/ml. Oocyte-sperm coincubation was carried out for 6–8 h at 39°C under 5% CO_2 in humidified air. After a brief wash in IVC medium, oocytes/putative zygotes were cultured (25–50 in 600 μl of IVC medium) for 2 days in four-well plates (Nunc). Cleaved embryos then were transferred into fresh IVC medium and cultured for a further 4 days. Putative zygotes and embryos were cultured at 39°C in humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. To assess fertilization parameters, oocytes/putative zygotes were fix-stained after IVF using the same procedure as that described above for assessment of the meiotic stage. Oocytes were considered to be penetrated when they had one or more (polyspermic) swollen sperm head(s) or male pronuclei with corresponding sperm tail(s).

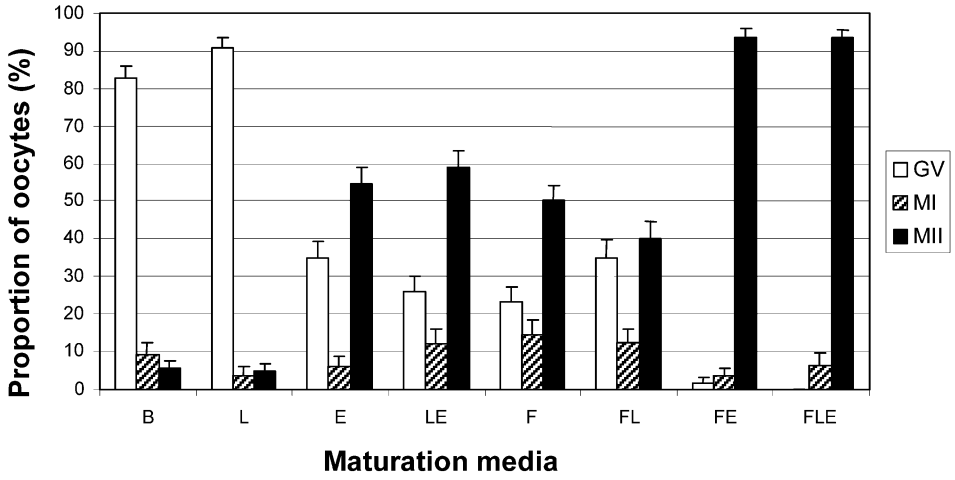

FIG. 1. Effect of hormones on gilt oocyte maturation: proportion of oocytes at the GV, MI, and MII stages following conventional culture for 44–48 h in basal medium (B) or medium supplemented with LH (L), EGF (E), or FSH (F), either alone or in combinations, examined under a phase-contrast microscope at ×400 magnification. Error bars represent the approximate SEMs estimated from a linear model fitted assuming binomial errors. Both EGF and FSH had statistically significant ($P < 0.001$) effects on the proportion of oocytes at the GV and MII stages.

Experiment 1

To determine the effects of FSH (F), LH (L), and EGF (E) on oocyte meiotic maturation and to compare the meiotic competencies of oocytes of different origin, oocytes collected from gilts and sows were cultured separately in the basic (B) and supplemented (F, L, E, LE, FL, FE, and FLE) maturation media. This was a full factorial experiment for three factors (FSH, LH, and EGF), each with two levels (presence or absence). At 44–48 h, all oocytes were fixed for examination of meiotic morphology. Four replicate dishes were used for each treatment combination, with a total of 1746 oocytes examined.

Experiment 2

To determine precisely the effects of hormones on the progression of meiotic maturation, oocytes of gilts and sows were synchronized by preincubation for 12 h with 5 μg/ml of CHX in the basic (B) or supplemented (F, L, E, LE, FL, FE, and FLE) maturation media. Synchronized oocytes then were cultured in the same media without CHX for up to 36 h. At 12, 24, and 36 h, oocytes were fixed for examination of meiotic morphology. Three replicate dishes were used for each treatment at each time point, with a total of 2113 oocytes examined.

Experiment 3

To determine the effects of CHX pretreatment and FSH on oocyte development after fertilization, half of each batch of oocytes were synchronized with CHX for 12 h as in experiment 2 and then matured in medium LE for 26–28 h or FLE for 36 h. The other half were matured conventionally in LE medium for 40–42 h or in FLE medium for 48 h. Culture timings were coordinated so that both sets of oocytes were harvested simultaneously for IVF with the same sperm preparation. The proportions of cleaved oocytes/zygotes were recorded 2 days after IVF. Cleaving embryos were cultured for a further 4 days, and the blastocyst formation rates were recorded. Some matured oocytes from all treatments were overmatured or treated with sperm-free IVF procedures for at least 2 days. Three replicates were performed for each treatment, with a total of 506 oocytes cultured and fertilized.

A number of oocytes matured with or without CHX pretreatment were fertilized using the IVF procedure described above or after various modifications (e.g., to sperm concentration, number of oocytes in fertilization droplet, IVF medium). They were examined for polyspermic fertilization after coincubation with sperm for various periods of 6–22 h. In total, 349 oocytes/zygotes were examined.

Statistical Analysis

The proportions of meiotically responding (maturing or matured) oocytes, fertilized oocytes, and developing embryos out of the known total numbers of oocytes cultured in single wells were analyzed by fitting a generalized linear model assuming binomial errors. The significances of the factors of FSH, LH, EGF, and CHX and their interactions were tested using an analysis-of-deviance table (GenStat 6.1, VSN International, Oxford, U.K.). Analyzed data are presented as mean proportions and approximate SEMs predicted from the fitted model. A probability of $P < 0.05$ was considered to be statistically significant.

RESULTS

Experiment 1

In the pig, MI is a relatively long component of the meiotic transition from GV to MII compared with other phases (the diakinesis of prophase I, prometaphase I, AI, and TI) [25]. Therefore, GV, MI, and MII were used as representative stages to indicate meiotic progression. As shown in Figures 1 and 2, more than 80% of oocytes from both gilts and sows remained at the GV stage after 44–48 h of culture in medium B, and less than 10% reached MII. Medium supplementation with LH had no effect ($P > 0.05$) on the maturation of oocytes from either gilts or sows. Significantly higher proportions of oocytes matured to MII in media F and E in both gilts (50.0% ± 4.2% and 54.8% ± 4.3%, respectively; $P < 0.001$) and sows (87.6% ± 3.4% and 78.8% ± 3.9%, respectively; $P < 0.001$) compared with those in the basic medium with or without LH. The rate of maturation of gilt oocytes to MII in media F and E was significantly lower than that of sow oocytes ($P < 0.001$); significantly more gilt oocytes (FSH, 23.4% ± 3.9%; EGF, 34.8% ± 4.6%) than sow oocytes (FSH, 4.1% ± 2.1%; EGF, 4.4% ± 2.0%; $P < 0.05$) stayed at GV. Combined supplementation with FSH and EGF resulted in an additive effect on nuclear maturation in gilt oocytes (93.7% ± 2.1%), which was comparable to that in sow oocytes (93.1% ± 2.8%). Addition of LH to medium containing either FSH or EGF (or both) had no further effect on the rates of nuclear maturation (Figs. 1 and 2).

Experiment 2

In our previous studies using CHX [25, 46], LH alone was used to supplement a basic maturation medium similar to the present one (0.57 mM cysteine added, but without any effect on nuclear maturation; data not shown), and meiotic progression was synchronized and highly predicable. In particular, almost all (>80%) meiotically competent oocytes reached MI by 14 h after removal of CHX, with none entering AI/TI; this proportion was maximal and comparable to that of oocytes that reached MII by 24 h [25, 46]. Therefore, in the present study, 14 and 24 h after CHX pretreatment were used as the key time points for assessing meiotic progression.

No significant difference was observed between gilt and sow oocytes in the rates of CHX-synchronized nuclear maturation in medium B (55.2% ± 5.6% and 58.8% ± 3.3%, respectively; not significant [NS]) or medium E (86.6% ±

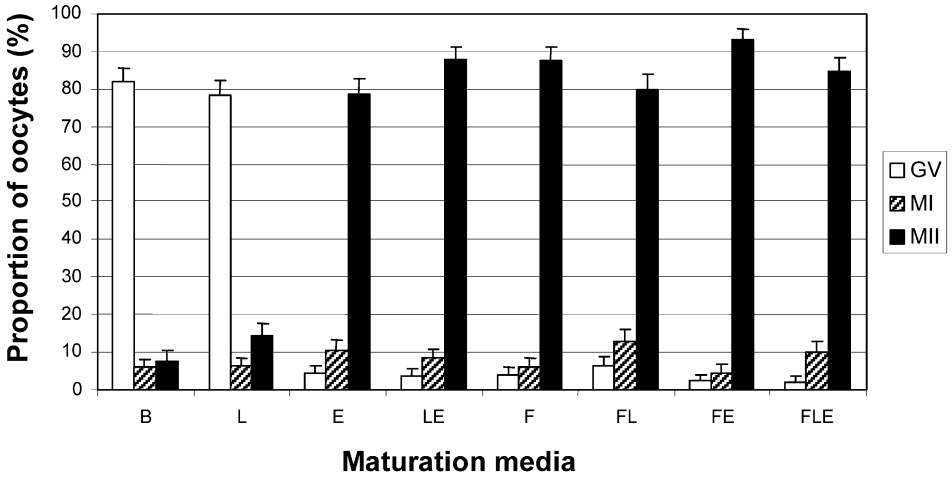

FIG. 2. Effect of hormones on sow oocyte maturation: proportion of oocytes at the GV, MI, and MII stages following conventional culture for 44–48 h in basal medium (B) or medium supplemented with LH (L), EGF (E), or FSH (F), either alone or in combinations, examined under a phase-contrast microscope at ×400 magnification. Error bars represent the approximate SEMs estimated from a linear model fitted assuming binomial errors. Both EGF and FSH had statistically significant ($P < 0.001$) effects on the proportion of oocytes at the GV and MII stages.

4.0% and 86.7% ± 7.8%, respectively; NS). Moreover, in contrast to conventional culture (experiment 1), pretreatment with CHX caused almost all oocytes to undergo GVBD (84–100%) regardless of culture media or oocyte origin, although the proportion of oocytes maturing to MII at 24 h was relatively low in the basal medium ($P < 0.05$). Therefore, data from gilt and sow oocytes (cultured separately) were pooled for further examination of meiotic progression (variances within pooled data remained low). As shown in Figures 3 and 4, the majority of oocytes had matured to MI and MII at 14 and 24 h of culture after CHX pretreatment, respectively, in media B, L, E, and LE. At 14 h, the rates of maturation to MI were similar in all media (78.8% ± 4.6%, 85.6% ± 3.6%, 88.9% ± 3.5%, and 83.8% ± 4.2% in media B, L, E, and LE, respectively; NS). In medium supplemented with FSH, a significantly lower proportion of oocytes matured to MI (43.8% ± 5.3%, $P < 0.001$), with a correspondingly higher proportion remaining at the GV stage (39.3% ± 5.9%, $P < 0.001$) until 14 h; only 48.4% of oocytes ($P < 0.001$) had reached MII by 24 h. However, a 12-h extension of culture (until 36 h) in medium F allowed the majority of oocytes to mature to MII (70.5% ± 2.9%) (Fig. 5). Addition of LH, EGF, or both to maturation medium in the presence of FSH had no significant effect on meiotic progression (Figs. 3–5).

Experiment 3

This experiment investigated whether CHX pretreatment and alteration of the speed of nuclear maturation by FSH would affect early embryo development after fertilization. To obtain similar nuclear maturation rates between control and treated oocytes before IVF, medium LE was chosen as the maturation medium, because neither medium B nor medium L stimulated maturation. As in most previous studies, LH was added, because it may provide general support for cytoplasmic maturation despite its lack of effect on nuclear maturation (experiment 2). Whereas the oocytes cultured without FSH were from sows, the three replicate batches cultured with FSH represented both gilts and sows, with at least one coming from gilts; variance between the batches in each treatment remained low (Fig. 6). As shown in Figure 6, the rates of normal cleavage 2 days after fertilization of CHX-pretreated oocytes were similar to those in untreated counterparts when matured with (71.3% ± 2.9% and 75.3% ± 3.1%, respectively; NS) or without (37.7% ± 3.0% and 43.0% ± 2.9%, respectively; NS) FSH. However, the frequencies of oocytes/zygotes developing to the blastocyst stage by Day 6 were significantly higher following CHX pretreatment than in untreated groups, both with (32.8% ± 2.0% and 10.3% ± 1.5%, respectively; $P < 0.001$) and without (16.7% ± 1.5% and 9.4% ± 1.2%, respectively; $P < 0.001$) FSH. Accordingly, a significantly higher proportion of Day 2-cleaved embryos from CHX-pretreated oocytes had developed to the blastocyst stage by Day 6 compared with untreated counterparts, both with (46.8% ± 4.9% and 14.3% ± 2.5%, respectively; $P < 0.001$) and without (44.7% ± 2.9% and 22.3% ± 1.4%, respectively; $P < 0.001$) FSH (data not shown in Fig. 6).

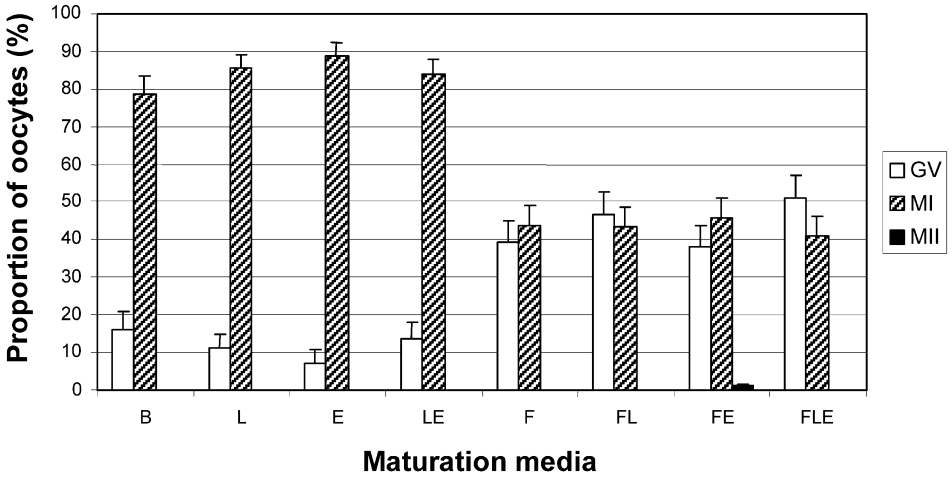

FIG. 3. Effect of hormones and CHX on porcine oocyte maturation: proportion of oocytes (from gilts and sows) at the GV, MI, and MII stages following culture for 14 h in basal medium (B) or medium supplemented with LH (L), EGF (E), or FSH (F), either alone or in combinations, preceded by preincubation with CHX for 12 h in the same medium and examined under a phase-contrast microscope at ×400 magnification. Error bars represent the approximate SEMs estimated from a linear model fitted assuming binomial errors. The FSH had statistically significant ($P < 0.001$) effects on the proportion of oocytes at the GV and MI stages.

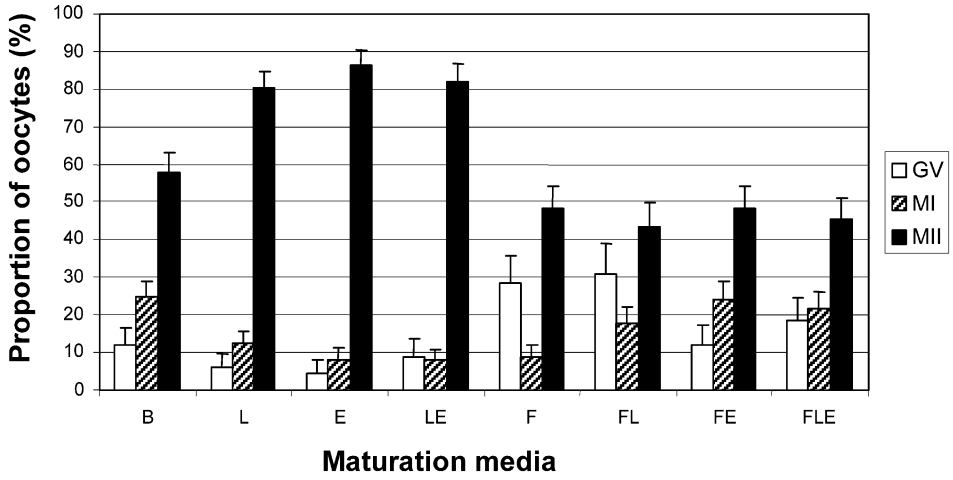

FIG. 4. Effect of hormones and CHX on porcine oocyte maturation: proportion of oocytes (from gilts and sows) at the GV, MI, and MII stages following culture for 24 h in basal medium (B) or medium supplemented with LH (L), EGF (E), or FSH (F), either alone or in combinations, preceded by preincubation with CHX for 12 h in the same medium and examined under a phase-contrast microscope at ×400 magnification. Error bars represent the approximate SEMs estimated from a linear model fitted assuming binomial errors. Basal and FSH-supplemented media had statistically significant ($P < 0.05$) effects on the proportion of oocytes at the MII stage.

Supplementation of the maturation medium with FSH in the presence of LH and EGF significantly increased the rate of cleavage ($P < 0.001$) in both CHX-pretreated and untreated oocytes. Moreover, a positive interaction was observed between CHX pretreatment and FSH, leading to a substantially improved rate of development to the blastocyst stage (32.8% ± 2.0%) when compared to the effect of each individually (10.3% ± 1.5% and 16.7% ± 1.5%, respectively; $P < 0.05$).

Figure 7 shows a representative group of embryos derived from CHX-pretreated gilt oocytes matured in the presence of FSH that had developed to the blastocyst stage at Day 6 after IVF. The cell count for all treatments was between 50 and 65 cells/embryo at Day 6 (NS). There appeared to be no difference between gilts and sows in the development of oocytes pretreated with CHX to the blastocyst stage after IVF. As shown in Table 1, the use of a variety of IVF protocols (variables are not specifically indicated but include IVF procedure and period of oocyte-sperm coincubation) resulted in a wide range ($P < 0.001$) of penetration and polyspermy rates in both CHX-pretreated and control oocytes. However, no difference from control was observed in either mean penetration rate (34.0% ± 1.6%; control, 29.5% ± 1.6%; NS) or mean polyspermy rate (14.3% ± 1.8%; control, 13.2% ± 1.9%; NS). The proportions of spontaneously parthenogenetically activated oocytes following overmaturation or with sperm-free IVF were very low (<5%), with no difference found between treatments.

DISCUSSION

Numerous studies have examined the effects of FSH, LH, and EGF on oocyte maturation, but very few have investigated their influence on the dynamics of meiotic progression in relation to cytoplasmic maturation. This may result from the lack of a suitable IVC system. Some of the terms used to describe effects on nuclear maturation are confusing. In the present study, we use *stimulate* for effects on the accumulated number of oocytes reaching nuclear maturation at a given time point (i.e., the effective rate of maturation), *accelerate* for identified effects on the speed of the nuclear maturation process, and *enhance* for effects on cytoplasmic maturation only. With both conventional and CHX-synchronized culture systems, the present study demonstrates that FSH and EGF, either alone or together, stimulate nuclear maturation in oocytes of both gilts and sows. However, whereas FSH and EGF have an additive effect on gilt oocytes, either FSH or EGF alone is sufficient to stimulate nuclear maturation in sow oocytes. Additionally, LH has no effect on meiotic resumption and does not

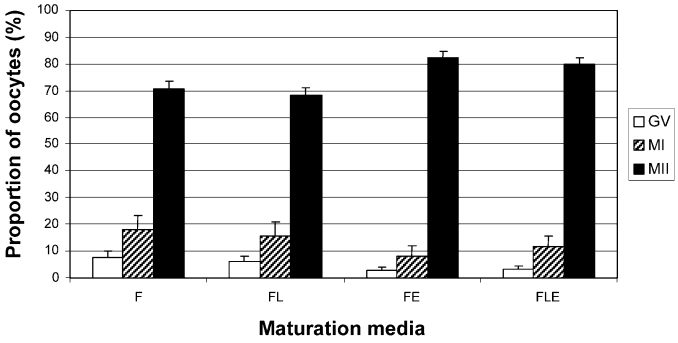

FIG. 5. Effect of hormones and CHX on porcine oocyte maturation: proportion of oocytes (from gilts and sows) at the GV, MI, and MII stages following culture for 36 h in medium supplemented with LH (L) and/or EGF (E) in the presence of FSH (F) preceded by preincubation with CHX for 12 h in the same medium and examined under a phase-contrast microscope at ×400 magnification. The error bars represent the approximate SEMs estimated from the linear model fitted assuming binomial errors. The EGF had statistically significant ($P < 0.05$) effects on the proportion of oocytes at the MII stage.

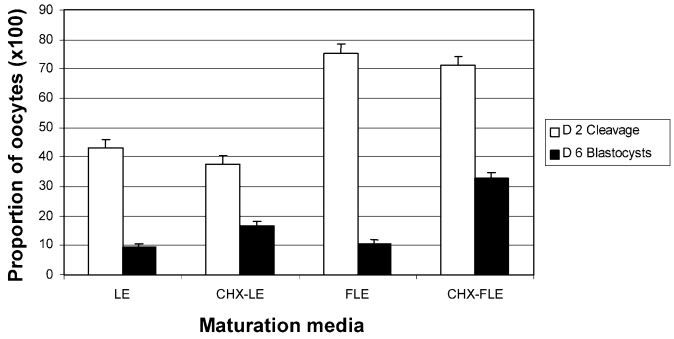

FIG. 6. Cleavage and blastocyst formation rates of oocytes from gilts and sows matured conventionally or after preincubation with CHX for 12 h with or without FSH in medium LE and then fertilized and cultured for 6 days. Error bars represent the approximate SEMs estimated from a linear model fitted assuming binomial errors. The CHX had a statistically significant ($P < 0.001$) effect on blastocyst rate at 6 days. The FSH had a statistically significant ($P < 0.001$) effect on both cleavage rate at 2 days and blastocyst rate at 6 days and interacted significantly ($P < 0.05$) with CHX for blastocyst rate at 6 days.

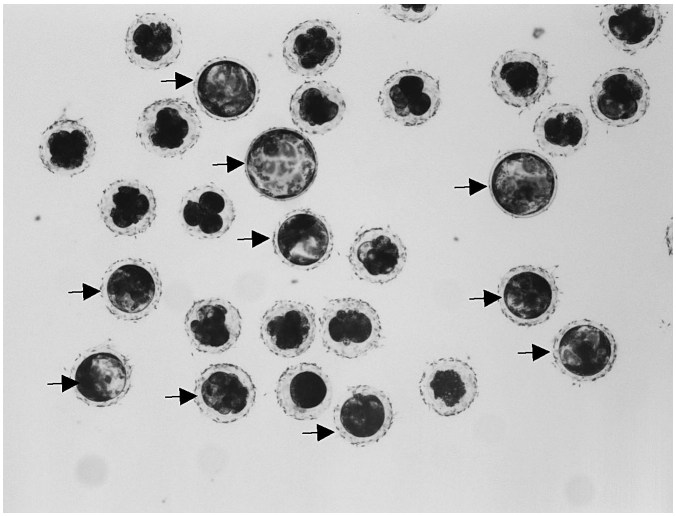

FIG. 7. Examples of Day 6 embryos derived from gilt oocytes matured after preincubation with CHX for 12 h with FSH in medium LE. A dish of 30 oocytes was cultured and fertilized (photograph taken at 5.5 days and ×50 magnification). Arrows indicate blastocysts.

interact with FSH or EGF, although it may influence later stages in CHX-pretreated oocytes (Fig. 4). Both FSH and EGF stimulate nuclear maturation, but their mechanisms apparently are different: FSH slows meiotic progression but supports maturation and even stimulates nuclear maturation by reducing its speed; EGF in the presence of FSH cannot significantly influence the speed of nuclear maturation.

The gonadotropin surge (LH/FSH) induces meiotic resumption of oocytes in preovulatory follicles in vivo [10]. For this reason, these hormones commonly are supplemented in the maturation medium, either alone or in combination. Earlier studies indicated that FSH and LH both stimulate nuclear maturation in pig oocytes [48], with medium supplementation for only the first half (20–22 h) of culture being enough to improve maturation rates considerably [49]. This approach has been adopted in most subsequent studies. In contrast, the present study shows that whereas FSH stimulates nuclear maturation, LH has virtually no effect (experiment 1). Nevertheless, this observation is not inconsistent with a maturational role for the gonadotropin surge in vivo, because this consists of an LH rise either coincident with or followed by a smaller FSH rise (for review, see [50]). Recent evidence shows very few, if any, LH receptors on porcine cumulus cells, at least in middle-sized follicles, although their formation may be stimulated by FSH [6, 51, 52]. Although the mechanisms resulting in resumption of meiosis in oocytes have yet to be defined in mammals, in vitro studies suggest that FSH, rather than LH, induces meiotic resumption [50, 52]. Early preparations of LH likely were less pure than those used in the present study and may have contained FSH. In contrast, the stimulatory effect of FSH on oocyte maturation is confirmed [53].

Because of their general stimulation of nuclear maturation, EGF and FSH would be expected to accelerate meiotic progression. However, this was not the case: EGF treatment caused no difference in the rate of maturation to MI as measured at 14 h after CHX pretreatment, and oocytes already had matured at maximal rates to MII by 24 h (experiment 2). As shown previously, pretreatment with CHX and culture in medium supplemented with LH did not alter the pace of nuclear maturation: Maturation remained highly synchronized and predicable at 36 h for GVI oocytes to mature to MII and at 24 h for those undergoing GVBD (at or beyond GVII) to mature to MII [25, 54]. Unexpectedly, FSH slowed meiotic progression and supported nuclear maturation. This effect of FSH also has been noted recently by Schoevers et al. [55], who observed that FSH retarded GVBD during the first half (20 h) of the culture period. However, their rates of maturation were not consistent among different experiments (no pretreatment for synchronization), and they did not investigate the interactions of FSH with other putative stimulators, such as EGF or LH. Whether FSH just postpones meiotic resumption (GVBD) or slows every phase of nuclear maturation remains to be investigated.

It is notable that FSH slows meiotic progression but stimulates the rate of nuclear maturation. This suggests an effect on cytoplasmic maturation that may be significant for subsequent embryo development. Because FSH can increase the concentration of cAMP in COCs [51], it may take longer for the oocyte to achieve the critical reduction in the concentration of cAMP that is necessary for meiotic resumption [51]. Additionally, FSH might act directly through receptors on the oocyte [56]. Positive effects of FSH on oocyte cytoplasmic maturation have been reported in many other studies [55, 57–59]. The present results suggest that improved cytoplasmic maturation may be associated with a decrease in the speed of meiotic progression and be reflected in a significant increase in the rate of cleavage after IVF. A decrease in the speed of nuclear maturation may facilitate cytoplasmic maturation by allowing the synthesis of specific proteins [58].

The present study demonstrates, to our knowledge for the first time, that pig oocytes pretreated with CHX to arrest meiosis temporarily can be fertilized successfully and will

TABLE 1. Effect of CHX pretreatment before maturation culture on fertilzation parameters of pig oocytes.[a]

	CHX-treated oocytes			Control oocytes		
Protocol	Examined	Penetrated	Polyspermic	Examined	Penetrated	Polyspermic
1	26	3	0	29	4	0
2	26	1	0	30	1	0
3	27	7	2	27	7	1
4	12	2	0	23	2	0
5	28	6	0	24	4	0
6	28	19	5	16	7	3
7	25	23	18	28	25	19
Total	172	61	25	177	50	23
Mean ± SEM (%)		34.0 ± 1.6	14.3 ± 1.8		29.5 ± 1.6	13.2 ± 1.9

[a] Gilt or sow oocytes were matured with or without CHX pretreatment and fertilized using various modifications (protocols 1–7) of the IVF procedure described in *Materials and Methods*. Mean percentage and approximate SEMs are predicted from a linear model fitted assuming binomial errors.

develop to the blastocyst stage at a higher frequency than untreated oocytes (experiment 3). This suggests that such pretreatment improves oocyte quality. It is possible that blastocyst yields from CHX-pretreated oocytes could be further increased by improvements to culture conditions: The development rate of untreated control oocytes was relatively low (10%) in the present study compared with those of other conventional cultures (e.g., 14–23% as reported by Marchal et al. [17]). The maturation medium influences subsequent embryo development (e.g., NCSU 23 may be better than Medium 199 [60]), and blastocyst yield is affected by features of the IVF protocol, such as boar identity, availability of frozen sperm, and ratio of medium volume or sperm to the number of oocytes [61–63].

Bovine oocytes treated with CHX before maturation retain full-term developmental competence, as demonstrated by the birth of calves [43, 45]. However, to our knowledge, no associated improvement in blastocyst yield has been observed, suggesting species-specific differences or a need for different levels or periods of exposure to CHX. Development to the blastocyst stage was compromised when bovine oocytes were pretreated with CHX at 10 μg/ml for 24 h, although fertilization and cleavage appeared to be normal [41, 43]. However, the development of CHX-pretreated oocytes was as good as that of untreated ones when maturation medium was supplemented with hormones [43], the period of pretreatment was reduced (≤18 h) [42], or the period of maturation after pretreatment was prolonged (≥24 h) [44].

The present results agree with those of Funahashi et al. [22, 23], who showed that synchronization of nuclear maturation (by dbcAMP or by omission of hormones) improved subsequent embryo development. The improvement in pig oocyte developmental competence brought about by CHX may result from synchronized nuclear maturation, because asynchronous maturation is associated with polyspermic fertilization resulting from suboptimal timing of insemination [54]. Pretreatment with CHX has been reported to reduce the rate of polyspermy in bovine oocytes [44]. In the present study, CHX pretreatment had no effect on this fertilization parameter when tested across a range of IVF protocols. More likely, CHX acts on oocytes either directly or through cumulus cells and improves meiotic and developmental competence during the blockage of GVBD: Almost all treated oocytes underwent GVBD on release from CHX, even without any further putative stimulators (experiment 2). Surprisingly, CHX pretreatment appeared to eliminate any intrinsic difference between the oocytes of gilts and sows not only in their meiotic competence but also in their subsequent development.

In conclusion, pig oocytes meiotically arrested by CHX before maturation not only retain but also improve their developmental competence. Supplementation with FSH stimulates the nuclear maturation rate but slows meiotic progression, implying an enhanced cytoplasmic maturation. Combining CHX-prematuration treatment and FSH supplementation of maturation medium results in an excellent blastocyst yield and has potential as a routine procedure both to improve pig IVP and to facilitate the application of novel animal biotechnologies.

ACKNOWLEDGMENTS

Highly purified porcine LH (lot no. AFP12389A) and FSH (lot no. AFP10640B) for the present study were obtained from NIDDK, the National Hormone and Peptide Program, through Dr. A.F. Parlow (Torrance, CA).

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