

Different hormonal requirements of pig oocyte–cumulus complexes during maturation *in vitro*

H. Funahashi, T. Cantley and B. N. Day*

Department of Animal Sciences, University of Missouri-Columbia, Columbia, MO 65211, USA

Cytoplasmic maturation as determined by male pronuclear formation following fertilization *in vitro* was examined in pig oocytes cultured under different hormonal conditions during either the first or second 20 h period of *in vitro* maturation. Exposure to several combinations of pregnant mares' serum gonadotrophin (PMSG) (10 iu ml^{-1}), hCG (10 iu ml^{-1}) and oestradiol ($1 \mu\text{g ml}^{-1}$) for a second 20 h period following culture in a medium supplemented with these hormones for 20 h did not result in differences among treatment groups in maturation rates, penetration rates or polyspermy rates. However, supplementation with PMSG and oestradiol for the last 20 h of culture reduced male pronuclear formation rates significantly. When oocyte–cumulus complexes were cultured in hormone-free media for 20 h after culture in several combinations of supplemental hormones for the first 20 h period, germinal vesicle breakdown rates and maturation rates were lower in oocytes previously exposed to oestradiol alone or no hormonal supplements (68–70% and 45–49%, respectively) than in oocytes previously exposed to PMSG or hCG (89–99% and 71–89%, respectively). Exposure of oocytes to oestradiol alone also reduced the penetration rate (61%) compared with PMSG or hCG (86–99%). Supplementation of media with PMSG alone or together with other hormones increased the male pronuclear formation rate (63–72%) compared with supplementation with oestradiol (33%) or no hormonal supplements (32%). The concentration of oestradiol in maturation medium decreased at filtration (to $216.5 \pm 72.6 \text{ ng ml}^{-1}$) before culture. Under paraffin oil, the concentration further decreased during equilibration (to $128.0 \pm 13.3 \text{ ng ml}^{-1}$), during the first 20 h of culture ($42.8 \pm 19.4 \text{ ng ml}^{-1}$) and also during the second 20 h period of culture without hormonal supplements ($0.925 \pm 0.544 \text{ ng ml}^{-1}$). The concentrations of progesterone in maturation media under paraffin oil were lower throughout maturation ($1.0 \pm 0.2 \text{ ng ml}^{-1}$ at 0 h to $6.9 \pm 1.5 \text{ ng ml}^{-1}$ after 40 h). These results demonstrate that at least two different hormonal conditions during maturation, which are the presence of PMSG during the first 20 h of culture and the absence of PMSG and oestradiol during the second 20 h of culture, are beneficial to meiotic and cytoplasmic maturation of pig oocytes. Furthermore, it was demonstrated that oocyte–cumulus complexes under paraffin oil were exposed to extremely low concentrations of steroids during maturation.

Introduction

Addition of suitable hormonal supplementation into maturation media enhances nuclear maturation and cumulus expansion of pig oocyte–cumulus complexes (Meinecke and Meinecke-Tillmenn, 1979; Hillensjo and Channing, 1980; Minato and Toyoda, 1982; Yoshida *et al.*, 1989; Prochazka *et al.*, 1991). Pig follicular oocyte–cumulus complexes have been cultured in media supplemented with a combination of hormones during maturation *in vitro*. Recently, we have also indicated that the

removal of hormonal supplements [pregnant mares' serum gonadotrophin (PMSG), hCG and oestradiol] from maturation media after 20 h of culture enhances cytoplasmic maturation, as determined by male pronuclear formation following fertilization *in vitro* (Funahashi and Day, 1993c). In control or superovulated pigs, the concentrations of gonadotrophins and steroids in follicular fluid change dramatically during preovulatory oocyte maturation (Moor, 1974; McNatty *et al.*, 1975; Hunter *et al.*, 1976; Ainsworth *et al.*, 1980; Lenton *et al.*, 1988), and, in particular, the concentration of oestrogen in prepubertal gilts declines rapidly between 4 and 16 h after hCG injection (Ainsworth *et al.*, 1980). There may therefore be different hormonal requirements of oocyte–cumulus complexes for

*Correspondence.
Received 10 August 1993.

meiotic and cytoplasmic maturation of pig oocytes. The present study was undertaken to examine the ability of pig oocytes to form the male pronucleus following fertilization *in vitro* when oocyte–cumulus complexes were cultured under the various hormonal conditions during the first and second half of maturation *in vitro*. Furthermore, we and other investigators have supplemented maturation media maintained under paraffin oil with a high concentration of oestradiol ($1 \mu\text{g ml}^{-1}$; Yoshida *et al.*, 1989, 1990, 1992; Wang *et al.*, 1991; Funahashi and Day, 1993a, b, c). It is known that paraffin oil can absorb lipid-soluble components of culture media (Miller and Pursel, 1987). However, steroid concentrations during maturation are not known. Thus, the concentrations of oestradiol and progesterone in maturation media were determined during culture of pig oocyte–cumulus complexes.

Materials and Methods

Culture media

As described by Funahashi and Day (1993c), the basic medium (mM199) was Medium 199 (Earle's salts; Gibco Laboratories, Life Technologies Inc, Grand Island, NY) supplemented with $3.05 \text{ mmol D-glucose l}^{-1}$, $2.92 \text{ mmol calcium lactate l}^{-1}$, $0.91 \text{ mmol sodium pyruvate l}^{-1}$, $75 \mu\text{g potassium penicillin G ml}^{-1}$ (Sigma Chemical Co., St Louis, MO) and $50 \mu\text{g streptomycin sulfate ml}^{-1}$. The medium for oocyte maturation was mM199 supplemented with hormones: $10 \text{ iu PMSG ml}^{-1}$ (Intervet America Inc, DE); $10 \text{ iu hCG ml}^{-1}$ (LyphoMed Inc, Rosemont, IL); $1 \mu\text{g oestradiol ml}^{-1}$ (Sigma Chemical Co) and 10% (v/v) porcine follicular fluid (PFF). PFF was collected from superficial porcine follicles, 3–6 mm in diameter. Thereafter, PFF was filtered using 1.20 and $0.45 \mu\text{m}$ syringe filters (Gelman Sciences, Ann Arbor, MI) and stored at -20°C until used. All media used in the present study were filtered using a $0.20 \mu\text{m}$ syringe filter (Schleicher and Schuell, Inc, Keene, NH) immediately before making droplets in culture dishes.

Preparation and culture of oocyte–cumulus complexes

Ovaries were collected and transported as described previously by Funahashi and Day (1993c). Oocyte–cumulus complexes were aspirated from follicles 3–6 mm in diameter. Complexes with uniform ooplasm and a compact cumulus cell mass were collected in modified Dulbecco's phosphate-buffered saline (mDPBS) (Funahashi and Day, 1993c) with 2% (v/v) newborn piglet serum and then washed three times with mM199 containing 10% PFF supplemented with hormonal supplements (PMSG, hCG and oestradiol) in Expt 1, or with mM199 supplemented with 10% PFF only in Expt 2.

In Expt 1 ten oocyte–cumulus complexes were transferred to a droplet of $100 \mu\text{l}$ of mM199 containing 10% PFF and hormonal supplements covered with warm paraffin oil in a polystyrene culture dish (Becton Dickinson Labware, Oxnard, CA), which had been equilibrated in a CO_2 incubator at 39°C for 3 h, and then cultured at 39°C in an atmosphere of 5% CO_2 in air. After 20 h of culture, the oocyte–cumulus complexes were removed from the medium and cultured in an equilibrated

droplet of $100 \mu\text{l}$ of mM199 containing 10% PFF supplemented (1) with PMSG, hCG and oestradiol, (2) with PMSG and hCG, (3) with PMSG and oestradiol, (4) with hCG and oestradiol, (5) with PMSG, (6) with hCG, (7) with oestradiol, or (8) with no hormonal supplements for an additional 20 h period after washing oocyte–cumulus complexes three times in each new medium. All media were covered with paraffin oil and equilibrated in a CO_2 incubator at 39°C for 3 h before the start of culture.

In Expt 2, ten oocyte–cumulus complexes were cultured in $100 \mu\text{l}$ of media supplemented with various combinations of hormones as described above for the first 20 h period of culture for maturation. Thereafter, the oocyte–cumulus complexes were cultured in mM199 containing 10% PFF for a further 20 h period following washing three times in the fresh medium. All media were covered with paraffin oil and equilibrated in a CO_2 incubator at 39°C for 3 h.

Analysis of oestradiol and progesterone in culture media

To determine the concentration of oestradiol and progesterone in culture media during maturation, 10 and 50 pig oocyte–cumulus complexes were cultured in a droplet of $100 \mu\text{l}$ and $500 \mu\text{l}$, respectively, of mM199 containing 10% PFF supplemented with PMSG, hCG and oestradiol under the presence or absence of 5 ml paraffin oil, respectively, for 20 h at 39°C in an atmosphere of 5% CO_2 in air. At the end of culture, oocyte–cumulus complexes were transferred into $100 \mu\text{l}$ and $500 \mu\text{l}$, respectively, of fresh mM199 containing 10% PFF supplemented with PMSG, hCG and oestradiol or no hormonal supplements and cultured for 20 h, under the presence or absence of 5 ml of paraffin oil, respectively. All culture media were equilibrated in a CO_2 incubator at 39°C for 3 h before the start of culture.

After 0 h, 20 h and 40 h of maturation culture, conditioned media were collected into 1.5 ml centrifuge tubes. Control samples were also collected before equilibration (-3 h of culture). All samples were stored in the freezer at -20°C until radioimmunological analysis. A radioimmunoassay described by Kesler *et al.* (1977) and validated for pigs by Redmer and Day (1981) was used to determine concentrations of oestradiol in media before and after culturing oocytes–cumulus complexes. Interassay and intra-assay coefficients of variation were 15.5% and 9.6%, respectively. Assay sensitivity was 4.1 pg ml^{-1} . Progesterone concentrations in conditioned media were measured by radioimmunoassay procedures described by Flowers *et al.* (1989). Mean interassay and intra-assay coefficients of variation were 10.9% and 7.7%, respectively. Assay sensitivity was 0.2 ng ml^{-1} .

Sperm preparation and *in vitro* fertilization

Sperm-rich fractions (15 ml) were collected from a boar, kept at 20°C for 16 h and washed as described by Funahashi and Day (1993c). At the end of washing, the spermatozoa were resuspended at 2×10^8 cells ml^{-1} in mM199 at pH 7.8 and supplemented with 0.4% BSA. The sperm suspension was incubated for 90 min at 37°C in an atmosphere of 5% CO_2 in air.

Table 1. Effects of hormonal supplements between 20 and 40 h of *in vitro* maturation on sperm penetration and male pronuclear formation in pigs 12 h after insemination

Hormonal supplements ^a	Number of oocytes			Number (%) of polyspermic oocytes ^c	Number (%) of oocytes with male and female pronuclei ^{c,d}
	Examined	Matured (%)	Penetrated (%) ^b		
PMSG + hCG + oestradiol	93	83 (89)	69 (83)	45 (65)	12 (17)*
PMSG + hCG	108	100 (93)	81 (81)	57 (70)	36 (44)*,‡
PMSG + oestradiol	99	89 (90)	71 (80)	47 (66)	18 (25)*
hCG + oestradiol	88	78 (89)	57 (73)	33 (58)	15 (26)*
PMSG	96	90 (94)	83 (92)	67 (81)	28 (34)*,†
hCG	90	82 (91)	69 (84)	45 (65)	36 (52)‡
Oestradiol	106	93 (88)	67 (72)	44 (66)	11 (16)*
None	73	67 (92)	47 (70)	33 (70)	30 (64)‡

^aPMSG: 10 iu pregnant mares' serum gonadotrophin ml⁻¹; hCG: 10 iu human chorionic gonadotrophin ml⁻¹; oestradiol: 1 µg oestradiol ml⁻¹.

^bPercentage of oocytes matured.

^cPercentage of oocytes penetrated.

^dDifferent superscripts within columns denote significant differences ($P < 0.05$).

After 40 h of culture for maturation, oocytes were used for fertilization *in vitro*. After removing cumulus cells with mDPBS supplemented with 0.1% (w/v) hyaluronidase (Sigma Chemical Co), ten oocytes were washed three times with 50 µl of mM199 at pH 7.4 supplemented with 10 mmol caffeine sodium benzoate l⁻¹ and 4 mg BSA ml⁻¹, and then placed into a droplet of 50 µl mM199 under paraffin oil, which had been pre-equilibrated in a CO₂ incubator at 39°C for 3 h. The sperm concentration in preincubated medium (2×10^8 cells ml⁻¹) was diluted to 2×10^6 cells ml⁻¹ with mM199 at pH 7.8 and supplemented with 0.4% BSA (final sperm concentration of 1×10^6 cells ml⁻¹), and 50 µl of preincubated spermatozoa was added to 50 µl of medium containing oocytes. Oocytes were cultured with spermatozoa for 6 h at 39°C in an atmosphere of 5% CO₂ in air. The eggs were then transferred to 1 ml of fresh Whitten's medium supplemented with 1.5% BSA (Beckmann and Day, 1993) and cultured at 39°C in an atmosphere of 5% CO₂ in air for 6 h (12 h after insemination) or 12 h periods (18 h after insemination) in Expt 1 and for a 6 h period (12 h after insemination) in Expt 2.

Assessment of cytoplasmic maturation

At the end of culture, eggs were removed from the droplet and washed in 4 ml of mDPBS to remove supernumerary spermatozoa from the zona pellucida. Thereafter, eggs were stained with 1% (w/v) orcein and examined under a phase-contrast microscope (Funahashi and Day, 1993c).

Statistical analysis

Statistical analyses from four replicate trials for treatment comparisons were carried out by analysis of variance (ANOVA) and Fisher's protected least significant difference test using the STATVIEW (Abacus Concepts, Inc, Berkeley, CA) program. All percentage data were subjected to arc sine transformation before statistical analysis. Correlation coefficients were assessed

after Fisher's R to Z transformation. The mean concentrations of oestradiol and progesterone in media were expressed as means \pm SD. Probability of $P < 0.05$ was considered to be statistically significant.

Results

Effects of hormonal conditions during the second half of maturation *in vitro*

There were no differences ($P > 0.05$) among treatment groups in maturation rates 12 and 18 h after insemination ($90.3 \pm 7.0\%$ and $94.1 \pm 6.4\%$, respectively), penetration rates ($80.8 \pm 18.1\%$ and $84.9 \pm 19.0\%$, respectively) or polyspermy rates ($60.8 \pm 29.5\%$ and $74.1 \pm 23.3\%$, respectively), but groups were different ($P < 0.001$) in rates of male pronuclear formation (Tables 1 and 2). The highest proportion of male pronuclei was observed in media with no hormonal supplement, although inclusion of hCG or PMSG with hCG did not significantly reduce this proportion (Fig. 1). Male pronuclear formation rates in pig oocytes that were exposed to oestradiol alone or in combination with other hormonal supplements during the second half of the culture period were significantly lower ($16\text{--}26\%$ at 12 h, and $31\text{--}39\%$ at 18 h after insemination) than those cultured in media containing hCG or no hormonal supplements (52 and 64% at 12 h, and 67 and 70% at 18 h after insemination, respectively). Supplementation with PMSG in combination with hCG did not decrease the ability of oocytes to form a male pronucleus (44% at 12 h and 58% at 18 h after insemination) compared with supplementation with hCG alone or no hormonal supplements (see above), but the pronuclear rate was reduced when PMSG was supplemented alone (34% at 12 h and 44% at 18 h). Although there were no correlations ($P > 0.05$) among meiotic maturation, sperm penetration and male pronuclear formation, there was a high correlation ($P < 0.0001$) between male pronuclear formation rates 12 and 18 h after insemination.

Table 2. Effects of hormonal supplements between 20 and 40 h of *in vitro* maturation on sperm penetration and male pronuclear formation in pigs 18 h after insemination

Hormonal supplements ^a	Number of oocytes			Number (%) of polyspermic oocytes ^c	Number (%) of oocytes with male and female pronuclei ^{c,d}
	Examined	Matured (%)	Penetrated (%) ^b		
PMSG + hCG + oestradiol	72	65 (90)	57 (88)	44 (77)	22 (38)*,†
PMSG + hCG	81	77 (95)	73 (95)	57 (78)	42 (58)*,‡
PMSG + oestradiol	81	73 (90)	57 (78)	39 (68)	22 (39)*
hCG + oestradiol	59	57 (97)	47 (82)	37 (79)	16 (34)*,†
PMSG	74	70 (95)	61 (87)	54 (89)	27 (44)*
hCG	66	65 (98)	48 (74)	39 (81)	32 (67)‡
Oestradiol	78	75 (96)	55 (73)	37 (67)	16 (31)†
None	57	54 (95)	44 (81)	34 (77)	31 (70)‡

^aPMSG: 10 iu pregnant mares' serum gonadotrophin ml⁻¹; hCG: 10 iu human chorionic gonadotrophin ml⁻¹; oestradiol: 1 µg oestradiol ml⁻¹.

^bPercentage of oocytes matured.

^cPercentage of oocytes penetrated.

^dDifferent superscripts within columns denote significant differences ($P < 0.05$).

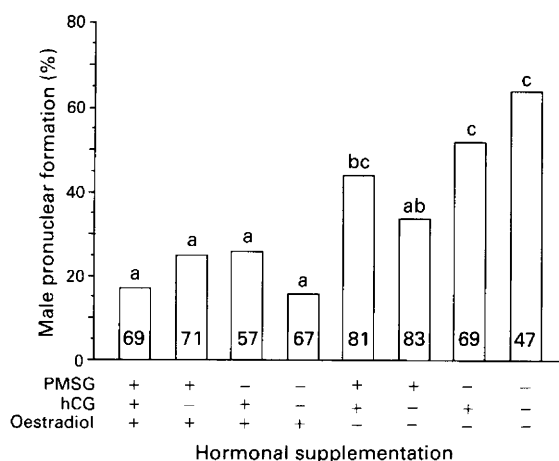


Fig. 1. Effects of hormonal supplements between 20 and 40 h of maturation *in vitro* on male pronuclear formation in pigs 12 h after insemination. Pig oocyte-cumulus complexes were exposed to several combinations of pregnant mares' serum gonadotrophin (PMSG) (10 iu ml⁻¹), hCG (10 iu ml⁻¹) and oestradiol (1 µg ml⁻¹) for a second 20 h period following culture in a medium supplemented with these hormones for 20 h. The numbers in the bars refer to the numbers of oocytes penetrated in the presence of the hormones indicated (see Table 1). Columns with different letters above the bars denote significant difference ($P < 0.05$).

Effects of hormonal conditions during the first half of maturation *in vitro*

There were differences ($P < 0.05$) among treatment groups in the percentage of oocytes with germinal vesicle breakdown, maturation rate, penetration rate and rate of male pronuclear formation, but groups were not different in polyspermy rate ($93.5 \pm 6.8\%$) 12 h after insemination (Table 3). There were beneficial effects of some hormones upon germinal vesicle breakdown, meiotic maturation, penetration and male pronuclear formation when compared with medium having no hormonal supplement. The germinal vesicle breakdown rates

and maturation rates were higher when exposed to PMSG, hCG or PMSG and hCG together (89–99% and 71–89%, respectively) than to oestradiol or no hormones (68–70% and 45–49%, respectively). Exposure of oocytes to oestradiol alone for the first 20 h period of culture resulted in a lower penetration rate (61%) compared with exposure to PMSG, hCG, or PMSG and hCG together (86–99%). Supplementation of oocytes with PMSG alone or together with other hormones during the first 20 h period of culture significantly increased the incidence of male pronuclear formation (63–72%) compared with supplementation with oestradiol (33%) or no hormones (32%). There were significant correlations between meiotic maturation rate and penetration rate ($P < 0.01$), between germinal vesicle breakdown rate and male pronuclear formation rate ($P < 0.0005$), between meiotic maturation rate and male pronuclear formation rate ($P < 0.0005$) and between sperm penetration rate and male pronuclear formation rate ($P < 0.025$).

Concentrations of oestradiol and progesterone in culture media during maturation *in vitro*

The concentration of oestradiol in maturation medium decreased dramatically from 1309.8 ± 70.8 ng ml⁻¹ to 216.5 ± 72.6 ng ml⁻¹ at filtration before culture. The oestradiol concentration was further reduced while under paraffin oil to 128.0 ± 13.3 ng ml⁻¹ by the end of equilibration and to 42.8 ± 19.4 ng ml⁻¹ after 20 h of culture (Fig. 2a), while culture without paraffin oil did not result in a lower oestradiol concentration (234.8 ± 47.4 ng ml⁻¹ and 225.0 ± 19.4 ng ml⁻¹, respectively). The concentrations of oestradiol at the end of the second 20 h period were 39.5 ± 11.4 ng ml⁻¹ and 228.3 ± 20.6 ng ml⁻¹ in mM199 containing 10% PFF supplemented with PMSG, hCG and oestradiol in the presence and absence of paraffin oil, respectively, and 0.925 ± 0.544 ng ml⁻¹ and 6.1 ± 3.645 ng ml⁻¹ in mM199 containing 10% PFF under the presence and absence of paraffin oil, respectively (Fig. 2a).

Table 3. Effects of hormonal supplements between 0 and 20 h of *in vitro* maturation on meiotic maturation, sperm penetration and male pronuclear formation in pigs 12 h after insemination

Hormonal supplements ^a	Number of oocytes				Number (%) of polyspermic oocytes ^{b,d}	Number (%) of oocytes with male and female pronuclei ^{b,d}
	Examined	GVBD (%) ^b	Matured (%) ^b	Penetrated (%) ^{b,c}		
PMSG + hCG + oestradiol	121	118 (98)*	107 (88)*	92 (86)* [†]	66 (72)	61 (66)*
PMSG + hCG	103	102 (99)*	88 (85)*	85 (97)* [†]	60 (71)	55 (65)*
PMSG + oestradiol	103	100 (97)*	91 (88)*	84 (92)* [†]	60 (71)	53 (63)*
hCG + oestradiol	120	110 (92)*	85 (71)*	77 (91)* [†]	52 (68)	42 (55)* [†]
PMSG	113	110 (97)*	101 (89)*	98 (97)* [†]	71 (72)	71 (72)*
hCG	120	107 (89)*	96 (80)*	95 (99) [†]	69 (73)	52 (55)* [†]
Oestradiol	121	85 (70) [†]	54 (45) [†]	33 (61) [‡]	22 (67)	11 (33) [†]
None	132	90 (68) [†]	65 (49) [†]	53 (82)*	38 (72)	17 (32) [†]

^aPMSG: 10 iu pregnant mares' serum gonadotrophin ml⁻¹; hCG: 10 iu human chorionic gonadotrophin ml⁻¹; oestradiol: 1 µg oestradiol ml⁻¹.

^bDifferent superscripts within columns denote significant differences ($P < 0.05$).

^cPercentage of oocytes matured.

^dPercentage of oocytes penetrated.

GVBD: germinal vesicle breakdown.

The concentrations of progesterone in maturation media under the presence of paraffin oil were not different throughout equilibration (from 3.6 ± 0.3 ng ml⁻¹ to 1.0 ± 0.2 ng ml⁻¹) and culture for maturation (2.4 ± 0.5 ng ml⁻¹ after 20 h and 5.3 ± 1.5 ng ml⁻¹ to 6.9 ± 1.5 ng ml⁻¹ after 40 h of culture), whereas in the absence of paraffin oil the concentrations increased during culture (3.0 ± 0.3 ng ml⁻¹ at the start of culture, 16.2 ± 5.4 ng ml⁻¹ after 20 h, and 46.1 ± 16.8 ng ml⁻¹ to 52.6 ± 16.1 ng ml⁻¹ after 40 h of culture; Fig. 2b).

Discussion

Meiotic maturation of pig oocytes is induced by culture with media containing PMSG, hCG or PMSG and hCG with or without oestradiol (Yoshida *et al.*, 1989). In our previous study, the exposure of oocyte-cumulus complexes to hormonal supplements (PMSG, hCG and oestradiol) for only 2 h enhanced germinal vesicle breakdown and maturation of the oocytes, and nearly all of the oocytes showed meiotic maturation by exposure to hormones for 20 h or longer (Funahashi and Day, 1993c). The data in the present study demonstrate that the presence of PMSG or hCG or both substances in maturation medium during the primary 20 h period was sufficient for the accomplishment of germinal vesicle breakdown and meiotic maturation. Also, the addition of everted follicles in the presence of LH alone or together with FSH (Mattioli *et al.*, 1991), and supplementation of media with PFF and FSH (Naito *et al.*, 1988) enhances male pronuclear formation of the oocytes after fertilization *in vitro*. We also reported that removing hormonal supplements after 20 h of culture improves both cumulus expansion and male pronuclear formation (Funahashi and Day, 1993c). Since cytoplasmic maturation was achieved by exposure to PMSG alone or in combination with hCG in the presence or absence of oestradiol from 0 to 20 h of culture, the presence of PMSG during the

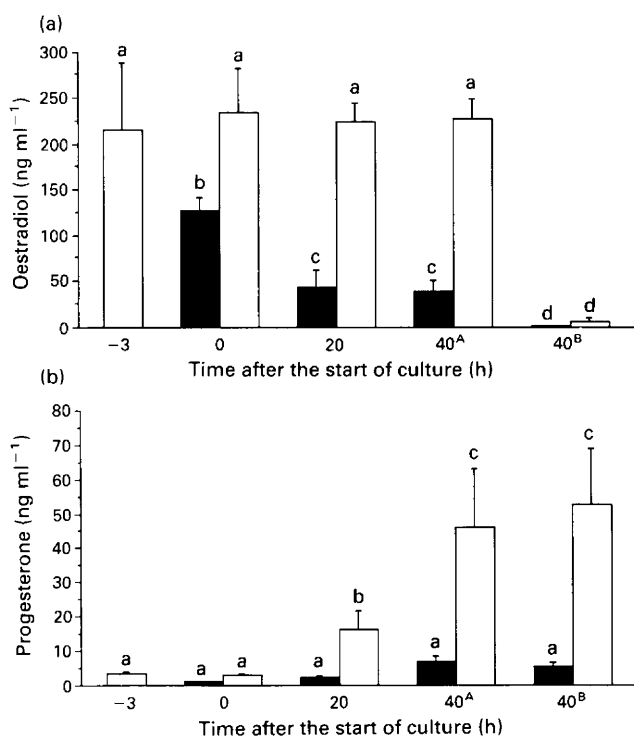


Fig. 2. The change in (a) oestradiol and (b) progesterone concentrations in media during culture of oocyte-cumulus complexes. Pig oocyte-cumulus complexes were cultured in mM199 medium containing 10% porcine follicular fluid supplemented with 10 iu pregnant mares' serum gonadotrophin (PMSG) ml⁻¹, 10 iu hCG ml⁻¹ and 1 µg oestradiol ml⁻¹ under the presence (■) or absence (□) of 5 ml paraffin oil for 20 h. Thereafter, the complexes were transferred into fresh mM199 containing 10% porcine follicular fluid supplemented with (column marked 40^A) or without (column marked 40^B) hormonal supplements for a further 20 h. Columns having different letters above the bars denote significant difference ($P < 0.05$).

initial 20 h period appears to promote both meiotic and cytoplasmic maturation.

In the present study, culture of oocyte–cumulus complexes in medium containing PMSG in the presence or absence of oestradiol during a second 20 h period of culture did not affect meiotic maturation and sperm penetration, but inhibited male pronuclear formation. A high correlation between male pronuclear formation rates 12 and 18 h after insemination indicates that these differences in male pronuclear formation rates were not due to a delay in the formation of the male pronucleus. The absence of PMSG and oestradiol during a second 20 h period of culture therefore appears to be beneficial for cytoplasmic maturation. This result is not inconsistent with our previous observation (Funahashi and Day, 1993c). A high incidence of decondensed spermatozoa was obtained after fertilization *in vitro* when pig oocytes maintained a high degree of intercellular coupling with surrounding cumulus cells until a later stage of maturation (Mattioli *et al.*, 1988). Whereas LH or hCG does not reduce intercellular coupling between cumulus cells and oocytes (Moor and Cran, 1980), LH increases the uptake of [³H]uridine in oocyte–cumulus complexes after 26 h of culture (Mattioli *et al.*, 1991). In contrast, FSH promotes the uncoupling of the cumulus cells from the oocytes and stimulates the cumulus expansion significantly (Moor and Cran, 1980; Eppig, 1982). The existence of PMSG, containing both FSH and LH activity during the later 20 h period of culture, may reduce the ability of oocytes to form a male pronucleus owing to increased uncoupling between somatic cells and oocytes.

We and other investigators have supplemented maturation medium with a high dose ($1 \mu\text{g ml}^{-1}$) of oestradiol and cultured oocyte–cumulus complexes under paraffin oil (Yoshida *et al.*, 1989, 1990, 1992; Wang *et al.*, 1991; Funahashi and Day, 1993a, b, c). Owing to the high steroid-binding ability of the syringe filter (Racowsky and McGaughey, 1982) and absorbing capability of paraffin oil (Miller and Pursel, 1987), the high concentration of supplemented oestradiol was reduced to concentrations similar to follicular concentrations (Ainsworth *et al.*, 1980) by changing to a medium without hormonal supplements. The concentration of progesterone was also reduced in medium under paraffin oil. Mattioli *et al.* (1988) reported that co-culture of oocyte–cumulus complexes with everted follicles in medium supplemented with progesterone resulted in an increase in male pronuclear formation. In the present study, oocyte–cumulus complexes that were cultured under paraffin oil were not exposed to high concentrations of progesterone during maturation but, even so, a high incidence of male pronuclear formation was achieved with suitable gonadotrophin conditions. Exposure to high concentrations of progesterone therefore does not seem to promote cytoplasmic maturation directly.

Recent reports indicate that glutathione is an important factor for male pronuclear formation of pig oocytes (see Niwa, 1993). Further studies will be needed to determine the relationship between hormonal conditions during oocyte maturation and glutathione synthesis in oocyte–cumulus complexes.

In the study reported here, hormonal conditions during maturation did not affect the incidence of polyspermic penetration. Since the number and nature of release of cortical granules *in vitro* differs from that *in vivo* (Cran and Cheng, 1986), the culture conditions may be expected to contribute to

this abnormal response at fertilization. However, polyspermic penetration of pig oocytes following fertilization *in vitro* has been inhibited by treatment of spermatozoa with macromolecules secreted by oviductal cells before insemination (Nagai and Moor, 1990) or with porcine follicular fluid (Funahashi and Day, 1993b).

In summary, the data presented here define two different hormonal conditions during maturation *in vitro* that result in improved cytoplasmic maturation of pig oocytes, namely, presence of PMSG during the first 20 h of culture and the absence of PMSG and oestradiol during the second 20 h of culture. It has also been determined that culture of oocyte–cumulus complexes under paraffin oil results in a marked reduction in the concentrations of steroids in the culture medium.

The authors thank Monfort Pork Inc for their donation of ovaries and B. Nichols for secretarial assistance with the preparation of this manuscript. H. Funahashi is the recipient of a University of Missouri Miller Fund postdoctoral fellowship. This manuscript was funded in part by Farmland Industries and is a contribution from the Missouri Agricultural Experimental Station: Journal Series Number 11 972.

References

- Ainsworth L., Tsang BK, Downey BR, Marcus GJ and Armstrong DT (1980) Interrelationships between follicular fluid steroid levels, gonadotropic stimuli, and oocyte maturation during preovulatory development of porcine follicles *Biology of Reproduction* **23** 621–627
- Beckmann LS and Day BN (1993) Effect of media NaCl concentration and osmolarity on culture of the early stage porcine embryo and viability of embryos cultured in a selected superior medium *Theriogenology* **39** 611–622
- Cran DG and Cheng WTK (1986) The cortical reaction in pig oocytes during *in vivo* and *in vitro* fertilization *Gamete Research* **13** 241–251
- Eppig JJ (1982) The relationship between parthenogenetic embryonic development and cumulus cell–oocyte intercellular coupling during oocyte meiotic maturation *Gamete Research* **5** 229–237
- Flowers B, Cantley TC, Martin MJ and Day BN (1989) Effect of elevated ambient temperatures on puberty in gilts *Journal of Animal Science* **67** 779–784
- Funahashi H and Day BN (1993a) Effects of different serum supplements in maturation medium on meiotic and cytoplasmic maturation of pig oocytes *Theriogenology* **39** 965–973
- Funahashi H and Day BN (1993b) Effects of follicular fluid at fertilization *in vitro* on sperm penetration in pig oocytes *Journal of Reproduction and Fertility* **99** 97–103
- Funahashi H and Day BN (1993c) Effects of the duration of exposure to supplemental hormones on cytoplasmic maturation of pig oocytes *in vitro* *Journal of Reproduction and Fertility* **98** 179–185
- Hillensjo T and Channing CP (1980) Gonadotrophin stimulation of steroidogenesis and cellular dispersion in cultured porcine cumuli oophori *Gamete Research* **3** 233–240
- Hunter RHF, Cook B and Baker TG (1976) Dissociation of response to injected gonadotropin between the Graafian follicle and oocyte in pigs *Nature* **260** 156–158
- Kesler DJ, Garverick HA, Youngquist RS, Elmore RG and Bierschwal CJ (1977) Effect of days postpartum and endogenous reproductive hormones on GnRH-induced LH release in daily cows *Journal of Animal Science* **45** 797–803
- Lenton EA, King H, Thomas EJ, Smith SK, McLachlan RI, MacNeil S and Cooke ID (1988) The endocrine environment of the human oocyte *Journal of Reproduction and Fertility* **82** 827–841
- McNatty KP, Hunter WM, McNeilly AS and Sawers RS (1975) Changes in the concentration of pituitary and steroid hormones in the follicular fluid of human graafian follicles throughout the menstrual cycle *Journal of Endocrinology* **64** 555–571
- Mattioli M, Galeati G, Bacci ML and Seren E (1988) Follicular factors influence oocyte fertilizability by modulating the intercellular cooperation between cumulus cells and oocyte *Gamete Research* **21** 223–232

- Mattioli M, Bacci ML, Galeati G and Seren E (1991) Effects of LH and FSH on the maturation of pig oocytes *in vitro* *Theriogenology* **36** 95–105
- Meinecke B and Meinecke-Tillmann S (1979) Effects of gonadotropins on oocyte maturation and progesterone production by porcine ovarian follicles cultured *in vitro* *Theriogenology* **11** 351–365
- Miller KF and Pursel VG (1987) Absorption of compounds in medium by the oil covering microdrop cultures *Gamete Research* **17** 57–61
- Minato Y and Toyoda Y (1982) Induction of cumulus expansion and maturation division of porcine oocyte–cumulus complexes *in vitro* *Japanese Journal of Zootechnological Science* **53** 480–487
- Moor RM (1974) The ovarian follicle of the sheep: inhibition of oestrogen secretion by luteinizing hormone *Journal of Endocrinology* **61** 455–463
- Moor RM and Cran DG (1980) Intercellular coupling in mammalian oocytes. In *Development in Mammals* pp 3–37 Ed. MH Johnson. Elsevier North-Holland, Amsterdam
- Nagai T and Moor RM (1990) Effect of oviduct cells on the incidence of polyspermy in pig eggs fertilized *in vitro* *Molecular Reproduction and Development* **26** 377–382
- Naito K, Fukuda Y and Toyoda Y (1988) Effects of porcine follicular fluid on male pronucleus formation in porcine oocytes matured *in vitro* *Gamete Research* **21** 289–295
- Niwa K (1993) Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in the pig *Journal of Reproduction and Fertility Supplement* **48** 49–59
- Prochazka R, Nagyova E, Rimkeviovova Z, Nagai T, Kikuchi K and Motlik J (1991) Lack of effect of oocyctectomy on expansion of the porcine cumulus *Journal of Reproduction and Fertility* **93** 569–576
- Racowsky C and McGaughey RW (1982) In the absence of protein, estradiol suppresses meiosis of porcine oocytes *in vitro* *Journal of Experimental Zoology* **224** 103–110
- Redmer DA and Day BN (1981) Ovarian activity and hormonal patterns in gilts fed allyl trenbolone *Journal of Animal Science* **53** 1088–1094
- Wang WH, Niwa K and Okuda K (1991) *In-vitro* penetration of pig oocytes matured in culture by frozen–thawed ejaculated spermatozoa *Journal of Reproduction and Fertility* **93** 491–496
- Yoshida M, Bamba K and Kojima Y (1989) Effects of gonadotropins and estradiol-17 β on the timing of nuclear maturation and cumulus mass expansion in pig oocytes cultured *in vitro* *Japanese Journal of Animal Reproduction* **35** 86–91
- Yoshida M, Ishizaki Y and Kawagishi H (1990) Blastocyst formation by pig embryos resulting from *in-vitro* fertilization of oocytes matured *in vitro* *Journal of Reproduction and Fertility* **88** 1–8
- Yoshida M, Ishizaki Y, Kawagishi H, Bamba K and Kojima Y (1992) Effects of pig follicular fluid on maturation of pig oocytes *in vitro* and on their subsequent fertilizing and developmental capacity *in vitro* *Journal of Reproduction and Fertility* **95** 481–488