Zygote **14** (August), pp. 189–199. © 2006 Cambridge University Press
doi:10.1017/S0967199406003765 Printed in the United Kingdom

The effect of FF-MAS on porcine cumulus–oocyte complex maturation, fertilization and pronucleus formation *in vitro*

Inger Faerge[1], *Frantisek Strejcek*[2], *Jozef Laurincik*[2,3], *Detlef Rath*[3], *Heiner Niemann*[3], *Karl Schellander*[4], *Christine Rosenkranz*[5], *Poul Maddox Hyttel*[6] *and Christian Grøndahl*[1]

Novo Nordisk A/S, Copenhagen, Denmark, Constantine the Philosopher University, Nitra, Slovak Republic, Research Institute of Animal Production, Nitra, Slovak Republic, Institute of Animal Breeding Science, University of Bonn, Bonn, Germany, University of Veterinary Medicine, Vienna, Austria, and Veterinary and Agricultural University, Copenhagen, Denmark

Date submitted: 13.12.05. Date accepted: 13.01.06

Summary

Follicular fluid meiosis-activating sterol (FF-MAS) has been isolated from the follicular fluid (FF) of several species including man. FF-MAS increases the quality of *in vitro* oocyte maturation, and thus the developmental potential of oocytes exposed to FF-MAS during *in vitro* maturation is improved. The aim of the present study was to investigate the effects of FF-MAS on porcine oocyte maturation and pronucleus formation *in vitro*. Porcine cumulus–oocyte complexes (COCs) were isolated from abattoir ovaries and *in vitro* matured for 48 h in NCSU 37 medium supplemented with 1 mg/l cysteine, 10 ng/ml epidermal growth factor and 50 μM 2-mercaptoethanol with or without 10% porcine follicular fluid (pFF). For the first 22 h, 1 mM db-cAMP and 10 I.E PMSG/hCG was added. The medium was supplemented with 1 μM, 3 μM, 10 μM, 30 μM or 100 μM FF-MAS dissolved in ethanol. After maturation the COCs were denuded mechanically using a fine glass pipette under constant pH and *in vitro* fertilized with fresh semen (5×10^5 spermatozoa/ml). The presumptive zygotes were evaluated 18 h after fertilization. The addition of pFF increased the monospermic as well as the polyspermic penetration of oocytes. In the absence of pFF, the addition of FF-MAS decreased the polyspermic penetration rate, whereas FF-MAS in combination with pFF decreased monospermic and increased polyspermic penetration. The degeneration rate of ova decreased in the presence of FF-MAS irrespective of the presence or absence of pFF. In the absence of pFF, FF-MAS at 3–10 μM increased the number of zygotes with advanced maternal pronuclear stages. In supraphysiological doses, i.e. 30–100 μM, FF-MAS dose-dependently and reversibly inhibited nuclear maturation in the absence of pFF.

Keywords: FF-MAS, Pig, Pronucleus formation, Sterols

All correspondence to: Inger Faerge, DVM, PhD, Institute of Basic Veterinary Sciences, Groennegaardsvej 7, 1870 Frb C, Copenhagen, Denmark. Tel: +45 35283882. Fax: +45 35282548. e-mail: if@kvl.dk
[1]Fertility Team, Novo Nordisk A/S, Copenhagen, Denmark.
[2]Constantine the Philosopher University, Nitra, Slovak Republic.
[3]Research Institute of Animal Production, Nitra, Slovak Republic.
[4]Faculty of Agriculture, Institute of Animal Breeding Science, University of Bonn, Bonn, Germany.
[5]University of Veterinary Medicine, Vienna, Austria.
[6]Veterinary and Agricultural University, Copenhagen, Denmark.

Introduction

In mammalian oocytes meiosis is initiated in fetal life and arrested in the prophase of first meiotic division. It is generally accepted that gonadotrophins induce the resumption of oocyte maturation by triggering the production of paracrine hormones in the somatic cell compartment surrounding the oocyte. Under these physiological conditions, a positive signal(s) mediated by gonadotrophins overcomes the upholding/inhibitory mechanism(s) present in the follicular environment. In contrast, when fully grown oocytes are removed from their follicles, the process of meiotic resumption occurs

spontaneously, a phenomenon described 70 years ago (Pincus & Enzmann, 1935).

Recently a group of 4,4-dimethylsterols has been isolated from human follicular fluid (FF) that are capable of inducing resumption of meiosis in prophase-arrested oocytes and have thus been designated meiosis-activating sterols (MAS). These sterols are intermediates in the cholesterol biosynthesis from lanosterol (Schroepfer, 1982), a synthesis occurring in most tissue, though without accumulation due to rapid conversion of the intermediates to cholesterol. In testis tissue and in ovarian preovulatory follicles, however, an accumulation of these sterols takes place and, specifically, the sterol 4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol has been isolated from human FF and termed follicular fluid meiosis-activating sterol (FF-MAS) (Byskov *et al.*, 1995). FF-MAS is converted from lanosterol by the CYP 51 gene product cytochrome P450 14α-demethylase (Stromstedt *et al.*, 1996), an enzyme under the control of gonadotrophins, and thus an accumulation of FF-MAS takes place in the ovary and the cumulus–oocyte complex (COC). FF-MAS affects the nuclear as well as the cytoplasmic maturation of immature rodent oocytes exposed *in vitro* (Grøndahl *et al.*, 1998; Hegele-Hartung *et al.*, 1999; Bivens *et al.*, 2004), thus leading to improved fertilization rate (Hegele-Hartung *et al.*, 1998) and subsequent pre-implantational development (Bivens *et al.*, 2004). The concentration of FF-MAS in the preovulatory FF of various species is in the same low micromolar range, i.e. 0.2 μM in pig and mare and approximately 1.6 μM in human FF. Addition of FF-MAS to immature oocytes from human patients suffering from polycystic ovarian syndrome increased the survival rate of the oocytes, and also improved the maturation rate of immature human oocytes donated from women undergoing intracytoplasmic sperm injection (Cavilla *et al.*, 2001). Together, these data suggest that FF-MAS improves the outcome of maturation *in vitro* of immature oocyte originating from various species, apparently by protecting mammalian oocytes from precocious chromatid segregation (Cukurcam *et al.*, 2003).

Porcine embryos produced by *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) can develop to blastocysts (Mattioli *et al.*, 1989). Significant differences in morphology and numbers of cells observed in IVC blastocysts compared with their *in vivo* counterparts suggest that the quality of the *in vitro* systems is still inferior to the *in vivo* condition. Despite the considerable progress made in the *in vitro* production of pig embryos by the use of IVM and IVF, the overall efficiency, possibly caused by poor cytoplasmic maturation leading to polyspermic penetration, remains low and is thus a problem for such *in vitro* systems. Since the somatic cell compartment surrounding the oocyte is known to play a role in reducing polyspermy, and since FF-MAS most likely has a somatic cell origin (Byskov *et al.*, 1997) besides having an effect on cytoplasmic maturation, the present study was performed to elucidate the effect of exposure of porcine COCs to FF-MAS on *in vitro* maturation, male and female pronucleus formation and polyspermy rate.

Materials and methods

Chemicals were purchased from Sigma Chemical (St Louis, MO) unless otherwise indicated.

In vitro production of porcine embryos

In vitro produced embryos were obtained after IVM of oocytes followed by IVF and IVC as previously described (Rath *et al.*, 1999). Briefly, ovaries were collected from prepubertal gilts slaughtered at a nearby abattoir and transported to the laboratory within 1 h in a pre-warmed thermos at 20–25 °C. Follicles with a diameter of 2–5 mm were punctured with a needle and the follicular content aspirated into 50 ml Falcon tubes with a flow rate set to 20 ml/min. Aspirates were allowed to settle for 10 min at room temperature. Subsequently, they were washed twice in mPBS (Dulbecco's phosphate-buffered saline, supplemented with 1% newborn calf serum), and then distributed into 100 mm Petri dishes. Under a stereomicroscope, COCs were evaluated and only intact COCs with at least three cumulus cell layers and evenly granulated ooplasm were selected into 50 mm Petri dishes, pre-filled with mPBS.

For IVM, oocytes were transferred into bovine serum albumin (BSA)-free NCSU 37 (Petters & Wells, 1993) supplemented with 6 mM L-cysteine, 10 ng/ml epidermal growth factor and 50 μM β-mercaptoethanol with or without 10% (v/v) porcine FF (pFF), subsequently designated FF^+ and FF^- media. For the first 24 h, 1 mM db-cAMP and 10 IE PMSG/hCG were added, together with either 1 μM, 3 μM, 10 μM, 30 μM or 100 μM FF-MAS dissolved in ethanol. In the control experiments adequate amounts of ethanol were added into the culture media. To assess the reversibility of a high dosage of FF-MAS on oocyte degeneration, nuclear maturation and sperm penetration, in some experiments the oocytes were cultured for the first 24 h in FF^- culture medium enriched with 100 μM FF-MAS. This regimen was followed by 24 h culture of the oocytes in FF-MAS-free medium in FF^+ and FF^- media, respectively. The results were then compared with those obtained after 48 h culture of the oocytes in NCSU 37 serum-free medium supplemented with 100 μM FF-MAS.

https://doi.org/10.1017/S0967199406003765 Published online by Cambridge University Press

Table 1 Influence of pFF and FF-MAS on sperm penetration rates

Medium	Monospermy N/n	%	Polyspermy N/n	%	Non-fertilized N/n	%
Control FF$^+$	223/80	35.87	223/41	18.39	223/67	30.04
1 μM FF$^+$	77/29	37.66	77/10	12.99	77/35	45.45
3 μM FF$^+$	122/50	40.98	122/22	18.03	122/38	31.15
10 μM FF$^+$	119/29	24.37	119/32	26.89	119/34	28.57
30 μM FF$^+$	130/33	25.38	130/36	27.69	130/31	23.85
100 μM FF$^+$	96/22	22.92	96/20	20.83	96/26	27.08
Control FF$^-$	113/17	15.04	113/13	11.50	113/51	45.13
1 μM FF$^-$	102/16	15.69	102/12	11.76	102/42	41.18
3 μM FF$^-$	101/24	23.76	101/4	3.96	101/45	44.55
10 μM FF$^-$	94/14	14.89	94/3	3.19	94/44	46.81
30 μM FF$^-$	118/3	2.54	118/13	11.02	118/40	33.90
100 μM FF$^-$	104/3	2.88	104/4	3.85	104/19	18.27

Control FF$^+$, NCSU 37 supplemented with pFF; control FF$^-$, NCSU 37 without pFF; N, total number of ova evaluated; n, calculated from all evaluated ova.

For IVF, matured COCs with maximally expanded intact cumulus cells were mechanically denuded and placed into TALP fertilization medium (Rath *et al.*, 1999) in 90 μl drops, each containing 50 oocytes. Frozen epididymal semen (0.75 ml) was thawed, diluted in 10 ml pre warmed (38 °C) Androhep and washed once (400 *g*, 3 min). The supernatant was discharged and the remaining pellet resuspended in 430 μl mTALP. The final concentration was adjusted to 1500 sperm per oocyte dissolved in 10 μl mTALP.

Sperm penetration and pronucleus development

At 16–18 h post-fertilization ova were fixed in acetic acid alcohol (1:3 v/v) and 24 h later stained with aceto-orcein (1% v/v). The specimens were evaluated under a microscope at ×1200 and the sperm penetration into the ooplasm and paternal and maternal pronucleus formation were recorded. Monospermic ova were classified into five developmental categories (PN1 to PN5) according to the criteria used previously (Laurincik *et al.*, 1994). Oocytes were classified as being normally fertilized when chromosomes, sperm head or two pronuclei with a nearby sperm tail could be identified within the ooplasm. In category PN1 the ooplasm was completely penetrated by the spermatozoon and the second meiotic division had resumed. Decondensation of the chromosomes began to appear in category PN2, the sperm tail was detached and the second polar body was extruded. Category PN3 was characterized by further decondensation and the appearance of the nuclear envelope. The second polar body was located close to the female pronucleus. In category PN4 decondensation of the chromosomes was completed. The spherical pronuclei were surrounded by a complete envelope and reached maximum size in category PN5. Ova with either aberrant chromatin, without chromatin or without distinctly recognizable pronuclei were evaluated as degenerative.

Statistical analyses

All experiments comprised 18 replicates, involving a total of 2418 ova. For the statistical analyses the test for the equality of the parameters of two binomial distributions was used. The results were considered significant at $p < 0.05$.

Results

The influence of ethanol

In present study no statistically significant influence of ethanol at the concentrations used was observed on any criteria described below.

Sperm penetration

Comparison of FF$^+$ and FF$^-$ media showed that pFF significantly improved the number of oocytes penetrated with single sperm (15% vs 36%, $p < 0.05$; Table 1, Fig. 1). When FF-MAS was added to FF$^+$ medium no effect was observed at 1 and 3 μM, whereas at 10–100 μM the monospermy rate was reduced to from 35% to 23–25% ($p < 0.05$). In FF$^-$ medium 3 μM FF-MAS increased the monospermy rate from 15% to 24% ($p < 0.05$), whereas higher concentrations of FF-MAS (30 and 100 μM) decreased the rate of monospermic penetration from 15% to 3% ($p < 0.05$).

The presence of pFF increased polyspermic penetration from 12% to 18% ($p < 0.05$) (Table 1, Fig. 2) and the addition of 10 and 30 μM FF-MAS, respectively, increased the polyspermy rate further to 27% and

https://doi.org/10.1017/S0967199406003765 Published online by Cambridge University Press

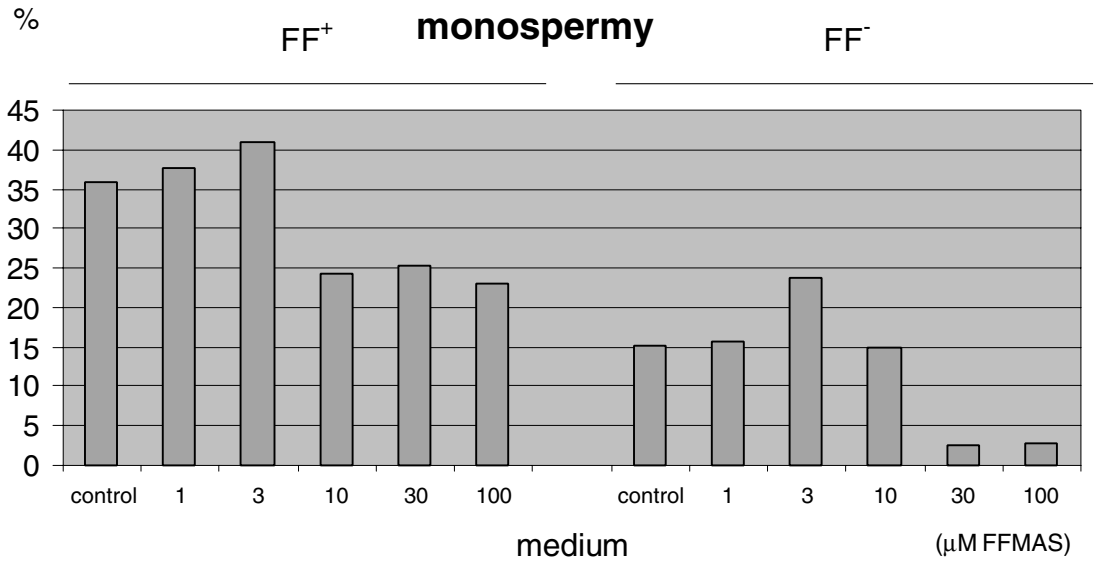

Figure 1 Influence of pFF and FF-MAS on monospermic penetration of oocytes. FF^+, presence of pFF; FF^-, absence of pFF. Statistical analysis (significance level $p < 0.05$): control FF^+ vs control FF^-, 10 µM/FF^+, 30 µM/FF^+, 100 µM/FF^+; 1 µM/FF^+ vs 10 µM/FF^+, 30 µM/FF^+, 100 µM/FF^+, 1 µM/FF^-; 3 µM/FF^+ vs 10 µM/FF^+, 30 µM/FF^+, 100 µM/FF^+, 3 µM/FF^-; 3 µM/FF^+ vs 10 µM/FF^+, 30 µM/FF^+, 100 µM/FF^+, 3 µM/FF^-; 30 µM/FF^+ vs 30 µM/FF^-; 100 µM/FF^+ vs 100 µM/FF^-; 30 µM/FF^-, 100 µM/FF^- vs control FF^-, 1 µM/FF^-, 3 µM/FF^-,10 µM/FF^-.

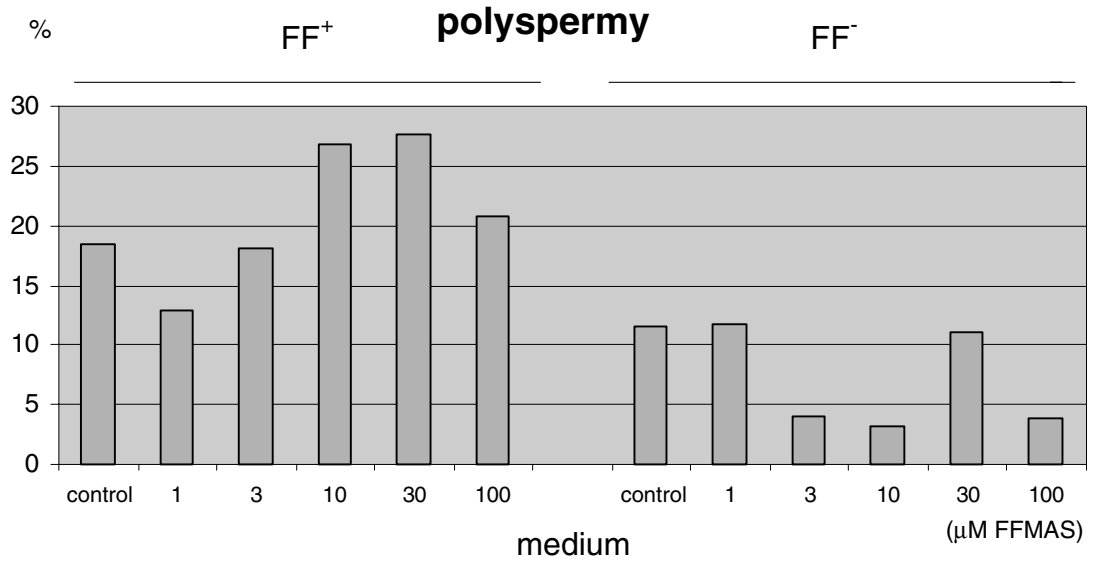

Figure 2 Influence of pFF and FF-MAS on polyspermic penetration of oocytes. FF^+, presence of pFF; FF^-, absence of pFF^-. Statistical analysis (significance level $p < 0.05$): control FF^+ vs control FF^-; control FF^+ vs 10 µM/FF^+, 30 µM/FF^+; 1 µM/FF^+, 3 µM/FF^+, 100 µM/FF^+ vs 10 µM/FF^+, 30 µM/FF^+; 3 µM/FF^+ vs 3 µM/FF^-; 10 µM/FF^+ vs 10 µM/FF^-; 30 µM/FF^+ vs 30 µM/FF^-; 100 µM/FF^+ vs 100 µM/FF^-; control FF^- vs 3 µM/FF^-, 10 µM/FF^-, 100 µM/FF^-; 1 µM/FF^- vs 3 µM/FF^-, 10 µM/FF^-, 100 µM/FF^-; 30 µM/FF^- vs 3 µM/FF^-, 10 µM/ FF^-, 100 µM/FF^-.

28% ($p < 0.05$). In FF^- medium 3 and 10 µM FF-MAS decreased the polyspermy rate from 12% to 3% and 4%, respectively ($p < 0.05$).

The addition of pFF decreased the non-fertilization rate (Table 1, Fig. 3) from 45% to 30% ($p < 0.05$). In the presence of pFF, FF-MAS increased the non-fertilization rate at 1 µM ($p < 0.05$). In the absence of pFF there was no effect of FF-MAS on the non-fertilization rate except at 100 µM, where a decrease from 45% to 19% ($p < 0.05$) was observed.

https://doi.org/10.1017/S0967199406003765 Published online by Cambridge University Press

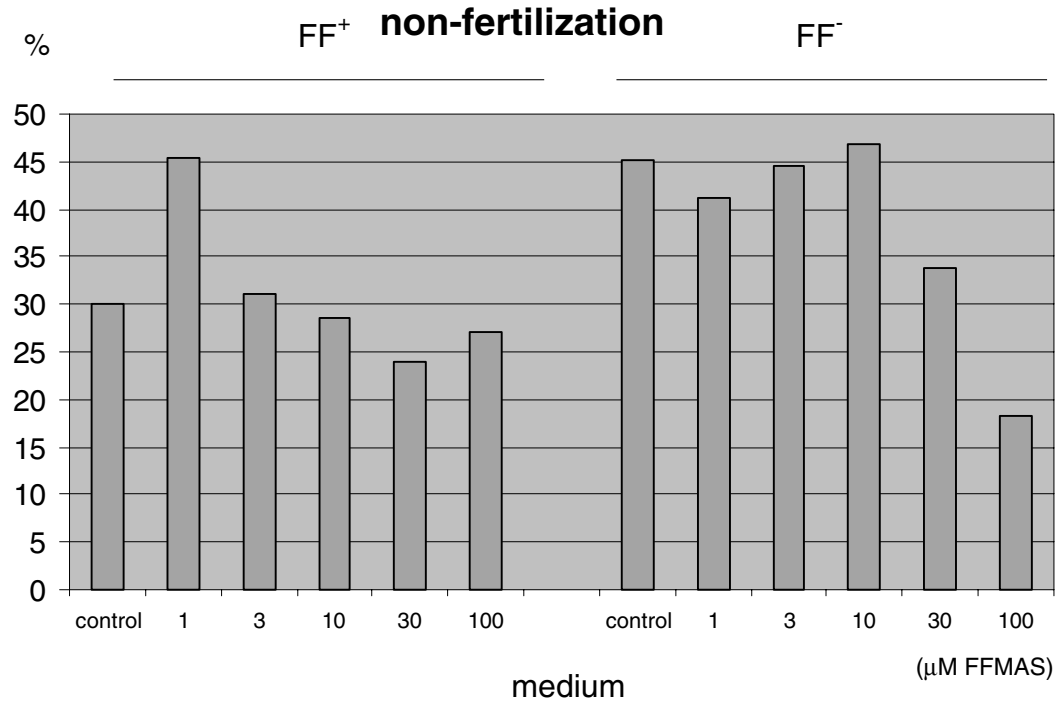

Figure 3 Influence of pFF and FF-MAS on the number of non-fertilized oocytes. FF^+, presence of pFF; FF^-, absence of pFF^-. Statistical analysis (significance level $p < 0.05$): control FF^+ vs control FF^-; 1 μM/FF^+ vs control FF^+, 3 μM/FF^+, 10 μM/FF^+, 30 μM/FF^+, 100 μM/FF^+; 3 μM/FF^+ vs 3 μM/FF^-; 10 μM/FF^+ vs 10 μM/FF^-; 30 μM/FF^+ vs 30 μM/FF^-; 100 μM/FF^+ vs 100 μM/FF^-; 100 μM/FF^- vs control FF^-, 1 μM/FF^-, 3 μM/FF^-, 10 μM/FF^-, 30 μM/FF^-.

Table 2 Influence of pFF and FF-MAS on degeneration and inhibition of nuclear maturation of ova

Medium	Degenerated N/n	%	Germinal vesicle N/n	%
Control FF^+	223/35	15.70	223/0	–
1 μM FF^+	77/3	3.90	77/0	–
3 μM FF^+	122/12	9.84	122/0	–
10 μM FF^+	119/23	19.33	119/1	0.84
30 μM FF^+	130/27	20.77	130/3	2.31
100 μM FF^+	96/22	22.92	96/6	6.25
Control FF^-	113/30	26.55	113/2	1.77
1 μM FF^-	102/17	16.67	102/15	14.71
3 μM FF^-	101/24	23.76	101/4	3.96
10 μM FF^-	94/23	24.47	94/10	10.64
30 μM FF^-	118/43	36.44	118/19	16.10
100 μM FF^-	104/27	25.96	104/51	49.04

Control FF^+, NCSU 37 supplemented with pFF; control FF^-, NCSU 37 without pFF; N, total number of ova evaluated; n, calculated from all evaluated ova.

Degeneration of oocytes

Addition of pFF decreased the degeneration rate of ova from 27% to 16% ($p < 0.05$) (Table 2, Fig. 4). FF-MAS dose-dependently decreased the degeneration rate in the presence of pFF ($p < 0.05$), whereas in FF^- medium 1 μM FF-MAS decreased the degeneration rate from 27% to 17% ($p < 0.05$). In FF^- medium, 30 μM FF-MAS increased the degeneration rate ($p < 0.05$).

Inhibition of nuclear maturation

In the absence of pFF, a dose-dependent inhibition of oocyte maturation rate induced by FF-MAS was observed after 44–46 h in culture ($p < 0.05$) (Table 2, Fig. 5), whereas in the presence of pFF only a slight inhibitory effect was observed.

Reversibility of the influence of high dosage of FF-MAS on degeneration, inhibition of nuclear maturation and sperm penetration of the ova

When control COCs were cultured for 48 h in the presence of 100 μM FF-MAS, a 49% germinal vesicle (GV) rate and a degeneration rate of 26% were observed (Table 3, Fig. 6). In the FF^- medium group the oocytes were cultured for 24 h in the presence of 100 μM FF-MAS and subsequently transferred to FF-MAS-free conditions in FF^- medium. In this group the GV rate was similar to that of the control group (44%) and the degeneration rate was 13%. When oocytes were transferred to FF-MAS-free medium in the presence of pFF, the FF^+ group, the GV rate was 25% and the degeneration rate was 8%. Thus, the degenerative effect of 100 μM FF-MAS was reversed in both FF^+ and FF^-

https://doi.org/10.1017/S0967199406003765 Published online by Cambridge University Press

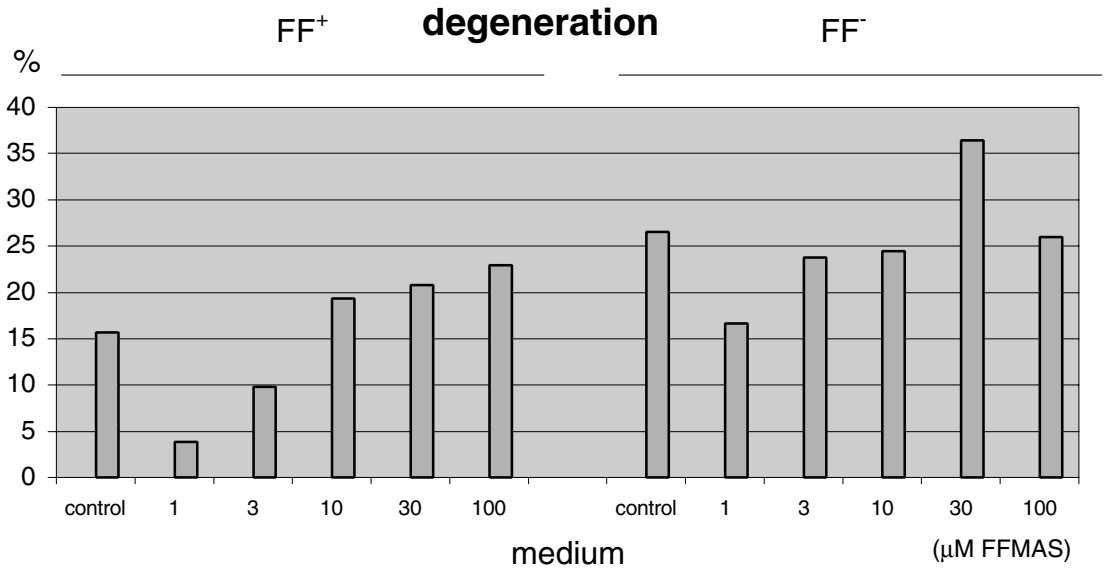

Figure 4 Influence of pFF and FF-MAS on the degeneration of ova. FF^+, presence of pFF; FF^-, absence of pFF^-. Statistical analysis (significance level $p < 0.05$): control FF^+ vs control FF^-, 1 μM/FF^+, 3 μM/FF^+; 1 μM/FF^+ vs 10 μM/FF^+, 30 μM/FF^+, 100 μM/FF^+, 1 μM/FF^-; 3 μM/FF^+ vs 10 μM/FF^+, 30 μM/FF^+, 100 μM/FF^+, 3 μM/FF^-; control FF^- vs 1 μM/FF^-, 30 μM/FF^-; 30 μM/FF^- vs control FF^-, 1 μM/FF^-, 3 μM/FF^-, 10 μM/FF^-, 100 μM/FF^-, 30 μM/FF^+.

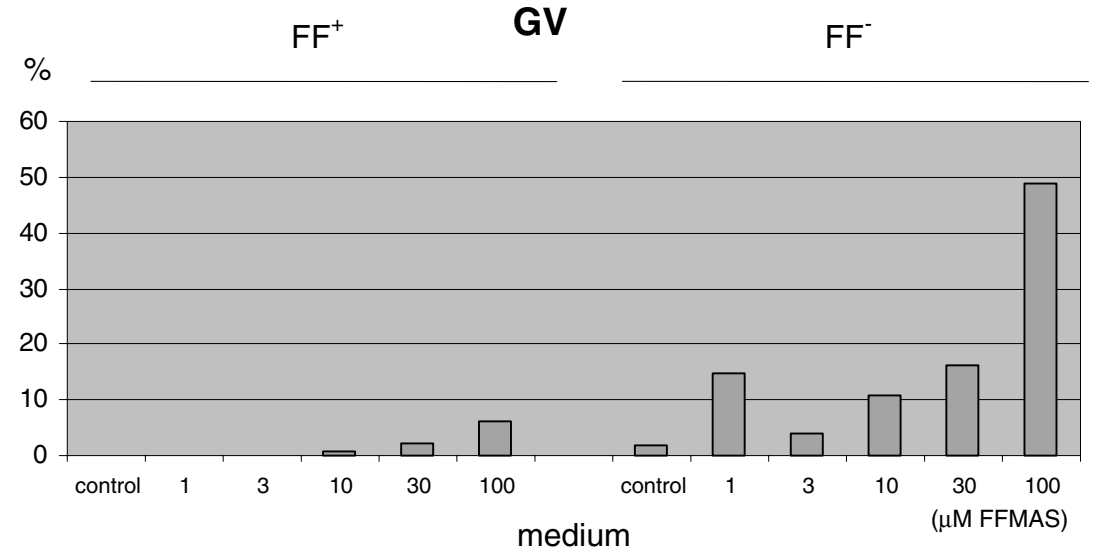

Figure 5 Influence of pFF and FF-MAS on the inhibition of nuclear maturation of the oocytes. FF^+, presence of pFF; FF^-, absence of pFF^-. Statistical analysis (significance level $p < 0.05$): control FF^+ vs control FF^-, 30 μM/FF^+, 100 μM/FF^+; 1 μM/FF^+ vs 1 μM/FF^-; 3 μM/FF^+ vs 3 μM/FF^-; 10 μM/FF^+ vs 10 μM/FF^-; 30 μM/FF^+ vs 30 μM/FF^-; 100 μM/FF^+ vs 1 μM/FF^+, 3 μM/FF^+, 10 μM/FF^+, 100 μM/FF^-; control FF^- vs 1 μM/FF^-, 10 μM/FF^-, 30 μM/FF^-, 100 μM/FF^-; 1 μM/FF^- vs 3 μM/FF^-; 3 μM/FF^- vs 1 μM/FF^-, 30 μM/FF^-; 100 μM/FF^- vs 1 μM/FF^-, 3 μM/FF^-, 10 μM/FF^-, 30 μM/FF^-, 100 μM/FF^+.

media (Table 3, Fig. 6), whereas the inhibition of nuclear maturation was reversible only in FF^+ medium.

Paternal pronucleus development

Porcine FF decreased the number of zygotes with retarded paternal pronucleus development (PN1–PN3) from 9% to 2% ($p < 0.05$) (Table 4, Fig. 7) and increased the number of advanced paternal pronuclear stages (PN4–PN5) from 6% to 39% ($p < 0.05$), which are typical for this time point of culture. In FF^+ medium, FF-MAS at 10–100 μM reduced the presence of advanced pronuclear stages from 34% to 19–23% ($p < 0.05$). In FF^- medium doses of 3 and 10 μM FF-MAS significantly increased the number of zygotes

https://doi.org/10.1017/S0967199406003765 Published online by Cambridge University Press

Table 3 Reversibility of the influence of a high dosage of FF-MAS on degeneration, inhibition of nuclear maturation and sperm penetration of ova

Criteria	Control N/n	%	FF^- N/n	%	FF^+ N/n	%
Degenerated	104/27	25.96	226/30	13.27	111/9	8.11
Germinal vesicle	104/51	49.04	226/99	43.81	111/28	25.23
Polyspermy	104/4	3.85	226/7	3.10	111/10	9.01
Monospermy	104/3	2.88	226/5	2.21	111/18	16.21

Control, oocytes were cultured for 48 h in NCSU 37 supplemented with 100 μM FF-MAS; FF^-, for the first 24 h the oocytes were cultured in NCSU 37 supplemented with 100 μM FF-MAS and for the next 24 h of culture the FF-MAS was omitted; FF^+, for the first 24 h the oocytes were cultured in NCSU 37 supplemented with 100 μM FF-MAS and for the next 24 h of culture the FF-MAS was omitted and NCSU 37 was supplemented with pFF; *N*, total number of ova evaluated; *n*, calculated from all evaluated ova.

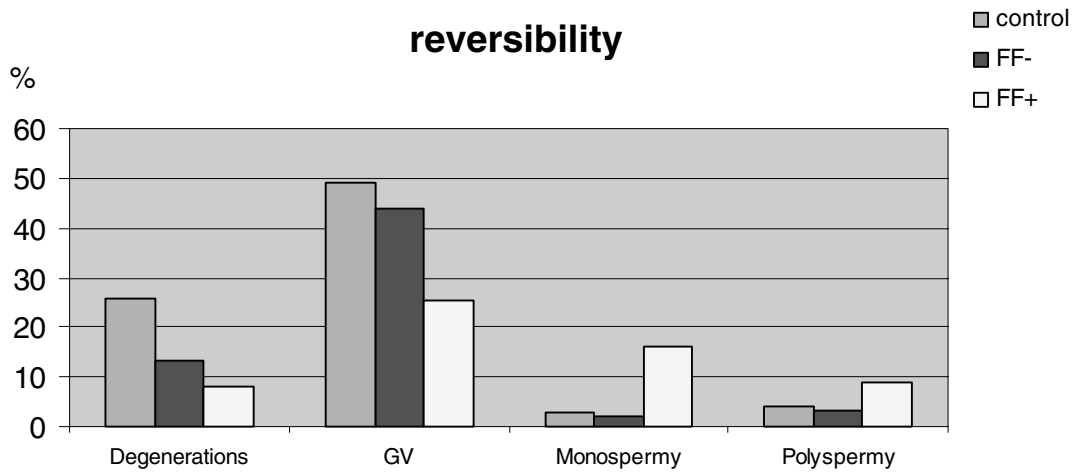

Figure 6 Reversibility of the influence of a high dosage of FF-MAS on degeneration, inhibition of nuclear maturation and sperm penetration of the ova. Control oocytes were cultured for 48 h in NCSU 37 supplemented with 100 μM FF-MAS. FF^-, for the first 24 h the oocytes were cultured in NCSU 37 supplemented with 100 μM FF-MAS and for the next 24 h of culture the FF-MAS was omitted. FF^+, for the first 24 h the oocytes were cultured in NCSU 37 supplemented with 100 μM FF-MAS and for the next 24 h of culture the FF-MAS was omitted and NCSU 37 was supplemented with pFF. Statistical analysis (significance level $p < 0.05$): degeneration: control vs FF^+, FF^-; GV: FF^+ vs control, FF^-; polyspermy: FF^+ vs FF^-; monospermy: FF^+ vs control, FF^-.

Table 4 Influence of pFF and FF-MAS on paternal pronucleus development

Medium	PN1–PN3 N/n	%	PN4–PN5 N/n	%
Control FF^+	223/5	2.24	223/75	33.63
1 μM FF^+	77/7	9.09	77/22	28.57
3 μM FF^+	122/12	9.84	122/38	31.15
10 μM FF^+	119/4	3.36	119/25	21.01
30 μM FF^+	130/3	2.31	130/30	23.08
100 μM FF^+	96/4	4.17	96/18	18.75
Control FF^-	113/10	8.85	113/7	6.19
1 μM FF^-	102/10	9.80	102/6	5.88
3 μM FF^-	101/13	12.87	101/11	10.89
10 μM FF^-	94/3	3.19	94/11	11.70
30 μM FF^-	118/3	2.54	118/0	–
100 μM FF^-	104/3	2.88	104/0	–

Control FF^+, NCSU 37 supplemented with pFF; control FF^-, NCSU 37 without pFF; *N*, total number of ova evaluated; *n*, calculated from all evaluated ova; PN, pronucleus.

with advanced pronuclear stages from 6% to 11% and 12% ($p < 0.05$), whereas higher doses of FF-MAS (30 and 100 μM) in FF^- medium blocked the development of higher developmental stages of paternal pronuclei completely.

Maternal pronucleus development

Porcine FF in the control medium did not influence the number of zygotes with PN1–PN3 (Table 5, Fig. 8), whereas the number of PN4–PN5 increased from 12% to 34% ($p < 0.05$). At 1 μM FF-MAS the number of PN1–PN3 stages in FF^+ increased from 2% to 9% ($p < 0.05$) and in FF^- medium 1 μM and 3 μM FF-MAS increased the PN1–PN3 stages from 4% to 10% ($p < 0.05$). At high concentrations of FF-MAS (30 and 100 μM) the normal development of maternal pronuclei was reduced in both FF^+ and FF^- media.

https://doi.org/10.1017/S0967199406003765 Published online by Cambridge University Press

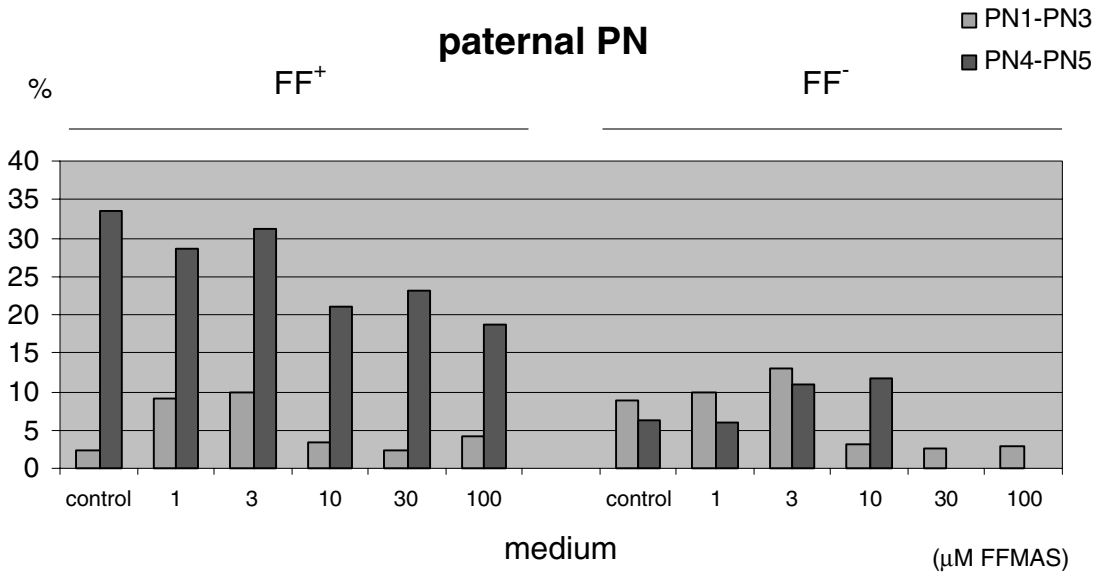

Figure 7 Influence of pFF and FF-MAS on paternal pronucleus development. FF^+, presence of pFF; FF^-, absence of pFF^-. Statistical analysis (significance level $p < 0.05$): **PN1–PN3**: control FF^+ vs control FF^-, 1 µM/FF^+, 3 µM/FF^+; 1 µM/FF^+, 3 µM/FF^+ vs control FF^+, 10 µM/FF^+, 30 µM/FF^+, 100 µM/FF^+; control FF^-, 1 µM/FF^-, 3 µM/FF^- vs 10 µM/FF^-, 30 µM/FF^-, 100 µM/FF^-; **PN4–PN5**: control FF^+ vs control FF^-, 10 µM/FF^+, 30 µM/FF^+, 100 µM/FF^+; 1 µM/FF^+ vs 1 µM/FF^-; 3 µM/FF^+ vs 3 µM/FF^-; 10 µM/FF^+ vs 10 µM/FF^-; 30 µM/FF^+ vs 30 µM/FF^-; 100 µM/FF^+ vs 100 µM/FF^-; control FF^-, 1 µM/FF^- vs 3 µM/FF^-, 10 µM/FF^-, 30 µM/FF^-, 100 µM/FF^-.

Table 5 Influence of pFF and FF-MAS on maternal pronucleus development

Medium	PN1–PN3 N/n	%	PN4–PN5 N/n	%
Control FF^+	223/4	1.79	223/76	34.08
1 µM FF^+	77/7	9.09	77/22	28.57
3 µM FF^+	122/4	3.28	122/46	37.70
10 µM FF^+	119/4	3.36	119/25	21.01
30 µM FF^+	130/3	2.31	130/30	23.08
100 µM FF^+	96/2	2.08	96/20	20.83
Control FF^-	113/4	3.54	113/13	11.50
1 µM FF^-	102/10	9.80	102/6	5.88
3 µM FF^-	101/10	9.90	101/14	13.86
10 µM FF^-	94/1	1.06	94/13	13.83
30 µM FF^-	118/3	2.54	118/0	–
100 µM FF^-	104/1	0.96	104/2	1.92

Control FF^+, NCSU 37 supplemented with pFF; control FF^-, NCSU 37 without pFF; N, total number of ova evaluated; n, calculated from all evaluated ova; PN, pronucleus.

Table 6 Influence of pFF and FF-MAS on synchronous development of paternal and maternal pronuclei

Medium	Synchronous pronuclei N/n	%
Control FF^+	223/75	33.63
1 µM FF^+	77/22	28.57
3 µM FF^+	122/38	31.15
10 µM FF^+	119/25	21.01
30 µM FF^+	130/30	23.08
100 µM FF^+	96/18	18.75
Control FF^-	113/7	6.19
1 µM FF^-	102/6	5.88
3 µM FF^-	101/11	10.89
10 µM FF^-	94/11	11.70
30 µM FF^-	118/0	0.00
100 µM FF^-	104/0	0.00

Control FF^+, NCSU 37 supplemented with pFF; control FF^-, NCSU 37 without pFF; N, total number of ova evaluated; n, calculated from all evaluated ova.

Synchrony of pronucleus development

The influence of pFF and different doses of FF-MAS on the proportion of advanced developmental stages of both paternal and maternal pronuclei is summarized in Table 6 and Fig. 9. The presence of pFF had a positive effect on the number of the zygotes with normal and synchronous pronucleus development (6% versus 34%, $p < 0.05$). In the absence of pFF there was a tendency towards a positive effect on the number of zygotes (6% vs 12%) – however, not significant – when FF-MAS was added at low doses (1–10 µM). High doses of FF-MAS (30 and 100 µM) significantly reduced the synchrony of pronuclei in both FF^+ and FF^- media ($p < 0.05$).

Discussion

In the present study the effect of FF-MAS on early porcine embryonic development *in vitro* was investigated.

https://doi.org/10.1017/S0967199406003765 Published online by Cambridge University Press

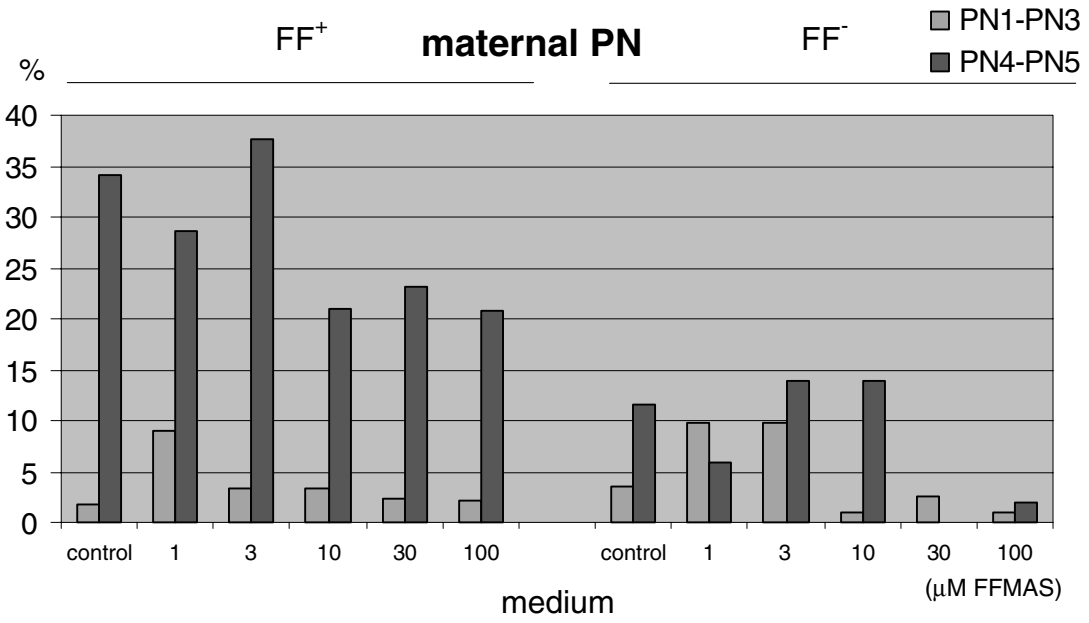

Figure 8 Influence of pFF and FF-MAS on maternal pronucleus development. FF^+, presence of pFF; FF^-, absence of pFF^-. Statistical analysis (significance level $p < 0.05$): **PN1–PN3**: 1 μM/FF^+ vs control FF^+, 30 μM/FF^+, 100 μM/FF^+; 3 μM/FF^+ vs 3 μM/FF^-; 1 μM/FF^-, 3 μM/FF^- vs 10 μM/FF^-, 30 μM/FF^-, 100 μM/FF^-, control FF^-; **PN4–PN5**: control FF^+ vs control FF^-, 10 μM/FF^+, 30 μM/FF^+, 100 μM/FF^+; 1 μM/FF^+ vs 1 μM/FF^-; 3 μM/FF^+ vs 10 μM/FF^+, 30 μM/FF^+, 100 μM/FF^+, 3 μM/FF^-; 30 μM/FF^- vs control FF^-, 1 μM/FF^-, 3 μM/FF^-, 10 μM/FF^-, 30 μM/FF^+; 100 μM/FF^- vs control FF^-, 3 μM/FF^-, 10 μM/FF^-, 100 μM/FF^+.

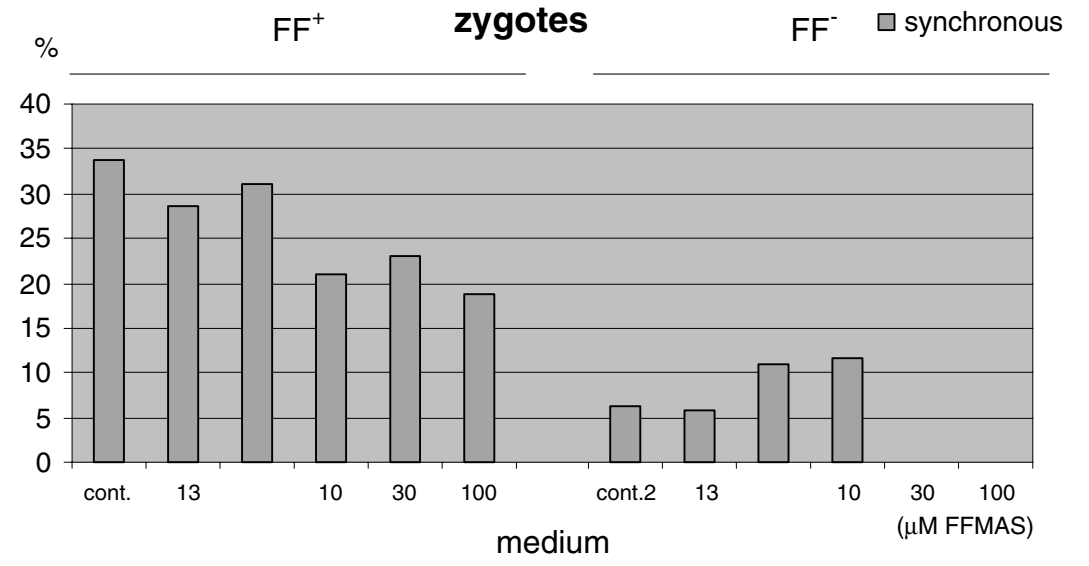

Figure 9 Influence of pFF and FF-MAS on synchronous development of paternal and maternal pronuclei. FF^+, presence of pFF; FF^-, absence of pFF^-. Statistical analysis (significance level $p < 0.05$): control FF^+ vs control FF^-, 10 μM/FF^+, 30 μM/FF^+, 100 μM/FF^+; 1 μM/FF^+ vs 1 μM/FF^-, 10 μM/FF^+, 30 μM/FF^+, 100 μM/FF^+; 3 μM/FF^+ vs 3 μM/FF^-, 10 μM/FF^+, 30 μM/FF^+, 100 μM/FF^+; 1 μM/FF^- vs 30 μM/FF^-, 100 μM/FF^-; 30 μM/FF^- vs control FF^-, 1 μM/FF^-, 3 μM/FF^-, 10 μM/FF^-, 30 μM/FF^+; 100 μM/FF^- vs control.

The results demonstrate that porcine COCs can be matured and fertilized in the absence of pFF and benefit from the presence of FF-MAS in the maturation medium.

Addition of pFF to the maturation medium has been demonstrated to enhance nuclear maturation *in vitro*, prevent polygyny and improve male pronucleus formation (Yoshida *et al.*, 1990, 1992; Naito

https://doi.org/10.1017/S0967199406003765 Published online by Cambridge University Press

et al., 1988). Similarly, we found that fertilization rate was higher in the presence of pFF than in its absence. Earlier studies have shown that *in vitro* matured porcine COCs display lower sperm penetration and cleavage rates compared with their *in vivo* counterparts (Laurincik *et al.*, 1994), probably due to insufficient maturation conditions. In addition to being a protein source, pFF is known to contain growth factors and hormones (Hsu *et al.*, 1987; Ainsworth *et al.*, 1980), thus possibly being able to partially compensate for the suboptimal *in vitro* conditions compared with the *in vivo* situation. In this context we also believe that the endogenous sterol content present in FF is active; however, we did not measure the concentration in this study.

Under *in vivo* conditions polyspermic fertilization appears to be reduced to a very low incidence by the female reproductive tract controlling the number of spermatozoa reaching the ovulated COC and by the polyspermic block displayed by the zona pellucida and oolemma. Polyspermy is one of the major problems of *in vitro* fertilization in the pig, and it appears that the block against polyspermy at the level of the zona pellucida and oolemma is not efficient in COCs matured *in vitro* (Coy *et al.*, 2002). In the present study the addition of FF-MAS to the maturation medium in the presence of pFF did not affect the fertilization rate, but addition of physiological doses of FF-MAS in the absence of pFF reduced the polyspermy rate. This suggests that FF-MAS may play a role in cytoplasmic maturation, possibly leading to improved efficiency of the zona pellucida block. It has previously been demonstrated that FF-MAS has a positive effect on cytoplasmic maturation of mouse oocytes leading to an improved fertilization rate (Hegele-Hartung *et al.*, 1998). We also found that FF-MAS at 1 μM, regardless of the presence or absence of pFF, decreased the degeneration rate of ova, an effect that is likely to be connected to improved cytoplasmic maturation.

The finding that FF-MAS could inhibit GV breakdown in a dose-dependent and reversible manner is surprising, since FF-MAS has so far exclusively been known as a pro-meiotic compound. It should be noted, however, that this effect was seen only at doses above the physiological range. A biological principle for the reversible inhibition of spontaneous oocyte maturation is highly desirable due to its wide range of applications in assisted reproductive technologies. Maintaining oocytes at GV stage without damaging their quality would allow synchronization of maturation and homogenization of the oocyte population. Compounds such as 6-DMAP, cycloheximide, roscovitine and butyrolactone I have been used to block the resumption of meiosis in porcine oocytes, though with varying efficiency (Le Beux *et al.*, 2003), and roscovitine treatment of porcine oocytes has resulted in abnormal metaphase II morphology with aberrant meiotic spindles and/or formation of cytoplasmic microtubules (Ju *et al.*, 2003). The question of whether supraphysiological doses of FF-MAS can be used as a reversible inhibitor of oocyte maturation needs to be explored in more detail.

Factors affecting the ability of the oocyte to form a pronucleus are manifold and include hormonal conditions, follicular secretions, intracellular ionic strength and oxidative stress. The ability of the oocyte to form pronuclei after sperm penetration strongly depends on the presence of cumulus cells during maturation and fertilization (Mattioli *et al.*, 1988). It is likely that mammalian oocytes acquire the ability to break down the sperm nuclear envelope during cytoplasmic maturation. Thus, incomplete maturation or inadequate conditions result in partial or delayed spermatozoon decondensation. The cause of delayed male pronucleus formation may be a lower concentration of male nucleus growth factor in *in vitro* matured COCs (Hunter, 1967; Calvin *et al.*, 1986). In line with our results, Naito *et al.* 1988 found that the formation of male pronuclei increases when pFF is added. Further, this could be enhanced by the addition of FSH, and the authors suggest that the synergistic effect of pFF and FSH on male pronucleus formation is due to the probable presence of a substance in the pFF stimulating cytoplasmic development (Naito *et al.*, 1988). In this context, the finding that FSH affects male pronucleus formation is interesting, since FF-MAS production in the cumulus cells is stimulated mainly by FSH (Byskov *et al.*, 1997) and thus it could explain the increase in male pronucleus formation in our study after the addition of physiological doses of FF-MAS in the absence of pFF.

No difference in the rate of development of paternal and maternal pronuclei has been observed in *in vivo* developed porcine zygotes, indicating that the process of pronucleus formation is well synchronized *in vivo* in the pig (Laurincik *et al.*, 1995). In contrast, *in vitro* matured oocytes display retarded male and enhanced female pronucleus formation (Laurincik *et al.*, 1994). As expected, the addition of pFF increased the synchrony of male and female pronucleus formation, whereas there was only a small positive effect of FF-MAS on the synchrony of pronucleus formation in the absence of pFF.

In conclusion, we have demonstrated in this study that FF-MAS can affect porcine COC maturation, fertilization and early development in various ways. A desired effect of FF-MAS has been demonstrated on lowering the degeneration rate of ova and on polyspermy rate regardless of the presence or absence of pFF. However, it is clear from the data presented here that determination of the correct dose of FF-MAS is crucial in order to make proper conclusions concerning this compound.

Acknowledgement

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) and Bundesministerium für Wissenschaft und Verkehr der Republik Österreich (GZ 45.517/I-VI/B/7a/2002).

References

Ainsworth, L., Tsang, B.K., Downey, B., Marcus, G.J. & Armstrong, D.T. (1980). Interrelationships between follicular fluid steroid levels, gonadotrophic stimuli, and oocyte maturation during preovulatory development of porcine follicles. *Biol. Reprod.* **23**, 621–7.

Bivens, M., Lindenthal, B., O'Brien, M.J., Wigglesworth, K., Blume, T., Grondahl, C. & Eppig, J.J. (2004). A synthetic analogue of meiosis-activating sterol (FF-MAS) is a potent agonist promoting meiotic maturation and preimplantation development of mouse oocytes maturing *in vitro*. *Hum Reprod.* **19**, 2340–4.

Byskov, A.G., Andersen, C.Y., Nordholm, L., Thøgersen, H., Guoliang, X., Wassmann, O., Andersen, J.V., Guddal, E. & Roed, T. (1995). Chemical structure of sterols that activate oocyte meiosis. *Nature* **374**, 559–62.

Byskov, A.G., Andersen, C.Y., Hossaini, A. & Guoliang, X. (1997). Cumulus cells of oocyte–cumulus complexes secrete a meiosis-activating substance when stimulated with FSH. *Mol. Reprod. Dev.* **46**, 296–305.

Calvin, H., Grosshans, K. & Blake, E. (1986). Estimation and manipulation of glutathione levels in prepubertal mouse ovaries and ova: relevance to sperm nucleus transformation in the fertilized egg. *Gamete Res.* **14**, 265–75.

Cavilla, J.L., Kennedy, C.R., Baltsen, M., Klentzeris, L.D., Byskov, A.G. & Hartshorne, G.M. (2001). The effects of meiosis activating sterol on *in vitro* maturation and fertilization of human oocytes from stimulated and unstimulated ovaries. *Hum. Reprod.* **16**, 547–55.

Coy, P., Gadea, J., Romar, R., Matas, C. & Garcia, E. (2002). Effect of *in vitro* fertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and *in vitro* development in pigs. *Reproduction* **124**, 279–88.

Cukurcam, S., Hegele-Hartung, C. & Eichenlaub-Ritter, U. (2003). Meiosis-activating sterol protects oocytes from precocious chromosome segregation. *Hum. Reprod*. **18**, 1908–17.

Donnay, I., Faerge, I., Grondahl, C., Verhaeghe, B., Sayoud, H., Ponderato, N., Galli, C. & Lazzari, G. (2004). Effect of prematuration, meiosis activating sterol and enriched maturation medium on the nuclear maturation and competence to development of calf oocytes. *Theriogenology* **15**, 1093–107.

Grøndahl, C., Ottesen, J.L., Lessl, M., Faarup, P., Murray, A., Grønvald, F.C., Hegele-Hartung, C. & Ahnfelt-Rønne, I. (1998). Meiosis-activating sterol promotes resumption of meiosis in mouse oocytes cultured *in vitro* in contrast to related oxysterols. *Biol. Reprod.* **58**, 1297–302.

Hegele-Hartung, C., Lessl, M., Ottesen, J. & Grøndahl, C. (1998). Oocyte maturation can be induced by a synthetic meiosis activating sterol (MAS) leading to improvement of IVF rate in mice. *Hum. Reprod. Suppl.* **13**, 98.

Hegele-Hartung, C., Kuhnke, J., Lessl, M., Grøndahl, C., Ottesen, J., Beier, H.M., Eisner, S. & Eichenlaub-Ritter, U. (1999). Nuclear and cytoplasmic maturation of mouse oocytes after treatment with synthetic meiosis-activating sterol *in vitro*. *Biol. Reprod.* **61**, 1362–72.

Hsu, C., Holmes, S. & Hammond, J. (1987). Ovarian epidermal growth factor-like activity: concentrations in the porcine follicular fluid during follicular enlargement. *Gamete Res.* **147**, 242–7.

Hunter, R. (1967). Polyspermic fertilization in pigs during luteal phase of the estrous cycle. *J. Exp. Zool.* **165**, 451–66.

Ju, J.C., Tsay, C. & Ruan, C.W. (2003). Alterations and reversibility in the chromatin, cytoskeleton and development of pig oocytes treated with roscovitine. *Mol. Reprod. Dev*. **64**, 482–91.

Laurincik, J., Rath, D. & Niemann, H. (1994). Differences in pronucleus formation and first cleavage following *in vitro* fertilization between pig oocytes matured *in vivo* and *in vitro*. *J. Reprod. Fertil.* **102**, 277–84.

Laurincik, J., Hyttel, P. & Kopecny, V. (1995). DNA synthesis and pronucleus development in pig zygotes obtained *in vivo*: an autoradiographic and ultrastructural study. *Mol. Reprod. Dev.* **40**, 325–32.

Le Beux, G., Richard, F.J. & Sirard, M.A. (2003). Effect of cycloheximide, 6-DMAP, roscovitine and butyrolactone I on resumption of meiosis in porcine oocytes. *Theriogenology* **1**, 1049–58.

Mattioli, M., Galeati, G. & Seren, E. (1988). Effect of follicle somatic cells during pig oocyte maturation on egg penetration and male pronucleus formation. *Gamete Res.* **20**, 177–83.

Mattioli, M., Bacci, M.L., Galeati, G. & Seren, E. (1989). Developmental competence of pig oocytes matured and fertilized *in vitro*. *Theriogenology* **31**, 1201–7.

Naito, K., Fukuda, Y. & Toyodam, Y. (1988). Effects of porcine follicular fluid on male pronucleus formation in porcine oocytes matured *in vitro*. *Gamete Res.* **21**, 289–95.

Petters, R.M. & Wells, K.D. (1993). Culture of pig embryos. *J. Reprod. Fertil. Suppl.* **48**, 61–73.

Pincus, G. & Enzmann, E.V. (1935). The comparative behavior of mammalian eggs *in vivo* and *in vitro*. I. The activation of ovarian eggs. *J. Exp. Med.* **62**, 655–75.

Rath, D., Long, C.R., Dobrinsky, J., Welsh, G., Schreier, L.L. & Johnson, L. (1999). *In vitro* production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. *J. Anim. Sci.* **77**, 3346–52.

Schroepfer, G.J. (1982). Sterol biosynthesis. *Annu. Rev. Biochem.* **51,** 555–85.

Stromstedt, M., Rozman, D. & Waterman, M.R. (1996). The ubiquitously expressed human CYP51 encodes lanosterol 14α-demethylase, a cytochrome P450 whose expression is regulated by oxysterols. *Arch. Biochem. Biophys.* **329**, 73–81.

Yoshida, M., Ishizaki, Y. & Kawagishi, H. (1990). Blastocyst formation by pig embryos resulting from *in vitro* fertilization of oocytes matured *in vitro*. *J. Reprod. Fertil.* **88**, 1–8.

Yoshida, M., Ishizaki, Y., Kawagishi, H., Bamba, K. & 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*. *J. Reprod. Fertil.* **95**, 481–8.