Differences in pronucleus formation and first cleavage following *in vitro* fertilization between pig oocytes matured *in vivo* and *in vitro*

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To elucidate the developmental differences occurring after *in vitro* fertilization (IVF) of pig oocytes matured either *in vitro* ($n = 1934$) or *in vivo* ($n = 1128$), the present experiment investigated the morphological changes from penetration to the two-cell stage. Oocytes were examined every 2–4 h from 2 to 32 h after *in vitro* insemination to study sperm penetration, male and female pronucleus formation, synkaryosis and first cleavage. The penetration rate was significantly higher ($P < 0.05$) for *in vivo* matured oocytes (69.8%) than for *in vitro* matured oocytes (35.0%). Penetration of spermatozoa into the ooplasm was first recorded 6 h (*in vitro* matured oocytes) and 4 h (*in vivo* matured oocytes) after addition of the spermatozoa to the oocytes. For both *in vivo* and *in vitro* matured oocytes, 2 h were required for sperm head decondensation. However, maximum sperm head decondensation occurred 2 h later in *in vitro* matured oocytes. Within 6 h, $41.7 \pm 5.6\%$ of the *in vivo* matured oocytes had completed second meiotic division, whereas only $20.8 \pm 6.5\%$ of the *in vitro* matured oocytes reached this developmental stage ($P < 0.01$). For *in vitro* matured oocytes, male pronucleus formation was retarded 2–4 h after onset of insemination and development of the female pronucleus was enhanced compared with *in vivo* matured oocytes. Synchronized opposing pronuclei were observed 14 h after insemination in *in vitro* matured oocytes and after 8 h in *in vivo* matured oocytes. Synkaryosis was first observed at 16 and 18 h in *in vivo* and *in vitro* matured oocytes, respectively. First cleavage was observed 32 h (*in vitro* matured oocytes) and 28 h (*in vivo* matured oocytes) after insemination. It is concluded that under our IVF conditions, oocytes matured *in vitro* display lower penetration and cleavage rates and asynchronous pronucleus development, as well as delayed cleavage, compared with oocytes matured *in vivo*.

Introduction

Pig oocytes matured *in vivo* and obtained by aspiration from pre-ovulatory follicles can be fertilized *in vitro* using fresh boar semen (Cheng *et al.*, 1986; Yoshida, 1987; Yoshida *et al.*, 1990; Rath, 1992) or frozen–thawed epididymal spermatozoa (Nagai *et al.*, 1988). Similarly, it has been shown that *in vitro* matured pig oocytes can be fertilized *in vitro* (Mattioli *et al.*, 1988a; Yoshida *et al.*, 1993). However, polyspermy (Nagai *et al.*, 1984; Cheng, 1985; Mattioli *et al.*, 1988a) and irregular male pronucleus formation (Motlik and Fulka, 1974; Nagai *et al.*, 1984; Mattioli *et al.*, 1988a; Naito *et al.*, 1988; Wang *et al.*, 1991) perturb early embryonic development and limit potential practical applications of IVF technology. *In vivo*, communication between cumulus cells and oocytes in pig (Motlik and Fulka, 1986; Mattioli *et al.*, 1988a, b) and bovine (Laurincik *et al.*, 1992a, b) cumulus–oocyte complexes is required for proper maturation of the respective oocytes and plays a crucial role in regular early embryonic development (Mattioli *et al.*,

1988b). *In vitro*, inappropriate culture conditions lead to an abnormal distribution of the cortical granules (Mattioli *et al.*, 1988a; Hyttel *et al.*, 1988a; Nagai *et al.*, 1988; Nagai and Moor, 1990; Yoshida *et al.*, 1990). The reduced or delayed release of specific substances from the granules is thought to be responsible for the establishment of an incomplete zona block (Cran and Cheng, 1986).

The influence of media on nuclear maturation of pig oocytes cultured *in vitro* has been reported by Tsafirri and Channing (1975), Sato *et al.* (1977), Racowsky and McGaughey (1982), Eng *et al.* (1986) and Yoshida *et al.* (1989). However, culture conditions for a complete and synchronous maturation of both nuclear and cytoplasmic compartments in pig oocytes have only partly been explored (Tsafirri *et al.*, 1976; Meinecke and Meinecke-Tillmann, 1979; Motlik and Fulka, 1986; Moor *et al.*, 1990; Moor, 1993). Low rates of male pronucleus formation in pig oocytes matured and fertilized *in vitro* persist (Iritani *et al.*, 1978; Nagai *et al.*, 1984; Mattioli *et al.*, 1988b; Yoshida *et al.*, 1990).

Follicular fluid has been demonstrated to have profound effects on male pronucleus formation (Naito *et al.*, 1988; Naito and Toyoda, 1992), as well as follicular cells (Mattioli *et al.*,

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1988b; Zheng and Sirard, 1992; Ding and Foxcroft, 1992), LH (Mattioli *et al.*, 1991), pregnant mares' serum gonadotrophin (PMSG), hCG and oestradiol (Funahashi and Day, 1993), and glutathione and cysteine (Yoshida *et al.*, 1992b, 1993; Naito and Toyoda, 1992). Similar findings were observed when cumulus cells surrounded the oocyte during fertilization (Wang *et al.*, 1991; Niwa, 1993).

Analysis of pig oocytes within the first 10 h after *in vitro* fertilization showed that sperm penetration begins 3 h after insemination. This was followed by sperm head decondensation and nuclear maturation up to anaphase II in 50% of penetrated oocytes within 5 h after insemination. Both male and female pronuclei were present 8 h after insemination (Ding *et al.*, 1992).

The objective of the present study was to compare the sequence of morphological development of both male and female pronuclei during the complete first embryonic cell cycle after *in vitro* fertilization of pig cumulus–oocyte complexes matured either *in vivo* or *in vitro*.

Materials and Methods

Sources of oocytes

In vitro matured oocytes. Ovaries were collected from prepubertal gilts at a local abattoir and within 1 h after slaughter were transported at room temperature to the laboratory. Follicles 2–5 mm in diameter were punctured; cumulus–oocyte complexes were collected in modified Dulbecco's phosphate buffered saline (PBS); and the aspirated fluid was examined at $\times 10$ –60 magnification. Cumulus–oocyte complexes were washed once and then placed into preincubated (39°C, 5% CO₂ in air, humidified atmosphere) TCM 199 (Sigma, St Louis, MO) supplemented with 1 µg purified FSH ml⁻¹ (UCB, l'Alleude), 0.1 mg l-glutamine ml⁻¹ (Sigma), 25 mmol Hepes l⁻¹ (Sigma), 10% new born calf serum (NBCS) (Boehringer, Mannheim) and 5 mg Gentamicin ml⁻¹ (Sigma) (maturation medium). Oocytes (five per microdrop) with firmly attached cumulus cell layers and an evenly granulated cytoplasm were selected for cultivation in maturation medium using 50 µl microdrops covered with preincubated (39°C, 5% CO₂ in air, and humidified atmosphere) silicone oil. Forty-eight hours later cumulus–oocyte complexes were evaluated for cumulus expansion and integrity of ooplasm (Rath, 1992).

In vivo matured oocytes. Prepubertal gilts were stimulated with 1500 iu PMSG (Intergonan; Vemie, Kempen), followed by 500 iu hCG (Ekluton; Vemie) 72 h later. The gilts were slaughtered to collect the oocytes 38 h thereafter. Follicles of 5–10 mm diameter were punctured and matured cumulus–oocyte complexes were preincubated for 2 h in TCM 199 supplemented with 0.1 mg l-glutamine ml⁻¹, 10% fetal calf serum (FCS) (Boehringer, Mannheim) and 10 µg dibekacin sulfate (Sigma) (Rath, 1992).

Semen preparation

Sperm rich fractions (20–30 ml) of ejaculates were collected from two German Landrace boars of proven fertility into

prewarmed (38°C) collection tubes by the gloved hand method. Immediately after collection, samples from both boars were mixed and diluted with Androhep extender (Minitüb, Tiefenbach) 1:1 (v:v) and treated for capacitation as described by Rath (1992). Briefly, semen samples were centrifuged, at 550 g for 5 min, twice and the pellets were resuspended with capacitation medium (modified TCM 199, pH 7.8). The concentration of spermatozoa was adjusted to 2×10^8 spermatozoa ml⁻¹ and the samples were incubated in humidified air for about 3.5 h at 39°C and 5% CO₂.

In vitro fertilization

Before *in vitro* fertilization, cumulus–oocyte complexes were denuded mechanically using a fine glass pipette and subsequently washed in fertilization medium (TCM 199), supplemented with 0.1 mg l-glutamine ml⁻¹, 10% FCS, 2.0 mmol caffeine l⁻¹ (Sigma) and 10 µg dibekacin sulfate. During denudation, the pH of the medium was kept constant using a special CO₂ chamber (5%), covering the whole microscope. Oocytes were fertilized in 5 ml fertilization medium (30 oocytes per Petri dish) containing 5×10^5 spermatozoa ml⁻¹.

Evaluation of events after insemination

From 4 to 32 h after *in vitro* insemination, oocytes from both groups were fixed at 2–4 h intervals in acetic alcohol (1:3 v:v) and were stained 24 h later with aceto-orcin (1% w/v). The preparations were evaluated under a microscope at $\times 1200$ and the sequence of sperm penetration into the ooplasm, male and female pronucleus formation and synkaryosis were recorded. Monospermic ova were classified into six developmental categories (PN1 to PN5 plus synkaryosis) according to the criteria for bovine oocytes given by Xu and Greve (1988). 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 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. At this time both pronuclei were close together and synkaryosis was initiated.

Statistical analyses

All experiments comprised 12 replicates involving a total of 1934 *in vitro* and 1124 *in vivo* matured oocytes. All data were tested for normal distribution (SAS Univariate procedure; SAS Institute Inc. Cary, NC). In cases of normal distribution data were analysed by the Chi square test and were considered

Table 1. Sperm penetration rates in oocytes matured *in vivo* or *in vitro*

Source of oocytes	<i>In vitro</i> matured oocytes	<i>In vivo</i> matured oocytes
Total (n)	1699	986
Penetration (n)	595	688
Penetration (%)	35.0 ^a	69.8 ^b
Monospermic (n/n)	485/595	649/688
Monospermic (%)	81.5 ^a	94.3 ^b
Polyspermic (n/n)	110/595	39/688
Polyspermic (%)	18.5 ^a	5.7 ^b

^{a,b}P < 0.05.

significant at *P* < 0.05. In all other cases data were tested for significance by the Kruskal–Wallis-test (Gogolok *et al.*, 1992). Data are presented as means ± SEM.

Results

Cumulus expansion

Cumulus corona layers from oocytes matured *in vivo* were completely expanded in all the cumulus–oocyte complexes of the experiment. Similarly, *in vitro* matured cumulus layers were fully expanded, but the dark rim of non-expanded corona radiata was still visible in more than half of the oocytes. At the end of the maturation period, enlargement of the perivitelline space and homogeneous ooplasm were common features for oocytes of both groups.

Sperm penetration

In vivo matured cumulus–oocyte complexes had a higher (*P* < 0.05) penetration rate than *in vitro* matured cumulus–oocyte complexes. The overall mean difference was 34.8% (Table 1). *In vivo* matured oocytes showed the first evidence of sperm penetration 4 h after insemination (Table 2). Penetration rate increased during the following 6 h and remained constant until the end of the observation period. In contrast, *in vitro* matured oocytes were first penetrated 6 h after insemination and reached a maximum after an additional 2 h. This plateau remained constant until the end of the observation period but on a lower level (35.2% versus 85.3%, *P* < 0.01) than that of *in vivo* matured oocytes. The rate of polyspermy increased significantly in *in vitro* matured oocytes (*P* < 0.05; Table 1). Polygyny was observed in only eight oocytes of both groups.

Pronucleus formation

Six hours after insemination 66.0 ± 7.8% of *in vitro* and 54.2 ± 4.2% of *in vivo* matured oocytes were in male pronucleus stage 1 (MPN1) (Fig. 1). However, at this time, the number of *in vivo* matured oocytes (45.8 ± 4.2%) at stage MPN2 was greater than the number of *in vitro* matured oocytes (13.2 ± 5.9%) (*P* < 0.05). *In vitro* matured oocytes showed a delayed sperm head decondensation of approximately 2 h.

Within the first 6 h after insemination (Fig. 1) 41.7 ± 5.6% of the *in vivo* matured oocytes had completed the second meiotic division (FPN2), whereas only 20.8 ± 6.5% of the *in vitro* matured oocytes reached this developmental stage (*P* < 0.01). In contrast to *in vivo* matured oocytes, 11.1 ± 5.9% of the *in vitro* matured oocytes had developed to FPN4 and 8.3 ± 4.3% to FPN5 by this time (Figs 2 and 3).

Late pronuclei stages (PN4 and PN5) developed in a synchronized manner in *in vivo* matured oocytes (Figs 2 and 3).

Table 2. Time sequence of penetration and frequencies of normal and abnormal fertilization at different time intervals after insemination *in vitro*

Time (h) ^a	Oocytes (n)	<i>In vitro</i> matured oocytes		Oocytes (n)	<i>In vivo</i> matured oocytes	
		Penetration Monospermic (%) ^b	Polyspermic (%) ^b		Penetration Monospermic (%) ^b	Polyspermic (%) ^b
4	56	0	0	60	3.3	0
6	200	11.5 ^c	0	76	30.2 ^d	0
8	191	35.0 ^c	2.0	73	58.9 ^d	0
10	79	36.7 ^c	7.7	73	83.5 ^e	10.9
12	107	40.1 ^c	9	71	81.6 ^e	2.8
14	126	27.7 ^c	8.7	75	84.0 ^d	4.0
16	121	34.7 ^c	9.2	71	85.9 ^d	2.8
18	110	36.3 ^c	6.5	74	85.1 ^d	5.4
20	114	37.7 ^c	11.5	70	80.0 ^d	7.2
22	140	40.7 ^c	7.1	63	84.1 ^e	3.3
24	100	38.0 ^c	9.0	65	76.9 ^d	4.7
28	120	35.8 ^c	13.4	65	80.0 ^e	6.2
32	125	35.2 ^c	10.4	75	85.3 ^e	8.1

^aHour after insemination; ^bcalculated from all fertilized oocytes.^{c,d}P < 0.05, ^eP < 0.01.

Fig. 1. (a, b) Male and (c, d) female pronucleus development ((a,c) PN1 and (b,d) PN2) of porcine oocytes matured *in vitro* or *in vivo* and fertilized *in vitro*. Oocytes matured *in vitro* (—) *in vivo* (---)

	$P \leq 0.05$	0.01	0.001
Male PN <i>in vitro</i> versus <i>in vivo</i>	■	■	■
Female PN <i>in vitro</i> versus <i>in vivo</i>	+	++	+++
Male PN versus female PN <i>in vitro</i>	*	**	***
Male PN versus female PN <i>in vivo</i>		••	

Male pronucleus formation was delayed in most of the *in vitro* matured oocytes between 6 and 12 h after insemination, but was accelerated between 12 and 14 h and was similar to that of *in vivo* matured oocytes by this time (Figs 1 and 2). Both pronuclei developed in a well-synchronized manner between 16 and 32 h after insemination (Figs 2 and 3).

Synkaryosis and first cleavage

Synkaryosis (Fig. 3) was first observed 16 h or 18 h after insemination in *in vivo* and *in vitro* matured oocytes, respectively. *In vivo* matured oocytes reached the maximum percentage (33.3%) of synkaryosis 20 h after insemination, whereas synkaryosis was delayed in *in vitro* matured oocytes for about 4 h. A second peak in synkaryosis was reached in both groups after 32 h. The percentage of synkaryosis was significantly lower (23.9% versus 30.8%; $P < 0.05$) for *in vivo* matured oocytes because most ($52 \pm 7.3\%$) had already progressed to first cleavage (Fig. 3).

Discussion

The results presented in this study show that there are distinct differences in the ability of *in vivo* and *in vitro* matured pig

oocytes to form female and male pronuclei and undergo first cleavage. Fertilization events were related to the onset of oestrus (Thibault, 1967), or were observed after pretreatment with hCG (Hunter, 1972) or after superovulation (Laurincik *et al.*, 1994). Time-dependent events after fertilization *in vivo* were described in cows (Hyttel *et al.*, 1988b), rabbits (Zamboni and Mastroianni, 1966) and mice (Edwards and Gates, 1959) and for the first 10 h after *in vitro* fertilization in pigs (Ding *et al.*, 1992).

Our study provides the first comparative analysis describing the time-dependent events of pronucleus development throughout the first embryonic cell cycle in oocytes obtained from *in vitro* and *in vivo* maturation. However, results from the *in vitro* matured oocytes can only be related to our *in vitro* maturation system.

The distinct differences between *in vitro* and *in vivo* matured oocytes could be related to the lower fertilization rates after *in vitro* fertilization in this species compared with cattle (see Brackett and Zuelke, 1993).

In vivo fertilization is thought to occur soon after the arrival of the matured oocytes in the oviduct already harbouring capacitated spermatozoa (Thibault, 1967; Hunter, 1972; Laurincik *et al.*, 1993, 1994). Under the conditions of our experiment, sperm penetration for *in vivo* matured cumulus-oocyte complexes was similar to that in non-stimulated

Fig. 2. (a, b) Male and (c, d) female pronucleus development ((a,c) PN3 and (b,d) PN4) of porcine oocytes matured *in vitro* or *in vivo* and fertilized *in vitro*. Oocytes matured *in vitro* (—) *in vivo* (---)

	$P \leq 0.05$	0.01	0.001
Male PN <i>in vitro</i> versus <i>in vivo</i>	■	■■	■■■
Female PN <i>in vitro</i> versus <i>in vivo</i>	+	++	+++
Male PN versus female PN <i>in vitro</i>	*	**	***

(Hunter, 1972) and stimulated (Laurincik *et al.*, 1994) gilts, demonstrating the physiological similarity of the three different sources of oocytes. In contrast to *in vivo* conditions as well as to data obtained after *in vitro* fertilization (Ding *et al.*, 1992), our data show that penetration was delayed after *in vitro* maturation of cumulus-oocyte complexes. Since semen was prepared identically in both groups of our study, it is suggested that insufficient maturation conditions (Xu and Greve, 1988) or an incomplete expanded corona layer (Laurincik *et al.*, 1992a, b) rather than deficiencies of the spermatozoa *in vitro* capacitation system (Cran and Cheng, 1986) were responsible for the delay and the lower penetration rate.

After fusion of the male and female gamete, the ovum is activated and a series of nuclear changes in both the fertilizing spermatozoon and the maternal chromosomes occurs. In the oviduct (Hunter, 1972; Cran and Cheng, 1986) and under our experimental conditions, *in vivo* matured oocytes required 2 h for sperm head decondensation and an additional 2–4 h to develop into opposing pronuclei. This process occurred in a well-synchronized manner in both the paternal and maternal pronucleus, which is similar to findings in cattle (Hyttel *et al.*, 1988b), hamsters (Wright and Longo, 1988) and humans (Lassalle and Testart, 1991).

A similar developmental sequence was observed after *in vitro* fertilization of pig oocytes by Ding *et al.* (1992) but no

differentiation between *in vivo* and *in vitro* matured oocytes was made.

In our experiment delayed male pronucleus formation during the first 14 h was observed, which supports the view that the concentration of male pronucleus growth factor (MPGF) was lower in *in vitro* matured cumulus-oocyte complexes (Hunter, 1967; Calvin *et al.*, 1986). For *in vitro* matured oocytes it was shown that female pronucleus formation was accelerated at the same time, indicating that this is independent of MPGF as suggested by Yanagimachi (1988). Male and female pronucleus development were found to be well synchronized after 16 h, indicating that, up to this point, the male pronucleus had undergone an accelerated development. Subsequently, first cleavage in *in vitro* matured oocytes was delayed for 4 h compared with *in vivo* matured oocytes, suggesting that the first cell cycle was delayed.

Polyspermic penetration is one of the major causes of the failure of morphologically uniform and intact gametes to develop normally, leading to irregular early embryonic development upon syngamy (Bomsel-Helmreich, 1961; Nagai *et al.*, 1984; Cheng, 1985; Mattioli *et al.*, 1988b; Nagai and Moor, 1990; Wang *et al.*, 1991; Zheng and Sirard, 1992). Excessive numbers of spermatozoa at the site of fertilization have been shown to increase the probability of polyspermic penetration (Hunter and Léglise, 1971). Cran and Cheng (1985, 1986)

Fig. 3. (a) Male and (b) female pronucleus development (PN5), (c) synkaryosis and (d) first cleavage of *in vitro* and *in vivo* matured oocytes fertilized *in vitro*. Oocytes matured *in vitro* (—) *in vivo* (---)

	$P \leq 0.05$	0.01	0.001
Male PN <i>in vitro</i> versus <i>in vivo</i>	■	■ ■ ■	■ ■ ■
Female PN <i>in vitro</i> versus <i>in vivo</i>		+	+
Male PN versus female PN <i>in vitro</i>		**	***
Syngamy <i>in vitro</i> versus <i>in vivo</i>	▲		
First cleavage <i>in vitro</i> versus <i>in vivo</i>		XX	XXX

observed a higher incidence of polyspermy followed by abnormal cleavage for *in vitro* fertilized oocytes compared with *in vivo* fertilized oocytes. We showed that the reduction of the number of spermatozoa per fertilizable oocyte raises the likelihood of normal fertilization considerably (Rath, 1992). Therefore, the number of spermatozoa was reduced in our experiment and the low incidence of polyspermy resembled the physiological condition (Hunter, 1967, 1972; Laurincik *et al.*, 1994). Our data suggest that the block against polyspermy at the level of zona or oolemma was not equally functional in *in vivo* and *in vitro* matured cumulus-oocyte complexes.

When compared with data reported earlier (Hunter, 1972), cleavage was found to be delayed by approximately 8–12 h under our experimental conditions. A similar finding was observed for *in vivo* matured oocytes (Yoshida, 1987) which could indicate nuclear or cytoplasmic insufficiencies (Hunter, 1990). Our results show that *in vitro* matured oocytes possessed less developmental competence than did *in vivo* matured oocytes. It has yet to be determined whether this can be attributed to the partly asynchronous pronuclei development or to a general incompetence of the ooplasm (Yoshida, 1992b).

The incidence of polygyny is mainly thought to be attributed to fertilization of immature or aged oocytes (Bedford,

1982). The time required to reach complete nuclear maturation *in vitro* varies considerably among immature oocytes (Sato *et al.*, 1978; Yoshida *et al.*, 1989) and the frequency of chromosomal abnormalities has been found to be high (McGaughey and Polge, 1971). Polygyny mainly resulting from failure of the extrusion of the first or second polar body was rarely observed in our study, indicating that the age of the oocytes was appropriate for our maturation conditions.

In summary, analysis of *in vitro* and *in vivo* matured oocytes at various times after *in vitro* fertilization revealed six prominent stages of early development, as defined previously in cattle (Xu and Greve, 1988). Co-incubation of *in vitro* matured cumulus-oocyte complexes with capacitated spermatozoa resulted in lower penetration and cleavage rates, asynchronous pronucleus formation and delayed first cleavage compared with oocytes matured *in vivo*. It has to be noted, however, that data obtained for *in vitro* matured oocytes are heavily dependent on the method of maturation used and may change as culture conditions are optimized, for example, by supplementation with follicular fluid (Yoshida *et al.*, 1992a, b).

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