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

J. Laurincik¹, D. Rath²*, and H. Niemann²

¹Research Institute of Animal Production, Hlňovska 2, 94992 Nitra, Slovak Republic; and ²Institut für Tierzucht und Tierverhalten (FAI) Mariensee, 31535 Neustadt, Germany

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 ± 5.6% of the in vivo matured oocytes had completed second meiotic division, whereas only 20.8 ± 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., 1988b; 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 Tsafiri 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 (Tsafiri 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 (Britani 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., 1988b).

*Correspondence.
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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 ×10–60 magnification. Cumulus–oocyte complexes were washed once and then placed into preincubated (39°C, 5% CO2 in air, humidified atmosphere) TCN 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 TCN 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 × 10^6 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 × 10^6 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-orcein (1% w/v). The preparations were evaluated under a microscope at ×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

<table>
<thead>
<tr>
<th>Source of oocytes</th>
<th>In vitro matured oocytes</th>
<th>In vivo matured oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n)</td>
<td>1699</td>
<td>986</td>
</tr>
<tr>
<td>Penetration (%)</td>
<td>595</td>
<td>688</td>
</tr>
<tr>
<td>Monospermic (n/%)</td>
<td>35.0**</td>
<td>69.8**</td>
</tr>
<tr>
<td>Monospermic (%)</td>
<td>485/595</td>
<td>649/688</td>
</tr>
<tr>
<td>Polyspermic (%)</td>
<td>81.5**</td>
<td>94.3**</td>
</tr>
<tr>
<td>Polyspermic (%)</td>
<td>110/595</td>
<td>39/688</td>
</tr>
<tr>
<td>Penetration (%)</td>
<td>18.5**</td>
<td>5.7**</td>
</tr>
</tbody>
</table>

**P < 0.05.

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 polyspermia increased significantly in in vitro matured oocytes (P < 0.05; Table 1). Polygyny was observed in only eight oocytes of both groups.

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 homogeneus ooplasm were common features for oocytes of both groups.

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

<table>
<thead>
<tr>
<th>Time (h)**</th>
<th>Oocytes (n)</th>
<th>In vitro matured oocytes</th>
<th>Penetration</th>
<th>Monospermic (%)**</th>
<th>Polyspermic (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>56</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>200</td>
<td>11.5**</td>
<td>0</td>
<td>0</td>
<td>76</td>
</tr>
<tr>
<td>8</td>
<td>191</td>
<td>35.0**</td>
<td>2.0</td>
<td>2.0</td>
<td>73</td>
</tr>
<tr>
<td>10</td>
<td>79</td>
<td>36.7**</td>
<td>7.7</td>
<td>7.7</td>
<td>73</td>
</tr>
<tr>
<td>12</td>
<td>107</td>
<td>40.1**</td>
<td>9</td>
<td>9</td>
<td>71</td>
</tr>
<tr>
<td>14</td>
<td>126</td>
<td>27.7**</td>
<td>8.7</td>
<td>8.7</td>
<td>75</td>
</tr>
<tr>
<td>16</td>
<td>121</td>
<td>34.7**</td>
<td>9.2</td>
<td>9.2</td>
<td>71</td>
</tr>
<tr>
<td>18</td>
<td>110</td>
<td>36.3**</td>
<td>6.5</td>
<td>6.5</td>
<td>74</td>
</tr>
<tr>
<td>20</td>
<td>114</td>
<td>37.7**</td>
<td>11.5</td>
<td>11.5</td>
<td>70</td>
</tr>
<tr>
<td>22</td>
<td>140</td>
<td>40.7**</td>
<td>7.1</td>
<td>7.1</td>
<td>63</td>
</tr>
<tr>
<td>24</td>
<td>100</td>
<td>38.0**</td>
<td>9.0</td>
<td>9.0</td>
<td>65</td>
</tr>
<tr>
<td>28</td>
<td>120</td>
<td>35.8**</td>
<td>13.4</td>
<td>13.4</td>
<td>65</td>
</tr>
<tr>
<td>32</td>
<td>125</td>
<td>35.2**</td>
<td>10.4</td>
<td>10.4</td>
<td>75</td>
</tr>
</tbody>
</table>

*Hour after insemination; **calculated from all fertilized oocytes.

*P < 0.05, **P < 0.01.
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 ± 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., 1987), 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 oocytes.
Porcine pronucleus development

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 (— —)

(a)

\[
\text{Percentage} \quad 0 \quad 4 \quad 6 \quad 8 \quad 10 \quad 12 \quad 14 \quad 16 \quad 18 \quad 20 \quad 22 \quad 24 \quad 28 \quad 32
\]

(b)

\[
\text{Percentage} \quad 0 \quad 4 \quad 6 \quad 8 \quad 10 \quad 12 \quad 14 \quad 16 \quad 18 \quad 20 \quad 22 \quad 24 \quad 28 \quad 32
\]

(c)

\[
\text{Percentage} \quad 0 \quad 4 \quad 6 \quad 8 \quad 10 \quad 12 \quad 14 \quad 16 \quad 18 \quad 20 \quad 22 \quad 24 \quad 28 \quad 32
\]

(d)

\[
\text{Percentage} \quad 0 \quad 4 \quad 6 \quad 8 \quad 10 \quad 12 \quad 14 \quad 16 \quad 18 \quad 20 \quad 22 \quad 24 \quad 28 \quad 32
\]

(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, 1987; 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)
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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