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# MATURATION, FERTILIZATION AND COMPLETE DEVELOPMENT OF PORCINE OOCYTES MATURED UNDER DIFFERENT SYSTEMS

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Received for publication: June 4, 1998
Accepted: November 4, 1998

## ABSTRACT

This study was designed 1) to determine the effectiveness of 2 in vitro maturation systems commonly employed to produce nuclear and cytoplasmicly mature pig oocytes, 2) to assess the effects of boar, sperm concentration and maturation system on oocyte penetrability and male pronucleus formation and 3) to determine the ability of the in vitro matured oocytes to be fertilized in vivo by artificial insemination (AI) of sows. The differences examined between the 2 maturation systems included the culture medium (Waymouth vs TCM199), hormones, additives, culture conditions (static vs gentle agitation) presence or absence of porcine follicular fluid (PFF) and presence or absence of follicular shells. The results showed that nuclear maturation rate was similar in both systems (83.3 ± 3.5 vs 86.4 ± 2.5%), and intracellular content of glutathione was 5.21 ± 0.73 vs 3.5 ± 0.39 pmol/oocyte, although no correlation between these parameters was observed. The penetration rate and number of sperm cells per oocyte were dependent on the boar, maturation system and sperm concentration, but the rate of male pronuclear formation seemed to be influenced only by the boar and the maturation system but not by sperm concentration. In vivo fertilization of in vitro matured oocytes showed that both maturation systems could yield viable oocytes since 3 of 4 gilts and 2 of 4 gilts, respectively, became pregnant. Failure to become pregnant was not associated with inadequate oocyte maturation since control gilts, which received their own ovulated oocytes rather than in vitro matured oocytes at transfer, also did not become pregnant. We conclude that polyspermy may be an inherent problem in the IVF but not in the IVM systems.



Key words: In vitro maturation, oocyte, male pronucleus, glutathione, pig

Acknowledgments
The authors thank Dr. R.M. Moor for critical reading of the manuscript; Dr. N. Ouhibi and Dr. H. Funahashi for technical support in IVM and GSH assays, respectively; O. Zubillaga for collecting boar semen; the staff of the El Pozo slaughterhouse for supplying the ovaries; the Lorca CCA for providing the gilts; and Dr. A. Romar for statistical review. This research was supported by Comisión Interministerial de Ciencia y Tecnología (CICYT) (AGF96-1069) and by Programa Séneca (COM-17/96 EXP).

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0093-691X/99/$–see front matter
PII S0093-691X(99)00028-X

## INTRODUCTION

In recent years, an increasing number of studies on pig in vitro maturation (IVM) and in vitro fertilization (IVF) have been reported. The wide range of new technologies, including that in applied molecular genetics, has increased this interest. The production of viable porcine embryos in vitro is a prerequisite for the successful production of transgenic pigs.

To date, the efficiency of IVM/IVF techniques in the porcine species is lower than that obtained in other species such as ruminants. Two major problems are generally thought to be the cause of poor results: the low rate of MPN formation derived from inadequate IVM of oocytes (31,36,38,41) and the high incidence of polyspermy after IVF (6,9,35,42).

The problem of a low rate of MPN formation has been widely studied, and, in recent years, the results have been improved (11,12,16,32,34,55). Basic studies of cellular and molecular factors operating during oocyte maturation have contributed to this advance (19,26,46,56), and, currently, primarily 2 different systems have been employed. In the first method, whole sperm nuclear decondensation is achieved and MPN formation results by culturing the oocytes in the presence of abundant follicular tissue (1,13,26,29,35). In the second system, similar results were obtained by supplementing the medium with cysteine, a substrate of glutathione (GSH) synthesis, which is involved in sperm nucleus decondensation (44,52). There are other differences between the 2 systems such as the culture medium employed, presence or absence of PFF and static vs gentle agitation culture conditions. Nevertheless, at present, few comparative studies examining all the above parameters of each system are available (7). For example, the effectiveness of maturation media on MPN formation or on high penetration rates have been compared (14,47,54), but these studies have utilized basically the same IVM/IVF/IVC systems for the different media examined. In addition, observations on MPN formation after IVF of oocytes matured under different conditions have usually only focused on the oocyte. Recently, Xu et al. (49) have suggested the MPN formation may be determined not only by the maturational stage of the oocyte but also by the spermatozoa of individual boars.

In the development of pig reproductive biotechnology, the sperm factor, that is, the problem of polyspermy, remains to be elucidated, and attempts to improve the rate of monospermic penetration have been only partially successful (15,25,35,45). Three possible reasons for the low rate of monospermic penetration include an excessive number of spermatozoa reaching the oocyte during IVF, an inadequate in vitro maturation of oocytes and culture conditions affecting sperm-oocyte interaction during IVF. Several workers (3,17) have suggested procedures for lowering sperm numbers for IVF in order to reduce the number of sperm cells reaching the oocyte surface. In vivo, polyspermy is observed when an excessive number of viable spermatozoa are deposited into the oviducts (22). Therefore, studies have been conducted in which the goal was to provide an optimal number of fully capacitated spermatozoa near oocytes

at the time of fertilization(6,42,50). The second possible cause of high polyspermy, that is, inadequate in vitro maturation of oocytes that would render them incapable of developing cortical granule reaction (20,41), does not seem probable since high levels of polyspermy have also been observed with in vivo matured oocytes (4,5,6,8,10,21,37). However, despite this last observation, the key to demonstrating the normal ability of in vitro matured oocytes to be fertilized and to develop cortical granule reaction would be to fertilize them in vivo. Once the third possible cause of polyspermy was established, a study could be designed to examine and improve specific IVF conditions during sperm-oocyte interaction.

The present study was therefore designed 1) to determine the effectiveness of 2 maturation systems commonly employed to obtain oocytes of full nuclear an cytoplasmic maturity; 2) to establish the effects of boar, sperm concentration and maturation system on the penetrability of oocytes and on MPN formation; and 3) to asses the ability of in vitro matured oocytes to be fertilized in vivo by artificial insemination of sows.

## MATERIALS AND METHODS

### Oocyte Collection

Within 30 min of slaughter, ovaries from prepubertal gilts were transported to the laboratory in saline (0.9% w/v NaCl) containing 100 mg/L Kanamycin and 50 mg/L Polymyxin B sulfate (Sigma Chemicals, St. Louis, MO, USA) at 37°C. The ovaries were washed once in 0.04% Cetrimide (Sigma) solution and twice in saline. Cumulus-oocyte complexes were collected from nonatretic follicles (3 to 6 mm diameter) and washed twice in 35-mm plastic Petri dishes containing Dulbecco's phosphate buffered saline modified (PBSDm; Sigma) supplemented with 4 mg/mL polyvinyl alcohol. Then they were washed twice more in maturation medium previously equilibrated for a minimum 3 h at 38.5°C under 5% $CO_2$ in air.

### In Vitro Maturation Systems

Two maturation systems were used in this study. The first, System 1 (S1), corresponded with that described by Yoshida et al. (52) and employed Waymouth (Sigma) as maturation medium, which contained a higher concentration of cysteine (0.57mM) than others, PFF and culture under static conditions (without agitation). The only modifications consisted of using 3 droplets 100 µl of medium covered with paraffin oil per dish, 20 oocytes per droplet and a 44-h culture period. System 2 (S2) consisted of 2 mL of TCM 199 (Sigma), 30 oocytes and 2 everted theca shells per dish (35). The dishes were cultured on a mechanical platform providing gentle agitation. Medium 199 was supplemented with 10% fetal calf serum (FCS; Biological Industries, Beitz Haemek, Israel), 100 µg/ mL glutamine (Sigma), 75 µg/ mL L-ascorbic acid (Sigma), 5 µg/ mL myo-inositol (Sigma), 110 µg/ mL sodium pyruvate (Sigma),10 µg/ mL bovine apotransferrin (Sigma), 10 µg/ mL insulin (Sigma), 2 µg/ mL Vitamin $B_{12}$ and

gonadotrophins (0.3 IU/mL h-FSH and 0.3 IU/mL h-LH; Pergonal, Serono; Robert M. Moor, personal communication). Thus, the differences studied in the 2 systems included the medium, hormones, additives, static vs non static culture conditions, presence or absence of pFF, and presence or absence of follicular shells.

The common culture conditions for both systems were the temperature (38.5°C), atmosphere (5% $CO_2$ in air), culture period (44 h) and number of oocytes (120 per system per replicate).

Assay of Glutathione

Intracellular content of glutathione (GSH) was measured as described by Funahashi et al. (14). In vitro matured oocytes from each maturation system were pooled, washed in PBS, and then 30 oocytes per system per replicate were used for GSH assay. Cumulus cells were removed by pipetting. Five microliters of PBS containing those 30 oocytes were transferred to a 2-mL microfuge tube, and 5 μL of 1.25 M phosphoric acid (Sigma) were added. Samples were stored in a freezer (-20°C) until assayed. The content of GSH in the oocytes was determined by the DTNB-GSSG reductase recycling assay (2). The total amount of GSH measured was divided by the number of oocytes in the sample to obtain the content per oocyte.

In Vitro Fertilization

The in vitro fertilization medium TCM199 was supplemented as described previously (5). For insemination, sperm rich fractions were collected by the gloved hand method from 3 boars of proven fertility, based on previous IVF and AI assays (Boars A, B and C, Experiment 2) or from only one boar (Boar B, Experiment 3 and Boar A, Experiment 4) and diluted at 3 x $10^7$ cells/mL in Betsville Thawing Solution extender (BTS) (24). Only samples with satisfactory semen characteristics (motility > 70%, morphological abnormalities <15% and acrosome abnormalities <10%) were used. The sperm samples in Experiments 2 and 3 were washed, without previous storage, by centrifugation at 120xg for 3 min in order to remove heavy particles. The supernatant was concentrated (1250xg, 3 min) and the pellet was resuspended in M 199 to the desired final concentration (5 x$10^5$ and $10^6$/mL in Experiment 2; $10^4$, 5x $10^4$, 2.5 x $10^5$ and 5 x $10^5$ /mL in Experiment 3). A final volume of 100 μL was introduced into Petri dishes containing 2 mL of fertilization medium and 20 mature oocytes from the pool (n=120) of each IVF system. After 18 h, the cultured oocytes were fixed, stained with 1% (w/v) lacmoid and examined for evidence of sperm penetration under a phase contrast microscope (at x400 magnification).

Oocyte Transfers

Eleven crossbred pubertal gilts 5 to 6 mo of age were induced to ovulate with 1250 IU, imPMSG (Intervet, Salamanca, España) and 750 IU hCG (Intervet, Salamanca, España) 55 h later (5). After 43 h (1 h before transfer) the gilts were inseminated with

fresh diluted spermatozoa from Boar A. The ovulated oocytes were flushed out from the oviducts after exposure through mid-ventral incision under general anaesthesia, as we previously described (5), except in one gilt which was used as a surgery control and no flushing was carried out. The ovaries were examined, and the number of preovulatory follicles (whose content was aspirated), points of ovulation or corpora lutea as well as the number of oocytes recovered were noted. Immediately after flushing, 20 in vitro matured oocytes (Groups S1 and S2) or the own in vivo matured oocytes from 2 gilts (Control Group) were introduced into each oviduct by a Tom Cat Catheter (Sherwood Medical, St. Louis, MO, USA) connected to an insulin syringe containing PBSDm at 37°C. The catheter was later observed under a stereomicroscope in order to check that all the oocytes were transferred into the oviducts. The gilts were kept under the usual conditions, and 26 to 28 days after transfer pregnancy diagnosis was carried out by ultrasonography (Toshiba SAL 32B).

Experimental Design and Statistical Analysis

Experiment 1. This experiment was designed to compare the effectiveness of 2 maturation systems in obtaining fully matured oocytes. After the culture period, oocytes were randomly collected and evaluated for nuclear or cytoplasmic maturation. Nuclear maturation was evaluated as the rate of oocytes (n= 896) reaching Metaphase II (MII) in each system, as determined by fixing, staining and examination under phase contrast microscopy (at x400 magnification). Cytoplasmic maturation was measured as the intracellular concentration of glutathione (14) per oocyte (n=360). This experiment was conducted in 6 replicates.

Experiment 2. To evaluate the influence of the maturation system and of boar on oocyte penetrability and MPN formation, mature oocytes (n=954) from Systems 1 and 2 were fertilized in vitro with spermatozoa collected from 3 different boars (2 x 3 factorial). The rate of oocyte penetration, number of sperm per penetrated oocyte and rate of male pronuclear formation were evaluated for each boar in each maturation system. Only oocytes with at least 2 pronuclei (1 female and 1 male) were considered to have the ability to develop a male pronucleus. Sperm concentration was $5 \times 10^5$ cells/mL for Boar A and $1 \times 10^6$ cells/mL for Boars B and C. These concentrations were chosen on the basis of previous experiments, which indicated that these sperm concentrations yielded optimal ratios between penetration and polyspermy. Experiment 2 was conducted in 5 replicates, each on a different day, and data from each replicate were pooled.

Experiment 3. To determine if the sperm concentration affects male pronucleus formation, oocytes (n= 868) matured in Systems 1 and 2 were fertilized in vitro with spermatozoa from Boar B at different concentrations ($1 \times 10^4$, $5 \times 10^4$, $2.5 \times 10^5$ and $5 \times 10^5$ cells/mL). The penetration rate, number of sperm cells per oocyte and MPN formation were evaluated for each concentration with each oocyte maturation system (2 x 4 factorial). Experiment 3 was also conducted in 5 replicates, and data were pooled as in Experiment 2.

Experiment 4. This experiment was carried out to check the viability of in vitro matured oocytes in Systems 1 and 2. Gilts were divided into 3 groups: Group S1 (n=4) gilts received S1 oocytes; Group S2 (n=4) gilts received S2 oocytes, while Control group (n=2) gilts received their own ovulated oocytes at transfer. The single surgery control gilt (SC gilt) was operated on but the oviducts were not flushed and oocytes were not transferred. Gilts were considered pregnant if the first pregnancy diagnosis was positive. In this experiment, groups of 2 or 3 gilts underwent surgery on the same day, with Groups S1, S2 and C or SC represented by the 3 sows, and Groups S1 and S2 represented by the 2 sows.

Statistical Analysis. Glutathione content and nuclear maturation rate (data for all rates were modeled according to the binomial model of parameters) were analyzed by one-way analysis of variance (ANOVA, Experiment 1). Correlation between the MII rate and GSH content was analyzed by Pearson's correlation coefficient. The rate of oocyte penetration, number of sperm cells per penetrated oocyte and rate of male pronucleus formation were analyzed by two-way analysis of variance (ANOVA); the maturation system and individual boar in Experiment 2 as well as the system and sperm dose in Experiment 3 were analyzed using the Multivariate General Linear Models of Systat. When ANOVA revealed a significant effect, values were compared by the Tukey test. Differences were considered to be statistically different at $P<0.05$. Statistical analyses were not conducted for Experiment 4 and only descriptive results are reported.

## RESULTS

### Experiment 1

No significant differences between the 2 maturation systems could be observed for the rate of oocytes reaching the MII stage and for mean intracellular GSH content (pmol/oocyte). Means ± SEM were 83.3 ± 3.5 in System 1 versus 86.5 ± 2.5 in System 2 for the rate of MII and 5.2 ± 0.73 in System 1 versus 3.5 ± 0.39 in System 2 for GSH content.

Furthermore, from data shown in Table 1, corresponding to the results of each replicate, it can be observed there was not relationship between the higher rate of oocytes reaching MII after 44 h of incubation and the high intracellular glutathione content (and vice versa) in either of the maturation systems. Pearson's correlation coefficient between these parameters was not significant ($P= 0.55$).

### Experiment 2

No differences were observed between Group S1 and S2 oocytes (Table 2) with regard to the sperm penetration rates by boars with the highest fertility (Boars A and B). Boar C showed higher values ($P<0.001$) for this parameter in S2 than in S1 mature oocytes. The sperm:oocyte ratios confirm differences between systems as well as among boars. System 2 was associated with a higher number of sperm per oocyte in all cases.

Table 1. Rates of oocytes in Metaphase II and mean intracellular glutathione (GSH) content (pmol/oocyte) for each replicate within the 2 two maturation systems employed.

| | System 1 | | | System 2 | | |
|---|---|---|---|---|---|---|
| | Nuclear maturation | | GSH content | Nuclear maturation | | GSH content |
| Replicate | Oocytes examined | %Met II | (pmol/oocyte)[a] | Oocytes examined | %Met II | (pmol/oocyte)[a] |
| 1 | 71 | 70.42 | 4.48 | 66 | 77.27 | 4.28 |
| 2 | 81 | 79.03 | 6.48 | 68 | 94.4 | 2.28 |
| 3 | 90 | 90.09 | 7.91 | 60 | 81.6 | 4.38 |
| 4 | 72 | 86.15 | 5.59 | 65 | 87.73 | 4.45 |
| 5 | 85 | 80 | 3.14 | 87 | 87.39 | 2.74 |
| 6 | 69 | 94.2 | 3.71 | 62 | 90.32 | 2.90 |

Pearson's correlation coefficient between Metaphase II and GSH content: r= 0.19 (P= 0.55).
[a]Mean from 30 oocytes per system per replicate.

The ability to transform the sperm head into a pronucleus within the oocyte cytoplasm was also dependent on maturation system and boar (Table 2). The MPN formation rate was consistently higher for all three boars (A, B and C) in S1 oocytes than in S2 oocytes, but differences were significant only for Boar B. Within the same maturation system, oocyte ability to form MPN was higher for Boar C than for Boars A or B, although significant differences were not found between Boars B and C in S1 (Table 2) oocytes.

Table 2. Rate of sperm penetration (% PEN), mean number of spermatozoa per oocyte (S/O), rate of polyspermy (% POL) and rate of male pronucleus formation (% MPN) after IVF in oocytes matured in System 1 vs System 2 using 3 boars.

| System | Boar | n | % PEN | S/O | % POL | % MPN |
|---|---|---|---|---|---|---|
| S1 | A | 181 | 91.71±2.05[a] | 6.82±0.43[a] | 86.14±2.69[a] | 43.37±3.86[a] |
| | B | 141 | 97.16±1.4[a] | 9.83±0.59[b] | 92.7±2.23[ab] | 69.34±3.95[b] |
| | C | 156 | 41.67±3.96[b] | 3.16±0.42[c] | 59.38±6.19[c] | 73.21±5.97[b] |
| S2 | A | 141 | 99.29±0.71[a] | 12.79±0.62[d] | 97.14±1.41[b] | 34.29±4.03[a] |
| | B | 167 | 100[a] | 15.35±0.75[e] | 95.81±1.56[b] | 46.11±3.87[a] |
| | C | 168 | 82.74±2.92[c] | 4.79±0.4[ac] | 84.17±3.11[a] | 64.03±4.09[b] |
| Source of variability | | | | | | |
| Maturation system | | | <0.001 | <0.001 | <0.001 | <0.001 |
| Boar | | | <0.001 | <0.001 | <0.001 | <0.001 |
| System by boar | | | <0.001 | 0.002 | <0.001 | 0.142 |

% POL and % MPN are based on penetrated oocytes
[a-e] Different superscripts indicate significantly different values (P<0.05).

Experiment 3

Sperm concentration had highly influenced the penetration rate, mean number of spermatozoa per oocyte and rate of polyspermy. Nevertheless, male pronuclear formation did not depend on sperm concentration (P<0.621) in either of the maturation systems (Table 3).

Table 3. Rate of sperm penetration (% PEN), mean number of spermatozoa per oocyte (S/O) and rate of male pronuclear formation (% MPN) after IVF in S1 and S2 oocytes at different sperm concentrations (cells/mL).

| System | Cells/mL | n | % PEN | S/O | % POL | % MPN |
|---|---|---|---|---|---|---|
| S1 | 10.000 | 102 | 20.59±4.02$^{a}$ | 1.42±0.11$^{a}$ | 42.86±11.07$^{a}$ | 57.14±11.07$^{ab}$ |
| | 50.000 | 97 | 56.7±5.06b$^{c}$ | 2.89±0.56$^{ab}$ | 52.73±6.79$^{a}$ | 61.82±6.61$^{ab}$ |
| | 250.000 | 97 | 90.72±2.96$^{d}$ | 2.87±0.22$^{ab}$ | 70.11±4.94$^{ab}$ | 57.47±5.33$^{ab}$ |
| | 500.000 | 115 | 99.13±0.87$^{d}$ | 6.33±0.36$^{c}$ | 91.23±2.66$^{c}$ | 71.93±4.23$^{b}$ |
| S2 | 10.000 | 103 | 41.75±4.88$^{b}$ | 1.63±0.15$^{a}$ | 44.19±7.66$^{a}$ | 53.49±7.7$^{ab}$ |
| | 50.000 | 112 | 71.43±4.29$^{c}$ | 2.17±0.16$^{a}$ | 57.50±5.56$^{a}$ | 48.75±5.62$^{a}$ |
| | 250.000 | 121 | 94.21±2.13$^{d}$ | 3.85±0.26$^{b}$ | 80.7±3.71$^{bc}$ | 64.04±4.51$^{ab}$ |
| | 500.000 | 121 | 98.35±1.16$^{d}$ | 7.36±0.48$^{c}$ | 91.6±2.55$^{c}$ | 50.42±4.6$^{a}$ |
| Source of variability | | | | | | |
| Maturation system | | | <0.001 | 0.246 | 0.261 | 0.082 |
| Cells/mL | | | <0.001 | <0.001 | <0.001 | 0.621 |
| System by cells/mL | | | 0.004 | 0.093 | 0.617 | 0.027 |

% POL and % MPN from penetrated oocytes.

$^{a-e}$: Different superscripts indicate significantly different values (P<0.05)

Experiment 4

Results from this experiment are shown in Table 4. At the time the first pregnancy diagnosis was carried out (26 to 28 d post AI), 3, 2 and 0 gilts from Group S1, S2 and C, respectively, as well as the surgical control gilt were pregnant. However, at the second pregnancy diagnosis (35 d post insemination) only 1 S1 gilt and the SC gilt remained pregnant. 113 d after oocyte transfer, the gilt from the S1 group farrowed 7 healthy piglets, 6 females and 1 male.

Table 4. Results of surgical oocyte transfers in recipient gilts.

| Recipient gilt | Source of oocytes | No. of periovulatory follicles[a] | No. of oocytes recovered | No. of oocytes transferred | Pregnancy diagnosis |
|---|---|---|---|---|---|
| 1 | S1 | 12 | 10 | 39 | positive |
| 2 | S1 | 7 | 6 | 37 | positive |
| 3 | S1 | 25 | 25 | 34 | positive |
| 4 | S1 | 12 | 9 | 39 | negative |
| 5 | S2 | 15 | 14 | 40 | positive |
| 6 | S2 | 14 | 12 | 37 | positive |
| 7 | S2 | 27 | 27 | 40 | negative |
| 8 | S2 | 15 | 14 | 40 | negative |
| 9 | C | 17 | 16 | 16 | negative |
| 10 | C | 14 | 14 | 14 | negative |

S1= oocytes matured in System 1; S2= oocytes matured in System 2; C= in vivo matured oocytes.
[a] Includes preovulatory follicles and points of ovulation.

## DISCUSSION

In this study, the rate of oocytes at the MII stage after the in vitro maturation period is very close to those reported in previous studies (12,40,53), and both maturation systems were similarly effective. However, this finding is no longer very important since, with the adequate hormonal support, it is relatively easy for oocytes to resume meiosis in vitro under different conditions (33).

Cytoplasmic maturation, as measured by intracellular glutathione content of the oocytes, was also similar to that reported by other authors (14,54), although we analyzed groups of 30 oocytes as one value, while in other studies each oocyte represented 1value. Again, we not find significant differences between the 2 maturation systems employed in our study.

A relationship between nuclear and cytoplasmic maturation could not be found in our study nor in previous reports (personal observations, 54). We did not observe that a high rate of oocytes at the MII stage corresponded to high glutathione content, suggesting that nuclear and cytoplasmic maturation of the porcine oocyte are relatively independent physiological events and that requisites for both are not the same. Another possible interpretation could be that the selected parameters were not sufficiently accurate to assess nuclear and /or cytoplasmic maturation of oocytes.

There is no doubt that the rate of oocytes reaching the MII stage is an adequate parameter for assessing nuclear maturation but we think that glutathione content can not be used for assessing cytoplasmic maturation. When cysteine is added to the

culture medium the intracellular GSH content of the oocytes increases, either because the oocytes take up cysteine or, most probably, because the follicular and/or cumulus cells incorporate cysteine and the produced GSH enters the oocytes (39,44). It has also been demonstrated there are a correlation between the intracellular GSH content and the oocyte ability to decondense the sperm head and form the male pronucleus (54) and this is the reason why it has been suggested that GSH functions as the cytoplasmic male pronuclear growth factor (MPGF) in pig oocytes (50). However, other authors have shown that GSH depletion disrupts meiotic progression (57). Then, it could not be adequate the use of GSH content as a measure of the cytoplasmic maturation since it is also implicated in the nuclear events.

On the other hand, it has been suggested that the different evolution of spermatozoa after penetration reflects only a different degree of oocyte cytoplasmic maturation and is totally independent of the spermatozoa (30). In our opinion, MPN formation after IVF is dependent on several factors, the GSH content among them, which is necessary for sperm chromatin decondensation, and, as we have shown in Experiment 2, other factors such as boar and maturation system play an important role. Indeed, Xu et al. (49) also reported that sperm characteristics may have an impact on successful MPN formation. From our results, however, it could not be inferred that a boar with the highest rate of sperm penetration has the highest rate of MPN formation. On the contrary, lower rates of penetration tended to be correlated with increased rates of MPN formation, as occurred with spermatozoa from Boar C.

Thus, we could hypothesize that a certain amount of GSH is necessary for MPN formation; however, once the oocyte acquires an adequate minimum level other factors affect its subsequent viability. To date, the adequate amount of GSH necessary for cytoplasmic maturation has not been established, and it could be lower than the amounts obtained based on the addition of components to the maturation medium (18). It is possible that oocytes with lower but sufficient GSH content could have higher MPN rates when the semen of different boars is employed. It could therefore be inferred that suitable MII stage achievement, glutathione content and MPN formation are essential for a successful maturation system, but none of these parameters alone can be used to asses the viability of resulting oocytes.

In Experiment 3 we showed a biological dose-effect relationship between sperm concentration and both the rate of penetration and the mean number of spermatozoa per oocyte. We also observed that MPN formation was an independent event. Even with a high number of spermatozoa, the rate of oocytes with at least 1 MPN remained approximately the same in both maturation systems. So, the hypothesis that oocytes are limited in their ability to transform spermatozoa into male pronuclei, as has been previously suggested (5,50,51), could affect the development of additional spermatozoa entering an oocyte when a high sperm/oocyte ratio is used, but not the formation of at least 1 full-size MPN.

Finally, from the results in Experiment 4 it can be inferred the 2 commonly employed systems for pig oocyte maturation can produce viable oocytes, although as shown

previously, the final number of farrowed gilts and/or piglets born is low (18,29,55). In fact, the pregnancy failures were not associated with an inadequate maturational stage of the oocytes because the in vivo matured oocytes also did not result in pregnancies. Although embryo mortality can be due to either maternal or embryonic factors or to interactions between the 2, losses due to the inability of an embryo to develop normally usually occur in the first 2 wk of pregnancy (43), being the later losses most commonly due to maternal factors (23). Several other factors can be responsible for embryonic loss, including seasonal effects such heat stress and management practices (27, 28,48), since fertilization and implantation were assessed as normal at Day 26 when pregnancy was diagnosed in our study.

In conclusion, we found that differences in the rates of MPN formation in pig oocytes matured under 2 systems differed in the degree of cytoplasmic maturation and were also dependent on indivudual boar. It was found that both systems of in vitro maturation of porcine oocytes are adequate for producing viable embryos. Thus, polyspermy seems to be an inherent problem of to the in vitro fertilization conditions.

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