Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in pigs

K. Niwa

Division of Animal Science and Technology, Faculty of Agriculture, Okayama University, Okayama 700, Japan

*In vitro* maturation and *in vitro* fertilization techniques in pigs have progressed considerably in recent years. Many reports focus on the factors affecting *in vitro* maturation that lead to normal male pronuclear formation or monospermy after fertilization *in vitro*. It is suggested that pig follicular fluid (pFF), follicle somatic cells and various hormones are important factors for the maintenance of cytoplasmic maturation of oocytes *in vitro*, but that fetal calf serum (FCS), which is generally added to maturation medium, is detrimental. A series of experiments clearly indicate that the glutathione (GSH) content of matured oocytes increases greatly when maturation medium is supplemented with cysteine, a precursor of GSH, and the rates of male pronuclear formation increase in parallel with the increasing GSH content. To prevent polyspermy, conditions of maturation and of fertilization *in vitro* are important. Culture of oocytes in medium with FCS for the first 24 h and with BSA for the second 24 h decreases the incidence of polyspermy, without a significant effect on nuclear maturation. However, it has been shown that secretory macromolecules of the oviduct may reduce the incidence of polyspermy by interacting with fertilizing spermatozoa rather than with oocytes. A reduction of polyspermy by treating spermatozoa with pFF is also reported. In addition to the many improvements in the methodology of *in vitro* fertilization using unfrozen spermatozoa in pigs, techniques for fertilizing oocytes *in vitro* with frozen epididymal and ejaculated spermatozoa have also recently been developed.

Introduction

In pigs, as in many other species, immature oocytes liberated from ovarian follicles can resume meiosis and complete maturation in culture. Although the matured oocytes can be penetrated *in vitro* by spermatozoa under appropriate conditions, low rates of pronuclear formation and a high incidence of polyspermy have been reported by many workers. With increased interest in producing transgenic pigs (see Post *et al.*, 1990; Pursel *et al.*, 1990; Wieghart *et al.*, 1990), there is an increased demand for production of normal pig embryos *in vitro*. In the most recent reviews, relationships between oocyte maturation and male pronuclear formation (Moor *et al.*, 1990) or polyspermy (Hunter, 1990, 1991), and available methodology for *in vitro* fertilization (Parrish, 1991) have been discussed. Techniques for achieving normal fertilization *in vitro* of pig oocytes matured in culture have been improved. This review aims to summarize the latest findings from experiments on maturation of pig oocytes *in vitro* with special reference to normal formation of the male pronucleus and reduction in the incidence of polyspermy, and also on methodological improvements for *in vitro* fertilization.
Table 1. Nuclear maturation and male pronuclear formation of pig oocytes matured in pig follicular fluid (pFF) and fertilized in vitro

| Maturation medium | Gonadotrophins added | Nuclear maturation (%) | Male pronuclear formation (%) | Reference       |
|-------------------|----------------------|------------------------|-----------------------------|-----------------|
| mKRB              | —                    | 85                     | 39                          | Naito et al. (1988) |
|                   | FSH                  | 79                     | 20                          |                 |
|                   | hCG                  | 85                     | 21                          |                 |
| 100% pFF          | —                    | 36                     | 60                          | Naito et al. (1988) |
|                   | FSH                  | 89                     | 81                          |                 |
|                   | hCG                  | 85                     | 66                          |                 |
| mKRB              | FSH                  | 90–93                  | 16–28                       | Naito et al. (1990) |
| 100% pFF          | FSH                  | 93                     | 77                          |                 |
| 100% cFF          | FSH                  | 84                     | 77                          |                 |
| mTCM-199          | PMSG/hCG             | 66–69                  | 43–51                       | Yoshida et al. (1992b) |
| + 10% pFF         | PMSG/hCG             | 90                     | 59                          |                 |
| + 10% DA-1        | PMSG/hCG             | 65                     | 22                          |                 |
| + 10% DA-2        | PMSG/hCG             | 69                     | 39                          |                 |
| + 10% DA-3        | PMSG/hCG             | 89                     | 67                          |                 |
| mTCM-199 + 10% pFF| PMSG/hCG             | 92                     | 63                          | Yoshida et al. (1992a) |
| Waymouth + 10% pFF| PMSG/hCG             | 94                     | 91                          |                 |
| mTLP-PVA + 10% pFF| PMSG/hCG             | 88                     | 47                          |                 |

mKRB: modified Krebs–Ringer bicarbonate solution; cFF: cattle follicular fluid; mTCM-199: modified tissue culture medium-199; DA-1–DA-3: pFF fractions obtained by ion-exchange chromatography; mTLP-PVA: modified Tyrode’s solution with lactate, pyruvate and polyvinyl alcohol (Bavister, 1981). PMSG: pregnant mares’ serum gonadotrophin.

Oocyte Maturation and Male Pronuclear Formation

Effects of follicular fluid

The effects of pig follicular fluid (pFF) supplemented with gonadotrophins on maturation of pig oocytes in vitro and male pronuclear formation of the matured oocytes after in vitro fertilization have been examined (Table 1). Naito et al. (1988) reported that only 36% of pig oocytes with cumulus cells matured to metaphase II when they were cultured for 48 h in pFF obtained from medium-sized follicles, whereas 85–89% did so if FSH or hCG was added to the follicular fluid. After in vitro fertilization, the proportion of fertilized oocytes with a male pronucleus 18 h after insemination also increased to 81% if the oocytes were matured in pFF and FSH. When the fertilized oocytes were examined 48 h after insemination, 36% of oocytes matured in pFF were normally cleaved, indicating a higher developmental capacity with FSH than in oocytes matured in a modified Krebs–Ringer bicarbonate (mKRB) solution in which only 13% had cleaved (Naito et al., 1989). Yoshida et al. (1992a, b) failed to find a beneficial effect of whole, non-fractionated pFF, but confirmed the presence of a substance(s) in partially purified pFF that improves nuclear maturation, male pronuclear formation and normal development of pig oocytes in vitro (Yoshida et al., 1990, 1992b). The effective factors are also present in cattle follicular fluid and the efficacy of the follicular fluid in supporting normal cytoplasmic maturation is maintained even after heat treatment at 56°C for 30 min (Naito et al., 1990). However, recent data obtained by Yoshida et al. (1992b) indicate that the efficacy of pFF for maintaining nuclear maturation of pig oocytes is reduced after similar heat treatment, but heating still did not affect male pronuclear formation.
Maturation and fertilization in vitro in the pig

Table 2. Sperm penetration and male pronuclear formation of pig oocytes matured with follicle cells and fertilized in vitro

| Maturation medium | Condition of oocytes | Penetrated oocytes (%) | Male pronuclear formation (%) | Reference |
|-------------------|----------------------|------------------------|-----------------------------|-----------|
| mTCM-199          | With cumulus         | 47                     | 2                           | Mattioli et al. (1988a) |
| mTCM-199 (CM)     | With cumulus attached to everted follicle | 80 | 62 | |
| mTCM-199          | With cumulus         | 84                     | 45                          | Mattioli et al., (1988b) |
| CM                | With cumulus         | 56                     | 4                           | Zheng and Sirard (1992) |
| Ether extracted CM| With cumulus         | 83                     | 80                          |           |
| CM + androgens    | With cumulus         | 88                     | 48                          |           |
| CM + progesterone | With cumulus         | 53                     | 0                           |           |
| CM + oestradiol   | With cumulus         | 82                     | 62                          |           |
| mTCM-199          | With cumulus         | 51                     | 1                           |           |
| mTCM-199          | With cumulus and a portion of follicular wall | 79 | 6 | Ding and Foxcroft (1992) |
| mTCM-199          | With cumulus and follicular wall from 36-h small* | 80 | 67 | |
|                   | 36-h large*          | 86                     | 77                          |           |
|                   | 72-h small*          | 78                     | 53                          |           |
|                   | 72-h large*          | 87                     | 67                          |           |

mTCM-199, modified tissue culture medium-199.

*Everted follicles of 3.5–5.0 mm (36-h small) and 6.0–9.0 mm (36-h large) in diameter obtained from prepubertal gilts 36 h after an injection of equine chorionic gonadotrophin (eCG) and of 4.0–7.0 mm (72-h small) and 7.5–11.0 mm (72-h large) in diameter 72 h after eCG injections were used.

Although the effective substance(s) has not been identified, it has been suggested that an acidic substance(s) with a molecular mass between 10 and 200 kDa improves not only male pronuclear formation, but also nuclear maturation, normal (monogynic) fertilization and normal development of pig oocytes (Yoshida et al., 1992b). However, Naito et al. (1990) suggested that glycosaminoglycans and inhibin are strong candidates for the effective factors in pFF.

Recently, Naito et al. (1992) found that the activities of histone H1 kinase (H1K), which is considered to be similar to maturation-promoting factor (MPF) (Arion et al., 1988; Labbe et al., 1988), in pig oocytes at metaphase I and II are markedly lower when they were cultured in mKRB solution than in pFF. As it appears that low MPF activity is responsible for abnormal male pronuclear formation in mouse oocytes (Borsuk, 1991), the reduced incidence of male pronuclear formation in pig oocytes matured without pFF might be due to the lower H1K activity of the oocytes (Naito et al., 1992). However, it is not known how the substance(s) in pFF regulates cytoplasmic factor(s).

Effects of follicular cells

Recent reports indicate that pig oocytes matured in vitro in the presence of follicular cells can induce normal male pronuclear formation (Table 2). When pig oocytes with cumulus cells were cultured for
44–46 h (39°C) in tissue culture medium (TCM)-199 supplemented with 10% fetal calf serum (FCS), FSH, LH, oestradiol and prolactin, only 47% of the matured oocytes were penetrated and 2% of the penetrated oocytes had male pronuclei (Mattioli et al., 1988a). However, when cumulus-oocyte complexes connected to the whole wall of everted follicles were cultured in the same medium, the percentage of oocytes penetrated and the percentage of penetrated oocytes with male pronuclei increased to 80% and 62%, respectively. This beneficial effect was also observed when cumulus-oocyte complexes were cultured in conditioned medium from everted follicles, indicating that the effects of the follicles might be mediated by soluble factors. In further studies, it was indicated that the soluble factors can maintain a functional intercellular coupling between oocyte and cumulus cells, which is probably necessary for full cytoplasmic maturation (Mattioli et al., 1988a) and stabilizes the distribution of cortical granules (Galeati et al., 1991).

The active factor(s) in conditioned medium is soluble in ether and can be stimulated by addition of progesterone but not by oestradiol or androgens (Mattioli et al., 1988b). It was demonstrated that the oocytes matured using the everted follicle method can develop to the blastocyst stage after in vitro fertilization and establish a normal pregnancy resulting in the birth of live piglets (Mattioli et al., 1989).

Although protein synthesis between 24 and 36 h of maturation is an absolute necessity for the formation of the male pronucleus in pig oocytes cultured in the presence of everted follicle shells (Ding et al., 1992), it is not known whether or how follicle cells support protein synthesis of oocytes during maturation.

However, Zheng and Sirard (1992) and Ding and Foxcroft (1992) found that the presence of a portion of follicular wall with a complete granulosa cell layer during maturation increases the incidence of pig oocytes with male pronuclei but not of the penetration rate. When small or large follicular walls obtained from prepubertal gilts 36 or 72 h after treatment with equine chorionic gonadotrophin were used for coculture with cumulus-enclosed oocytes, follicular size, but not the time of obtaining follicular walls, significantly affected male pronuclear formation in penetrated oocytes (small: 60% versus large: 72%), an effect possibly mediated by nonsteroidal factors (Ding and Foxcroft, 1992).

Effects of serum and hormonal supplements

Media used for maturation of pig oocytes are generally supplemented with sera such as FCS (Mattioli et al., 1988a, b; Yoshida et al., 1990; Galeati et al., 1991; Wang et al., 1991), newborn calf serum (Nagai and Moor, 1990) and pig serum (Eng et al., 1986; Zheng and Sirard, 1992). However, it is reported that, when added to mKRB solution supplemented with FSH, FCS (5–100%) inhibits maturation of pig oocytes and cannot improve male pronuclear formation after sperm penetration in vitro (Naito et al., 1988). The most recent data obtained by Funahashi and Day (1993a) also showed that supplementation of 10% FCS or newborn piglet serum (NPS) in mTCM-199 appears to be detrimental to cytoplasmic maturation, as the proportion of penetrated oocytes with male pronuclei was lower in those after culture in FCS (28%) or NPS (28%) than in 10% pFF (59%) or 0.4% polyvinyl alcohol (54%).

Beneficial effects of gonadotrophins on nuclear maturation of pig oocytes and cumulus expansion were reported by Meinecke and Meinecke-Tillmann (1979). Racowski and McGaughey (1982) reported that oestradiol also stimulates the progression of maturation beyond metaphase I of pig oocytes in a dose-dependent manner. Thus pig oocytes are frequently cultured in maturation media supplemented with different combinations of gonadotrophins and oestradiol. According to Yoshida et al. (1989), PMSG, and hCG alone or in combination, irrespective of the addition of oestradiol, promote nuclear maturation of pig oocytes compared with oocytes with no hormones in the maturation medium (mTCM-199). However, cumulus expansion is observed only in the presence of PMSG, or in combinations with hCG or hCG plus oestradiol. Mattioli et al. (1991) showed that FSH and LH, separately or in combination, induced cumulus expansion and accelerate the resumption of meiosis of pig oocytes. However, the percentage of oocytes with male pronuclei after maturation in the presence of LH and penetrated in vitro was nearly twice that observed in those matured in FSH or with no hormones, indicating that LH seems to be a more effective gonadotrophin than FSH for both nuclear and cytoplasmic maturation of pig oocytes. Funahashi and Day (1993b) cultured pig oocyte–cumulus complexes in mTCM-199 supplemented with PMSG, hCG and oestradiol for various periods and then in hormone-free medium for a total of 40 h. They found that there were no differences in the proportions of oocytes matured (88–90%) and penetrated in vitro (96%) between those cultured in hormones for only 20 h or for the total 40 h, but that the degree of
cumulus expansion and the incidence of male pronuclear formation were greatly improved when oocyte–
cumulus complexes were cultured in a medium with hormones for 20 h followed by 20 h culture without
hormones.

Requirement for glutathiione

Yoshida et al. (1992a) found that the rate of male pronuclear formation was significantly higher in
oocytes matured in Waymouth MB 752/1 with or without 10% pPF than in those matured in mTCM-199
or mTLP-PVA (Table 1). Waymouth medium contains a higher concentration of glutathione (GSH) and
cysteine, a precursor of GSH, than does mTCM-199. It has been suggested that the synthesis of GSH
during oocyte maturation is a prerequisite for male pronuclear formation in mouse (Calvin et al., 1986)
and hamster (Perreault et al., 1988; Perreault, 1990) oocytes. Although permeability of plasma membranes
to GSH is low (De Felici et al., 1987), the intracellular concentration of GSH depends on the availability of
cysteine (Meister, 1983). Thus, Yoshida et al. (1992a) showed that the rates of male pronuclear formation
are significantly higher in pig oocytes matured in modified Tyrode’s solution with lactate, pyruvate and
polyvinyl alcohol (mTLP-PVA) containing cysteine with or without GSH (93–94%) than in GSH alone
(59%) or without both chemicals (20%). In a further experiment, Yoshida (1993) found that when pig
oocytes are cultured in Waymouth medium for 36 h, the addition of buthionine sulfoximine, an inhibitor of
GSH synthesis, to the medium 0 and 12 h after culture greatly reduced the rate of male pronuclear
formation of oocytes penetrated in vitro (1–2%) compared with no addition (90%) or the addition (78%) of
the inhibitor 24 h after culture. This finding indicates that there is an intimate relationship between GSH
synthesis during maturation of oocytes and male pronuclear formation following sperm penetration.
When a small volume of GSH solution (240 mmol l⁻¹) was micro-injected into pig oocytes matured in
mKRB solution, the rate of male pronuclear formation (52–53%) was higher than in non-injected oocytes
(17–23%) (Naito and Toyoda, 1992).

To examine whether cysteine acts as a substrate for GSH synthesis by pig oocytes, Yoshida et al.
(1993a) examined the effects of different concentrations (0–0.57 mmol l⁻¹) of cysteine added to mTLP-
PVA on GSH contents of matured oocytes. The results indicate that GSH contents per oocyte increase
from 4.6 mmol l⁻¹ with no cysteine to 17.2 mmol l⁻¹ with 0.57 mmol cysteine l⁻¹. The GSH content
observed with 0.57 mmol cysteine l⁻¹ was exactly the same as that found in oocytes matured in vivo.
Furthermore, the rate of male pronuclear formation increased in parallel with increasing GSH (Yoshida et
al., 1993a). Full viability of pig embryos derived from oocytes matured in vitro in the presence of a pPF
fraction and cysteine and fertilized in vitro has also been demonstrated (Yoshida et al., 1993b). These
results clearly indicate that the addition of cysteine as a substrate of GSH to the maturation medium is an
important factor for male pronuclear formation of pig oocytes after sperm penetration.

Conditions of fertilization in vitro and male pronuclear formation

Our recent results (Table 3) suggest that penetration in vitro with frozen–thawed ejaculated spermato-
zoa (Wang et al., 1991) and male pronuclear formation are mainly affected by the presence of cumulus
cells around the oocyte during fertilization in vitro rather than during maturation for 36 h in mTCM-199
supplemented with glucose, lactate, pyruvate, antibiotics, FCS, PMSG, hCG and oestradiol (M. Uchida,
W. H. Wang and K. Niwa, unpublished data). However, the mechanism by which cumulus cells regulate
male pronuclear formation is unknown.

Oocyte Maturation and Polyspermy

A high incidence of polyspermy is another major unsolved problem during fertilization of pig oocytes in
vitro. It is not known, however, whether this abnormality is due to the inadequate conditions for matu-
ration of oocytes or for fertilization in vitro. The persistence of the interaction between cumulus cells
and oocytes stabilizes the distribution of cortical granules; the interruption of this coupling induces
cortical granule migration under the cytoplasmic membrane, which may increase the frequency of their
Table 3. Effects of the presence (+) or absence (—) of cumulus cells during maturation or fertilization or both processes on penetration in vitro and male pronuclear formation of pig oocytes fertilized in vitro by frozen-thawed ejaculated spermatozoa*

| Cumulus cells during | Number of oocytes penetrated | Number of oocytes in penetrated oocytes |
|---------------------|-----------------------------|---------------------------------------|
| Maturation          | Number of oocytes matured   | Total (%)                             | With male polyspermic pronuclei (%) | Mean number of spermatozoa |
|                     |                             | (Total)                               | (%)                                 | in penetrated oocytes |
| (+)                 | 67                          | 55 (82)                               | 40 (73)                             | 46 (84)               | 4.5                     |
| (+)                 | 68                          | 38 (56)                               | 12 (32)                             | 25 (66)               | 2.5                     |
| (−)                 | 63                          | 33 (52)                               | 13 (39)                             | 17 (52)               | 1.7                     |

Data from M. Uchida, WH Wang and K. Niwa (unpublished).

*Wang et al. (1991).

exocytosis, reducing zona penetrability of oocytes (Galeati et al., 1991). Although the uncoupling between cumulus cells and oocytes occurs normally in vivo and in vitro by the end of maturation (Motlik et al., 1986), there have been no reports that clearly demonstrate that this uncoupling prevents polyspermy in pig oocytes matured in culture. The effects of maturation conditions on the rate of polyspermy have recently been examined. According to Zheng and Sirard (1992), when pig cumulus-oocyte complexes are cultured for 48 h in a maturation medium (mTCM-199) supplemented with BSA, cumulus expansion and nuclear maturation of oocytes are greatly inhibited; however, when complexes are cultured for the first 24 h in a medium with FCS and for the second 24 h in that medium with BSA, no such inhibitions are observed and the incidence of polyspermy and the mean number of penetrated spermatozoa per oocyte are significantly decreased.

Conditions of Fertilization In Vitro and Polyspermy

It has been suggested that the rate of polyspermy can be reduced by controlling the conditions of the medium used for treatment of spermatozoa and fertilization of oocytes (Table 4).

The conditions in the uterus and oviducts of oestrous pigs are considered to be the most favourable for prerequisite changes of spermatozoa that are necessary before penetration into an egg (Hunter, 1990). Shortly after ovulation, significant amounts of oviductal glycoproteins can bind firmly to the pig zona pellucida, and after contact with spermatozoa there is evidence of a limited hydrolysis of the structure by sperm protease acrosin (Brown and Cheng, 1986). Thus the consequences of interactions between pig spermatozoa or oocytes matured in vitro and oviduct cells before and during fertilization in vitro have been examined with particular reference to the block to polyspermy (Nagai and Moor, 1990). These authors showed that oviduct secretory macromolecules may reduce the incidence of polyspermy in pig oocytes fertilized in vitro by interacting with fertilizing spermatozoa rather than with oocytes. In standard procedures with no oviduct cell involvement, high rates of penetration (91%) were accompanied by equally high incidences of polyspermy (91%). Fertilization on oviduct cell monolayers, regardless of whether spermatozoa underwent preincubation with oviduct cells for 1 h, also did not reduce polyspermy (83–91%). However, when spermatozoa were preincubated for 2.5 h with oviduct cells, the rate of polyspermy was reduced to 58%, without a marked reduction in penetration rate (84%).

Recent studies by Funahashi and Day (1993c) indicate that, when pig spermatozoa are incubated for 1.5 h in mTCM-199 with 0.4% BSA before fertilization in vitro in the same medium, the addition of 0.01 and 0.1% pFF to the medium significantly reduces the incidence of polyspermy (63 and 46%, respectively) compared with medium without pFF (89%). Increasing the concentration of pFF to 1 and 10% results in a further reduction of polyspermy (0–20%), but this is accompanied by a marked reduction in penetration.
Table 4. Effects of various treatments of spermatozoa on the incidence of polyspermy of pig oocytes matured and fertilized in vitro

| Prefertilization treatment of spermatozoa | Condition during fertilization | Number of oocytes matured at examination | Number of oocytes penetrated (%) | Number of polyspermic oocytes (%) | Reference |
|-----------------------------------------|--------------------------------|----------------------------------------|----------------------------------|----------------------------------|-----------|
| Cultured with oviduct cells for         |                                |                                        |                                  |                                  |           |
| 0 h                                     | — Oviduct cells                | 77                                     | 70 (91)                          | 64 (91)                          | Nagai and Moor (1990)b                |
| 0 h                                     | + Oviduct cells                | 83                                     | 77 (95)                          | 70 (91)                          |                                      |
| 1 h                                     | + Oviduct cells                | 82                                     | 70 (95)                          | 58 (83)                          |                                      |
| 2.5 h                                   | + Oviduct cells                | 86                                     | 72 (84)                          | 42 (58)                          |                                      |
| 3.5 h                                   | + Oviduct cells                | 36                                     | 7 (19)                           | 1 (14)                           |                                      |
| Preincubated for 1.5 h in a medium with 0.4% BSA and pFF* at the concentration (%) of |                                |                                        |                                  |                                  | Funahashi and Day (1993c)            |
| 0                                       | — pFF                          | 55                                     | 44 (80)                          | 33 (77)                          |                                      |
| 0.01                                    | — pFF                          | 63                                     | 50 (79)                          | 29 (58)                          |                                      |
| 0.1                                     | — pFF                          | 59                                     | 47 (80)                          | 17 (36)                          |                                      |
| 1.0                                     | — pFF                          | 72                                     | 56 (78)                          | 18 (32)                          |                                      |
| 10.0                                    | — pFF                          | 84                                     | 31 (37)                          | 4 (13)                           |                                      |

*Percentage of oocytes penetrated.
*The data obtained from two separate series of trials in UK and Japan were combined except those in a 3.5 h period of coculture which were from trials in Japan.
*pFF: pig follicular fluid obtained from immature follicles.

rates (10–22%). When 10% FCS is substituted for BSA, the beneficial effects of pFF on polyspermy are blocked. It has been reported that the binding property of FCS used in in vitro fertilization reduces the available calcium such that the cortical reaction cannot be induced (Cran and Cheng, 1986). The data obtained by Funahashi and Day (1993c) also indicate that the addition of 0.1 and 1% pFF to mTCM-199 with 0.4% BSA during sperm preincubation alone reduces the incidence of polyspermy to 32–34% (Table 4).

Rath (1992) reported that there is a high correlation between the incidence of polyspermy and the absolute number of spermatozoa and oocytes present at fertilization in vitro: if the number of spermatozoa per oocyte is minimized, polyspermy could be minimized. However, these results should be carefully considered because minimizing the sperm:oocyte ratio may be accompanied by a reduction in penetration rate.

Methods for Sperm Capacitation and Fertilization In Vitro

Fresh ejaculated spermatozoa

As spermatozoa are usually obtained after boars have been killed, it is not possible to use epididymal spermatozoa collected repeatedly from the same boar. This problem was minimized, however, when the first successful fertilization in vitro of in vivo matured pig oocytes using ejaculated spermatozoa was reported by Cheng (1985). The method includes storing semen at 20°C for 16 h, washing spermatozoa with saline three times and preincubation of spermatozoa (2 × 10⁶ ml⁻¹) for 4–5 h at 37°C in TCM-199.
Fig. 1. Effect of heparin in the medium with 5 mmol caffeine l⁻¹ on penetration of pig oocytes in vitro by frozen-thawed ejaculated spermatozoa (25–50 × 10⁶ ml⁻¹). The number of oocytes used is given in parentheses. (Reproduced with permission from Wang et al., 1991.)

Fig. 2. Effects of the presence or absence of intact cumulus cells (CC) with or without extra granulosa cells (GC) on in vitro penetration of (■) immature or (□) in vitro matured pig oocytes by frozen-thawed ejaculated spermatozoa. Extra granulosa cells were cultured for 36 or 48 h at 39°C before placing them with oocytes. Although matured oocytes were inseminated by spermatozoa obtained from three boars, the data were combined because similar results were obtained among boars. Immature oocytes were inseminated by spermatozoa obtained from one boar. The number of oocytes used is given in parentheses. (Reproduced with permission from Wang et al., 1992.)

(pH 7.8) supplemented with 3.05 mmol glucose l⁻¹, 2.92 mmol calcium lactate l⁻¹, 0.91 mmol sodium pyruvate l⁻¹, 100 μg dibekacin sulfate ml⁻¹ and 12% FCS, for inducing sperm capacitation, and fertilizing in vivo matured oocytes with 0.1–1 × 10⁶ spermatozoa ml⁻¹ at 39°C in the TCM-199 (pH 7.4) supplemented with 2 mmol caffeine l⁻¹. This procedure is also effective for fertilization in vitro of in vitro matured oocytes (Mattioli et al., 1988a, b). Many of the further studies at different laboratories have used the same method with minor modifications.

Alternative methods for inducing capacitation of ejaculated spermatozoa were reported by Hamano and Toyoda (1986), who observed that sperm concentration during preincubation is an important factor as was observed in epididymal spermatozoa (Nagai et al., 1984). When ejaculated spermatozoa were preincubated for 4 h in mKRB solution at a concentration of 40 × 10⁶ cells ml⁻¹ and used to inseminate
matured oocytes at $5 \times 10^5$ spermatozoa ml$^{-1}$ in vitro in mKRB with 2 mmol caffeine ml$^{-1}$, a significantly higher proportion (100%) of oocytes was penetrated than after preincubation at $2.5-10 \times 10^6$ cells ml$^{-1}$ (25–57%). In a further experiment, conditioned mKRB solution obtained after preincubation of ejaculated spermatozoa at 20 or $40 \times 10^6$ cells ml$^{-1}$ for 2–4 h maintained penetration of oocytes by spermatozoa preincubated at $2.5 \times 10^6$ cells ml$^{-1}$ for 4 h in the conditioned medium (Hamano et al., 1989).

**Frozen spermatozoa**

The first successful in vitro fertilization of pig oocytes matured in vivo and in vitro with frozen spermatozoa was performed using almost the same procedures as those reported by Cheng (1985) for treatment of thawed spermatozoa (Nagai et al., 1988). When the oocytes were inseminated with frozen-thawed epididymal spermatozoa ($2.4 \times 10^7$ ml$^{-1}$) in 0.4 ml modified Tyrode's solution (Brackett and Oliphant, 1975) containing 10 mg BSA ml$^{-1}$ and 2 mmol caffeine ml$^{-1}$, a higher penetration rate was obtained 12 h after insemination in oocytes matured in vivo (79%) than in vitro (42%). The proportion of penetrated oocytes with male and female pronuclei was also higher in oocytes matured in vivo (83%) than in vitro (54%). However, none of the oocytes inseminated with frozen–thawed ejaculated spermatozoa was penetrated (Nagai et al., 1988).

Data from Wang et al. (1991) clearly indicate that frozen–thawed pig ejaculated spermatozoa can penetrate oocytes matured in vitro. The methods used for treatment of thawed spermatozoa and fertilization in vitro were essentially the same as described by Cheng (1985), except that the medium (mTCM-199) was supplemented with 10% rather than 12% FCS and that penicillin and streptomycin, rather than dibekacin, were used as the antibiotics. However, spermatozoa were not preincubated after thawing and washing because it has been reported that preincubation of frozen–thawed ejaculated spermatozoa results in a great reduction of sperm motility (Clarke and Johnson, 1987; Nagai et al., 1988). When oocytes were inseminated with various concentrations of spermatozoa, high penetration rates (85–89%) and increased incidence of polyspermy were obtained at $25–100 \times 10^5$ spermatozoa ml$^{-1}$. Wide variation in penetration rates (16–89%) was observed in oocytes inseminated in medium containing 5 mmol caffeine $^{-1}$ and at $25–50 \times 10^6$ spermatozoa ml$^{-1}$ obtained from six boars, regardless of sperm motility. At $25–50 \times 10^6$ spermatozoa ml$^{-1}$, penetration rates of oocytes depended on the concentration of caffeine in the medium. None of the oocytes was penetrated in the medium supplemented with heparin at 5–40 μg ml$^{-1}$. When heparin was included in the medium with 5 mmol caffeine $^{-1}$, it inhibited the efficacy of caffeine to promote sperm penetration of oocytes (Fig. 1), indicating that there is a species difference. Heparin effects are positive in cattle for inducing sperm capacitation and fertilization of oocytes in vitro (Parrish et al., 1988; Niwa and Ohgoda, 1988).

Wang et al. (1992) found that high proportions (79–94%) of cumulus-enclosed mature oocytes, but no immature oocytes, were penetrated by frozen–thawed ejaculated pig spermatozoa, regardless of the presence of extra granulosa cells. However, significantly higher penetration rates were always obtained in denuded mature and immature oocytes in the presence rather than in the absence of granulosa cells (Fig. 2). This ability of granulosa cells to support sperm capacitation was expressed fully when they were cultured for 36 h at 39°C before being added to the fertilization medium.

To avoid rapid reduction of motility during preincubation of frozen–thawed ejaculated spermatozoa, Zheng et al. (1992) isolated a motile sperm population immediately after thawing, using various fractionation techniques and succeeded in fertilizing in vitro pig oocytes matured in culture. When spermatozoa suspended in the upper fraction after slight centrifugation of thawed semen were used, the penetration rate of oocytes was similar to that by fresh spermatozoa, but the incidence of polyspermy was lower and the proportion of penetrated oocytes with two pronuclei was higher than in oocytes penetrated by fresh spermatozoa.

**Conclusions**

Although low rates of male pronuclear formation and a high incidence of polyspermy have been repeatedly reported in pig oocytes matured and fertilized in vitro, recent reports indicate that pFF, follicular somatic
cells, various hormones and cysteine are important factors for maintaining cytoplasmic maturation of oocytes that can lead to normal male pronuclear formation after fertilization in vitro. However, most studies have focused on the factors present during insemination for preventing polyspermy. Recent data have shown that the preincubation of spermatozoa either with oviduct cells or in a medium with pFF, and the use of a reduced number of spermatozoa at insemination, can lower the incidence of polyspermy. Techniques for fertilizing oocytes in vitro with frozen epididymal and ejaculated spermatozoa have also recently been reported. However, it is apparent that further improvements in the conditions used for in vitro maturation and fertilization are needed to ensure the production of larger numbers of normal pig embryos and, eventually, piglets.

The author is very grateful to B. N. Day for critical reading and editing of the manuscript and is also indebted to H. Funahashi and M. Yoshida for supplying their manuscripts in press, and to T. Nagai for valuable suggestions.

References

Arion D, Meijer L, Brizuela L and Beach D (1988) cdc2 is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF Cell 55 371–378

Bavister BD (1981) Substitution of a synthetic polymer for protein in a mammalian gamete culture system Journal of Experimental Zoology 217 45–51

Borsuk E (1991) Anucleate fragments of parthenogenetic eggs and of maturing oocytes contain complementary factors required for development of a male pronucleus Molecular Reproduction and Development 29 150–156

Brackett BG and Oliphant G (1975) Capacitation of rabbit spermatozoa in vitro Biology of Reproduction 12 260–274

Brown CR and Cheng WKT (1986) Changes in composition of the porcine zona pellucida during development of the oocyte to the 2- to 4-cell embryo Journal of Embryology and Experimental Morphology 92 183–191

Calvin H, Grosshans K and Blake ET (1986) Estimation and manipulation of glutathione levels in prepubertal mouse ovaries and ova: relevance to sperm nucleus transformation in the fertilized egg Gamete Research 14 265–275

Cheng WKT (1985) In Vitro Fertilization of Farm Animal Oocytes PhD thesis. Council for National Academic Awards, Cambridge

Clarke RN and Johnson LA (1987) Effect of liquid storage and cryopreservation of boar spermatozoa on acrosomal integrity and penetration of zona-free hamster ova in vitro Gamete Research 16 193–204

Cran DG and Cheng WKT (1986) The cortical reaction in pig oocytes during in vitro and in vitro fertilization Gamete Research 13 241–251

De Felici M, Dolci S and Siracusa G (1987) Involvement of thioldisulfide groups in the sperm of fully grown mouse oocytes to calcium-free medium Journal of Experimental Zoology 243 283–287

Ding J and Foxcroft GR (1992) Follicular heterogeneity and oocyte maturation in vitro in pigs Biology of Reproduction 47 648–655

Ding J, Moor RM and Foxcroft GR (1992) Effects of protein synthesis on maturation, sperm penetration, and pronuclear development in porcine oocytes Molecular Reproduction and Development 33 59–66

Eng LA, Kornegay ET, Huntington J and Wellman T (1986) Effects of incubation temperature and bicarbonate on maturation of pig oocytes in vitro Journal of Reproduction and Fertility 76 657–662

Funahashi H and Day BN (1993a) Effects of different serum supplements in maturation medium on meiotic and cytoplasmic maturation of pig oocytes Theriogenology 39 965–973

Funahashi H and Day BN (1993b) Effects of the duration of exposure to supplemental hormones on cytoplasmic maturation of pig oocytes in vitro Journal of Reproduction and Fertility 98 179–185

Funahashi H and Day BN (1993c) Effects of follicular fluid at fertilization in vitro on sperm penetration in pig oocytes Journal of Reproduction and Fertility 99 97–103

Galeati G, Modina S, Lauria A and Mattioli M (1991) Follicle somatic cells influence pig oocyte penetrability and cortical granule distribution Molecular Reproduction and Development 29 40–46

Hamano S and Toyoda Y (1986) In vitro fertilization of pig eggs with ejaculated spermatozoa preincubated at high sperm concentration Japanese Journal of Animal Reproduction 32 177–183

Hamano S, Naito K, Fukuda Y and Toyoda Y (1989) In vitro capacitation of boar ejaculated spermatozoa: effect of conditioned media prepared from preincubated sperm suspension Gamete Research 24 483–489

Hunter RHF (1990) Fertilization of pig eggs in vivo and in vitro Journal of Reproduction and Fertility Supplement 40 211–226

Hunter RHF (1991) Oviduct function in pigs, with particular reference to the pathological condition of polyspermy Molecular Reproduction and Development 29 385–391

Labbe JC, Lee MG, Nurse P, Picard A and Doree M (1988) Activation at M-phase of a protein kinase encoded by a starfish homologue of the cell cycle control gene cdc2* Nature 335 251–254

Mattioli M, Galeati G and Seren E (1988a) Effect of follicle somatic cells during pig oocyte maturation on egg penetrability and male pronucleus formation Gamete Research 20 177–183

Mattioli M, Galeati G, Bacci ML and Seren E (1988b) Follicular factors influence oocyte fertilizability by modulating the intercellular cooperation between cumulus cells and oocyte Gamete Research 21 223–232

Mattioli M, Bacci ML, Galeati G and Seren E (1989) Developmental competence of pig oocytes matured and fertilized in vitro Theriogenology 36 1201–1207

Mattioli M, Bacci ML, Galeati G and Seren E (1991) Effects of LH and FSH on the maturation of pig oocytes in vitro Theriogenology 36 95–105

Meinecke B and Meinecke-Tillmann S (1979) Effects of gonadotropins on oocyte maturation and progesterone production
Maturation and fertilization in vitro in the pig

by porcine ovarian follicles cultured in vitro Theriogenology 11 351–365

Meister A (1983) Selective modification of glutathione metabolism Science 220 472–477

Moor RM, Mattioli M, Ding J and Nagai T (1990) Maturation of pig oocytes in vivo and in vitro Journal of Reproduction and Fertility Supplement 40 197–210

Motlik J, Fulka J and Flechon JE (1986) Changes in intercellular coupling between pig oocytes and cumulus cells during maturation in vivo and in vitro Journal of Reproduction and Fertility 76 31–37

Nagai T and Moor RM (1990) Effect of oviduct cells on the incidence of polyspermy in pig eggs fertilized in vitro Molecular Reproduction and Development 26 377–382

Nagai T, Niwa K and Iritani A (1984) Effect of sperm concentration at preincubation in chemically defined medium on fertilization in vitro of pig follicular oocytes Journal of Reproduction and Fertility 70 271–275

Nagai T, Takahashi T, Masuda H, Shiyoa Y, Kuwayama M, Fukushima M, Iwashaki S and Hanada A (1988) In-vitro fertilization of pig oocytes by frozen boar spermatozoa Journal of Reproduction and Fertility 84 585–591

Naito K and Toyoda Y (1992) Effects of microinjection of glutathione on male pronucleus formation in porcine oocytes matured in vitro Journal of Reproduction and Fertility 38 173–178

Naito K, Fukuda Y and Toyoda Y (1988) Effects of porcine follicular fluid on male pronucleus formation in porcine oocytes matured in vitro Gannet Research 21 289–295

Naito K, Fukuda Y and Ishihashi I (1989) Developmental ability of porcine ova matured in porcine follicular fluid in vitro and fertilized in vitro Theriogenology 31 1049–1057

Naito K, Kosaka M, Fukuda Y, Ishihashi I and Toyoda Y (1990) Analysis of the factor(s) present in follicular fluids promoting male pronucleus formation ability of porcine follicular oocytes Japanese Journal of Animal Reproduction 36 213–218

Naito K, Daen FP and Toyoda Y (1992) Comparison of histone H1 kinase activity during meiotic maturation between two types of porcine oocytes matured in different media in vitro Biology of Reproduction 47 43–47

Niwa K and Obguda O (1988) Synergistic effect of caffeine and heparin on in vitro fertilization of cattle oocytes matured in culture Theriogenology 30 733–741

Parrish JJ (1991) Application of in vitro fertilization to domestic animals. In Elements of Mammalian Fertilization, (Vol. II) Practical Applications pp 111–132 Ed. FM Wassarman, CRC Press, Boca Raton

Parrish JJ, Susko-Parrish J, Winer MA and First NL (1988) Capacitation of bovine sperm by heparin Biology of Reproduction 38 1171–1180

Perreault SD (1990) Regulation of sperm nuclear reactivation during fertilization. In Fertilization in Mammals pp 285–296 Eds BD Bavister, J Cummins and ERS Rolden. Serono Symposia, Norwell

Perreault SD, Barbee RR and Slott VL (1988) Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes Developmental Biology 125 181–186

Post LE, Thomsen DK, Petrovskis EA, Meyer AL, Berlinski Pj and Wardley RC (1990) Genetic engineering of the pseudo-rabies virus genome to construct live vaccines Journal of Reproduction and Fertility Supplement 41 97–104

Pursel VG, Hammer RE, Bolt DJ, Palmiter RD and Brinster RL (1990) Integration, expression and germ-line transmission of growth-related genes in pigs Journal of Reproduction and Fertility Supplement 41 77–87

Racowsky C and McGaughey RW (1982) In the absence of protein, estradiol suppressed meiosis of porcine oocytes in vitro Journal of Experimental Zoology 224 103–110

Rath D (1992) Experiments to improve in vitro fertilization techniques for in vitro-matured porcine oocytes Theriogenology 37 885–896

Wang WH, Niwa K and Okuda K (1991) In-vitro penetration of pig oocytes matured in culture by frozen-thawed ejaculated spermatozoa Journal of Reproduction and Fertility 93 491–496

Wang WH, Uchida M and Niwa K (1992) Effects of follicle cells on in vitro penetration of pig oocytes by cryopreserved, ejaculated spermatozoa Journal of Reproduction and Development 38 125–131

Wieghart M, Hoover JL, McGrane MM, Hanson RW, Rottman FM, Holtzman SH, Wagener TE and Pinkert CA (1990) Production of transgenic pigs harbouring a rat phosphoenolpyruvate carboxykinase–bovine growth hormone fusion gene Journal of Reproduction and Fertility Supplement 41 89–96

Yoshida M (1993) Role of glutathione on maturation and fertilization of pig oocytes in vitro Molecular Reproduction and Development 38 76–81

Yoshida M, Bamba K and Kojima Y (1989) Effects of gonadotropins and estradiol-17β on the timing of nuclear maturation and cumulus mass expansion in pig oocytes cultured in vitro Japanese Journal of Animal Reproduction 35 86–91

Yoshida M, Ishizaki Y and Kawagishi H (1990) Blastocyst formation by pig embryos resulting from in-vitro fertilization of oocytes matured in vitro Journal of Reproduction and Fertility 88 1–8

Yoshida M, Ishigaki K and Pursel VG (1992a) Effect of maturation media on male pronucleus formation in pig oocytes matured in vitro Molecular Reproduction and Development 31 68–71

Yoshida M, Ishigaki K, Kawagishi H, Mamba K and Kojima Y (1992b) Effects of pig follicular fluid on maturation of pig oocytes in vitro and on their subsequent fertilizing and developmental capacity in vitro Journal of Reproduction and Fertility 95 481–488

Yoshida M, Ishigaki K, Nagai T, Chikyu M and Pursel VG (1993a) Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus Biology of Reproduction 49 89–94

Yoshida M, Mizoguchi Y, Ishigaki K, Kojima T and Nagai T (1993b) Birth of piglets derived from in vitro fertilization of pig oocytes matured in vitro Theriogenology 39 1303–1311

Zheng YS, Fiser P and Sirard MA (1992) The use of ejaculated boar semen after freezing in 2 or 6% glycerol for in vitro fertilization of porcine oocytes matured in vitro Theriogenology 38 1065–1075