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

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*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 | |
| | 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.

**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) |
| | With cumulus attached to everted follicle | 80 | 62 | |
| Follicle-conditioned mTCM-199 (CM) | With cumulus | 84 | 45 | |
| mTCM-199 | With cumulus | 56 | 4 | Mattioli *et al.*, (1988b) |
| CM | With cumulus | 83 | 80 | |
| Ether extracted CM | With cumulus | 88 | 48 | |
| CM + androgens | With cumulus | 53 | 0 | |
| CM + progesterone | With cumulus | 82 | 62 | |
| CM + oestradiol | With cumulus | 51 | 1 | |
| mTCM-199 | With cumulus | 79 | 6 | Zheng and Sirard (1992) |
| | With cumulus and a portion of follicular wall | 56 | 26 | |
| mTCM-199 | With cumulus and follicular wall from | | | Ding and Foxcroft (1992) |
| | 36-h small[a] | 80 | 67 | |
| | 36-h large[a] | 86 | 77 | |
| | 72-h small[a] | 78 | 53 | |
| | 72-h large[a] | 87 | 67 | |

mTCM-199, modified tissue culture medium-199.
[a]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.*, 1988b) 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 glutathione*

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% pFF 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 buthinine 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 pFF 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 spermatozoa (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 maturation 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[a]

| Cumulus cells during | | Number of oocytes matured | Number of oocytes penetrated | | Number of polyspermic oocytes (%) | Mean number of spermatozoa in penetrated oocytes |
|---|---|---|---|---|---|---|
| Maturation | Fertilization | | Total (%) | With male pronuclei (%) | | |
| + | + | 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).
[a]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 (%)[a] | Reference |
|---|---|---|---|---|---|
| Cultured with oviduct cells for | | | | | Nagai and Moor (1990)[b] |
| 0 h | −Oviduct cells | 77 | 70 (91) | 64 (91) | |
| 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[c] 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) | |

[a]Percentage of oocytes penetrated.
[b]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.
[c]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 ($2 \times 10^8$ ml$^{-1}$) for 4–5 h at 37°C in TCM-199

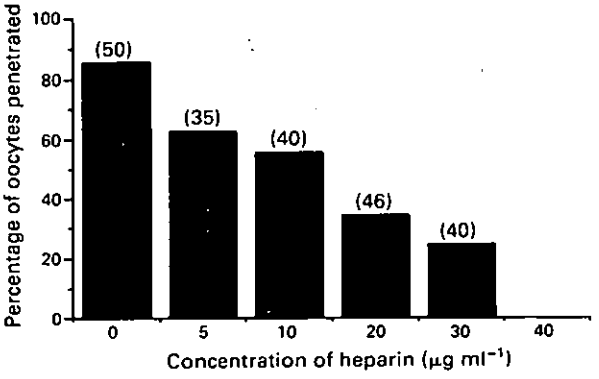


**Fig. 1.** Effect of heparin in the medium with 5 mmol caffeine $l^{-1}$ on penetration of pig oocytes *in vitro* by frozen–thawed ejaculated spermatozoa (25–50 × $10^6$ $ml^{-1}$). The number of oocytes used is given in parentheses. (Reproduced with permission from Wang *et al.*, 1991.)

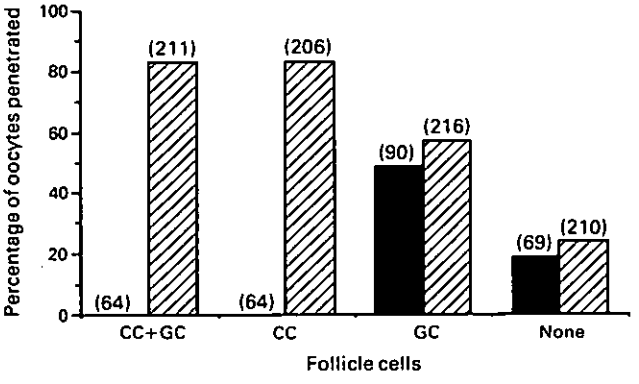


**Fig. 2.** Effects of the presence or absence of intact cumulus cells (CC) with or without extra granulosa cells (GC) on *in vitro* penetration of (■) immature or (▨) *in vitro* matured pig oocytes by frozen–thawed ejaculated spermatozoa. Extra granulosa cells were cultured for 36 or 48 h at 39°C before 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 × $10^6$ 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 × $10^6$ 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 $25–100 \times 10^6$ spermatozoa $ml^{-1}$. Wide variation in penetration rates (16–89%) was observed in oocytes inseminated in medium containing 5 mmol caffeine $l^{-1}$ 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 $l^{-1}$, 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.

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