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# Effects of Fetal Calf Serum, Pig Follicular Fluid, and Gonadotropins on Maturation of Pig Oocytes Cultured in Different Media

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**Abstract:** Pig follicular oocytes with cumulus cells were cultured in different maturation media, modified tissue culture medium 199 (TCM-199B) and BSA-free Whitten's medium (mWM), containing gonadotropins (10 IU eCG/ml + 10 IU hCG/ml) and/or 10% (v/v) fetal calf serum (FCS) or 10% (v/v) pig follicular fluid (PFF). After 48 h of culture, complete (degree +3) cumulus expansion was observed in oocytes cultured in both kinds of media supplemented with gonadotropins together with FCS or PFF. However, when oocytes were cultured in media with gonadotropins only, moderate (degree +2) cumulus expansion was observed in TCM-199B, while no (degree 0) cumulus expansion was observed in mWM. The proportions of metaphase II (M-II) oocytes were significantly ($P<0.01$) higher in both media in the presence of gonadotropins (61–95%) than in their absence (22–35%), irrespective of FCS or PFF supplementation. The addition of FCS or PFF to the media containing gonadotropins increased the proportion of oocytes reaching M-II in mWM but not in TCM-199B. When gonadotropin-containing media were used, the rate of male pronuclear formation in oocytes fertilized in vitro was significantly higher ($P<0.05$) in TCM-199B with PFF (70%) than with FCS (30%) although the value in PFF did not differ statistically from that (50%) in unsupplemented medium. In mWM, FCS did not support penetration of oocytes by spermatozoa, while PFF supported high rates of penetration (72%) but not male pronuclear formation. These results indicate that gonadotropins and/or FCS and/or PFF affect nuclear and cytoplasmic maturation of pig oocytes in different ways.

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Low rates of male pronuclear formation and a high incidence of polyspermy in pig follicular oocytes matured and fertilized *in vitro* have been reported by many workers [for review see 1]. Although rates of male pronuclear formation have been improved considerably by modification of culture conditions for maturation of oocytes, the efficient production of normal pig embryos derived from *in vitro* maturation and *in vitro* fertilization is still limited by a high incidence of polyspermic penetration [for review see 2–4]. Further information on the effects of individual components and factors in media on maturation and fertilization of pig oocytes *in vitro* is needed.

Serum, such as fetal calf serum (FCS), is usually added to oocyte maturation media [5–8], but it has been reported that FCS inhibits cytoplasmic maturation of pig oocytes by decreasing male pronuclear formation after *in vitro* fertilization [9, 10]. Replacing FCS by pig follicular fluid (PFF) or adding both FCS and PFF to maturation media was reported to improve the rates of nuclear maturation, male pronuclear formation and normal development of pig embryos [10–12]. However, most media used in these studies were complex, such as tissue culture medium (TCM)-199 [6, 8, 10, 13–15]. These media contain many components, including amino acids and vitamins, which make it difficult to identify the effects of individual components on *in vitro* maturation. Recently Funahashi *et al.* [16, 17] used modified Whitten's medium supplemented with PFF [18] to culture pig follicular oocytes and reported that, when oocytes were cultured in this medium which has a low

NaCl concentration, the cytoplasmic maturation was improved and the incidence of oocytes with male pronuclei and monospermy after *in vitro* fertilization was increased. It would appear that this medium is suitable for the culture of pig oocytes *in vitro* because it is a simple medium in which components can be defined. The present study was carried out to compare the potential of a defined simple medium (Whitten's medium) and a complex medium (TCM-199) to support maturation of pig oocytes *in vitro*. The abilities of FCS and PFF, in the presence/absence of gonadotropins, to promote cumulus expansion, nuclear maturation and cytoplasmic maturation were also examined in the two different media.

## Materials and Methods

*Media*

Two different media were used for the maturation of oocytes. One was a complex medium, designated as TCM-199B (pH 7.4) which was modified TCM-199 with Earle's salts (Gibco Laboratories, Grand Island, NY, USA) supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 75 μg potassium penicillin G/ml and 50 μg streptomycin sulphate/ml. This medium was essentially the same as that used by Wang *et al.* [19] except that calcium lactate was omitted. The other was a simple medium, BSA-free modified Whitten's medium (mWM; pH 7.4) composed of 87.95 mM NaCl, 4.83 mM KCl, 1.18 mM $KH_2PO_4$, 1.18 mM $MgSO_4$, 22.62 mM $NaHCO_3$, 5.56 mM D-glucose, 21.58 mM sodium lactate, 0.31 mM sodium pyruvate, 2.66 mM hemicalcium lactate, 80 μg potassium penicillin G/ml, 50 μg streptomycin sulphate/ml and 0.01 mg phenol red/ml [20]. Where noted below, these media were supplemented with 10% (v/v) heat-inactivated FCS, 10% (v/v) PFF and/or gonadotropins, 10 IU eCG (Serotropin; Teikoku-Zoki Co., Tokyo)/ml and 10 IU hCG (Puberogen; Sankyo Co., Tokyo)/ml. The PFF was prepared as described by Naito *et al.* [10]. Briefly, PFF was withdrawn from antral follicles (2 to 5 mm in diameter) of ovaries by the same procedures as in the collection of oocytes described below, centrifuged at 1,500 g for 15 min at room temperature, and the supernatant was stored at −20°C until used.

*Ovaries*

Ovaries were collected from maturing gilts at a local abattoir and transported to the laboratory within 1 to 1.5 h in 0.9% (w/v) NaCl solution containing 75 μg potassium penicillin G/ml and 50 μg streptomycin sulphate/ml at about 4°C for collection of PFF and at 37 to 39°C for collection of oocytes.

*Preparation of oocytes*

Oocytes were aspirated from antral follicles (2 to 5 mm in diameter) with an 18-gauge needle fixed to a 10-ml disposable syringe and washed four times with each of the maturation media. Ten oocytes surrounded by compact cumulus were transferred to a 100-μl drop of the same medium which had previously been covered with warm paraffin oil in a polystyrene culture dish (35 × 10 mm; Becton Dickinson Labware, Lincoln Park, NJ, USA) and equilibrated in an atmosphere of 5% $CO_2$ in air for about 3 h, and cultured for 48 h at 39°C under the same atmospheric conditions. After 48 h of culture, the degree of cumulus expansion and nuclear maturation of oocytes were examined. For *in vitro* fertilization, oocytes with cumulus cells were washed twice in TCM-199B (pH 7.8) with 10 mM caffeine-sodium benzoate (Sigma chemical Co., St. Louis, MO, USA), placed into 50 μl of the same medium, and kept in a $CO_2$ incubator (5% $CO_2$ in air at 39°C) for 30 min until spermatozoa were added for fertilization.

*Sperm preparation and in vitro fertilization*

Spermatozoa were prepared as described by Wang *et al.* [15]. Briefly, three 0.1-ml pellets of frozen ejaculated spermatozoa obtained from boars of proven fertility were thawed in 2 ml of TCM-199B (pH 7.8) at 37°C for 1 min. The medium had previously been equilibrated in an atmosphere of 5% $CO_2$ in air for about 3 h. After thawing, the spermatozoa were washed three times in the same medium by centrifugation at 550 g for 5 min. The sperm pellet was then resuspended to give a sperm concentration of 2–3 × $10^7$ cells/ml. A 50-μl aliquot of final sperm suspension was introduced into 50 μl of TCM-199B containing 10 mM caffeine that included the oocytes, and gametes were cultured at 39°C in an atmosphere of 5% $CO_2$ in air. This mixture had final concentrations of 1–1.5 × $10^7$ spermatozoa/ml and 5 mM caffeine.

*Assessment of cumulus expansion*

At the end of the culture for maturation, the degree of cumulus expansion was determined with a dissecting microscope. The degree of cumulus expansion was classified into four categories which were scored 0 (no expansion), +1 (separation of only the outermost layer of cumulus), +2 (moderate expansion involving the outer half of cumulus), and +3 (complete expansion including or except the corona radiata). This classification was originally described by Downs [21] for mouse oocytes.

*Assessment of nuclear maturation*

At the end of the culture for maturation, the oocytes

were freed from cumulus cells by treatment with Dulbecco's phosphate buffered saline (D-PBS; Sigma) containing 0.1% hyaluronidase from bovine testis (Sigma), followed by repeated passage through a fine pipette. The oocytes were then mounted, fixed for 48–72 h in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined under a phase-contrast microscope at a magnification of 200 × or 400 × for assessment of nuclear maturation. Oocytes at the stage of metaphase II (M-II) were regarded as mature.

*Assessment of sperm penetration*

Fourteen hours after insemination, the oocytes were freed from cumulus cells by repeated passage through a fine pipette, mounted, fixed and stained as described above for the assessment of nuclear maturation. The stained oocytes were examined under a phase-contrast microscope at a magnification of 200 × or 400 × for evidence of sperm penetration. Oocytes were considered penetrated when they contained one or more swollen sperm heads and/or male pronuclei and the corresponding sperm tails. When polyspermic oocytes contained at least one male pronucleus, they were classified as penetrated oocytes with male pronuclei.

*Statistical analysis*

The percentages of oocytes reaching M-II, penetration, male pronuclear formation and polyspermy were subjected to an arc-sine transformation, and the transformed values were analysed by one-way ANOVA. When ANOVA revealed a significant effect, the treatments were compared by Duncan's multiple range test. Tukey's studentized range tests were applied to compare mean numbers of spermatozoa in penetrated oocytes.

## Results

*Cumulus expansion*

No cumulus expansion was observed in oocytes cultured in the absence of gonadotropins, irrespective of supplementation with FCS or PFF in either medium (Table 1), but in media supplemented with gonadotropins together with FCS or PFF, most (78–85%) of the oocytes showed cumulus expansion to degree +3, regardless of the medium used. In TCM-199B with gonadotropins but without FCS or PFF supplementation, most (75%) of the oocytes had cumulus expanded to degree +2 and only 4% of the oocytes showed expansion to degree +3. In contrast, in mWM with gonadotropins but without FCS or PFF, most (81%) of the oocytes failed to show signs of cumulus expansion and the remainder only showed expansion to degree +1.

*Nuclear maturation*

When oocytes were cultured in TCM-199B, the proportions of oocytes which reached M-II were significantly ($P<0.01$) higher in the presence (92–95%) than in the absence (22–35%) of gonadotropins, irrespective of supplementation with FCS or PFF (Fig. 1A). When oo-

**Table 1.** Effects of gonadotropins (10 IU eCG/ml + 10 IU hCG/ml) and/or additives on cumulus expansion of pig oocytes cultured for 48 h in different maturation media[a]

| Media | Gonadotropins | Additive[c] | No. of oocytes examined | No. (%) of oocytes with cumulus expansion at various degrees[b] | | | |
|---|---|---|---|---|---|---|---|
| | | | | 0 | +1 | +2 | +3 |
| TCM-199B | − | − | 80 | 80 (100) | 0 (0) | 0 (0) | 0 (0) |
| | − | FCS | 80 | 80 (100) | 0 (0) | 0 (0) | 0 (0) |
| | − | PFF | 80 | 80 (100) | 0 (0) | 0 (0) | 0 (0) |
| | + | − | 80 | 2 (3) | 15 (19) | 60 (75) | 3 (4) |
| | + | FCS | 79 | 2 (3) | 1 (1) | 9 (11) | 67 (85) |
| | + | PFF | 78 | 3 (4) | 2 (3) | 12 (15) | 61 (78) |
| mWM[d] | − | − | 80 | 80 (100) | 0 (0) | 0 (0) | 0 (0) |
| | − | FCS | 80 | 80 (100) | 0 (0) | 0 (0) | 0 (0) |
| | − | PFF | 80 | 80 (100) | 0 (0) | 0 (0) | 0 (0) |
| | + | − | 80 | 65 (81) | 15 (19) | 0 (0) | 0 (0) |
| | + | FCS | 72 | 1 (1) | 4 (6) | 6 (8) | 61 (85) |
| | + | PFF | 78 | 2 (3) | 3 (4) | 7 (9) | 66 (85) |

[a] Experiments were repeated four times. [b] See text for classification of the degrees. [c] FCS: 10% fetal calf serum; PFF: 10% pig follicular fluid. [d] Modified Whitten's medium.

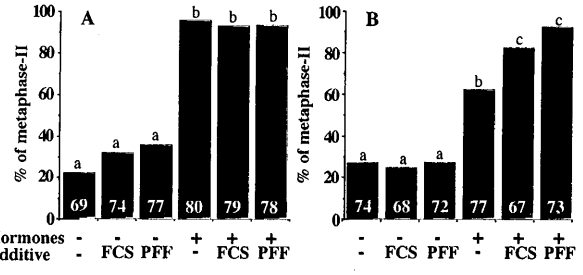


**Fig. 1.** Nuclear maturation (metaphase II) of pig oocytes cultured for 48 h in TCM-199B (A) and modified Whitten's medium (B) supplemented with (+) or without (−) gonadotropins (10 IU eCG/ml + 10 IU hCG/ml) and 10% fetal calf serum (FCS) or 10% pig follicular fluid (PFF). The height of the bars indicates the mean % of matured oocytes, and the total number of oocytes examined is given at the bottom of each bar. Different letters (a,b and c) on the bars denote significant differences (a-b, a-c, $P<0.01$; b-c, $P<0.05$). The experiments were repeated four times.

cytes were cultured in mWM, the proportions of oocytes which reached M-II were also significantly ($P<0.01$) higher in medium supplemented with (61–90%) than without (24–26%) gonadotropins (Fig. 1B). Since TCM-199B containing gonadotropins supported nuclear maturation in >90% of oocytes, it was not surprising that no additional stimulation was observed with FCS or PFF supplementation (Fig. 1A). In contrast, the addition of FCS or PFF to mWM containing gonadotropins did significantly ($P<0.05$) increase the proportion of M-II oocytes as compared to the value (61%) obtained in their absence (Fig. 1B).

*Sperm penetration*

Because the presence of gonadotropins was required for high proportions of cultured oocytes to reach M-II in either TCM-199B or mWM (Fig. 1), only oocytes cultured under these conditions were inseminated to examine sperm penetration *in vitro*. In oocytes matured in TCM-199B, there were no differences among the experimental groups in the rates of sperm penetration (83–92%) and polyspermy (87–88%) or the mean number of spermatozoa in penetrated oocytes (3.4–4.5) (Table 2). Although no significant differences in male pronuclear formation rates were observed between oocytes cultured without (50%) and with FCS (30%), or between those cultured without and with PFF (70%), the value for oocytes cultured in PFF-supplemented medium was significantly ($P<0.05$) higher than that for oocytes cultured in FCS-supplemented medium. On the other hand, when oocytes cultured in mWM were inseminated, a significantly ($P<0.05$) higher sperm penetration rate was observed in the oocytes cultured in the presence (72%) than in the absence (16%) of PFF; in contrast, no penetration was observed in the presence of FCS. The proportions of oocytes with male pronuclear formation (36–40%) and with polyspermy (50–66%) and the mean number of spermatozoa per penetrated oocyte (1.8–2.5) were similar in oocytes cultured in the presence or absence of PFF.

## Discussion

The results of the present study demonstrated that: 1) gonadotropins (eCG + hCG) in maturation media stimulate cumulus expansion and nuclear maturation of pig follicular oocytes, especially when either FCS or

**Table 2.** Effects of addition of 10% fetal calf serum (FCS) or 10% pig follicular fluid (PFF) into different maturation media containing gonadotropins (10 IU eCG/ml + 10 IU hCG/ml) on sperm penetration *in vitro* and male pronuclear formation of pig oocytes[a]

| Maturation media | Additive | No. of oocytes inseminated | No. of oocytes penetrated | | No. of polyspermic oocytes (%)[b] | Mean number of spermatozoa in penetrated oocytes |
|---|---|---|---|---|---|---|
| | | | Total (%) | with male pronuclei (%)[b] | | |
| TCM-199B | – | 65 | 60 (92) | 30 (50)[de] | 52 (87) | 4.1 |
| | FCS | 65 | 54 (83) | 16 (30)[d] | 47 (87) | 3.4 |
| | PFF | 67 | 59 (88) | 41 (70)[e] | 52 (88) | 4.5 |
| mWM[c] | – | 63 | 10 (16)[d] | 4 (40) | 5 (50) | 1.8 |
| | FCS | 71 | 0 (0)[d] | – | – | – |
| | PFF | 78 | 56 (72)[e] | 20 (36) | 37 (66) | 2.5 |

[a] Experiments were repeated four times separately in each maturation medium. [b] Percentage of the oocytes penetrated. [c] Modified Whitten's medium. [d, e] Values with different superscripts within each column in each maturation medium differ significantly ($P<0.05$).

PFF is present together in the media; 2) in the presence of gonadotropins, PFF promotes penetration of oocytes *in vitro* in mWM but not in TCM-199B; 3) male pronuclear formation is not promoted by PFF in either medium, although PFF can support higher male pronuclear formation than FCS in TCM-199B. Cumulus expansion and nuclear and cytoplasmic maturation of pig oocytes *in vitro* are therefore affected in different ways depending on the basic culture medium and supplements used.

Cumulus expansion usually refers to the dispersion of cumulus cells by mucification during oocyte maturation. Gonadotropins [22] and growth factors [21, 23] are known to enhance cumulus expansion of oocytes *in vitro*. In the present study, when the media were supplemented with gonadotropins together with FCS or PFF, complete cumulus expansion was observed in most oocytes in both media used (TCM-199B and mWM), but without supplementation with gonadotropins, cumulus expansion failed to occur in either medium, consistent with the observations by Yoshida *et al.* [24]. In the present study, noticeably different patterns of cumulus expansion were observed in TCM-199B and mWM containing gonadotropins only. Most of the oocytes cultured in TCM-199B expanded moderately, but no expansion was observed in most oocytes cultured in mWM. This indicates that some components in TCM-199B, perhaps amino acids, vitamins or trace constituents, may permit cumulus expansion induced by gonadotropins.

In the present study, the stimulatory effect of gonadotropins on nuclear maturation of oocytes was observed in both TCM-199B and mWM, as also reported by Yoshida *et al.* [24] and Mattioli *et al.* [25]. In the presence of gonadotropins, FCS and PFF were not necessary for nuclear maturation in TCM-199B, but there was a beneficial effect of FCS and PFF in mWM. This indicates that the components in FCS and PFF can affect nuclear maturation of the oocytes in a simple medium containing gonadotropins. Various hormones such as LH [26] and growth factors such as EGF [27] are found in FCS and PFF, and LH [25] and EGF [23, 28] have been reported to stimulate pig oocyte nuclear maturation *in vitro*.

In oocytes cultured in supplemented TCM-199B and mWM, no cumulus expansion was observed, although some oocytes (22–35%) could mature to M-II. These results are consistent with the results obtained in a serum-free medium by Wang and Niwa [28]. A similar phenomenon was also reported in mouse [21], but a high rate of nuclear maturation is always accompanied by cumulus expansion. Further experiments are necessary to examine whether there is a direct relationship between cumulus expansion and nuclear maturation in pig oocytes.

In the present study, very high penetration rates were obtained in oocytes matured in TCM-199B containing gonadotropins, regardless of the addition of FCS or PFF. In oocytes matured in mWM with gonadotropins, a high penetration rate (72%) was obtained only when the medium was supplemented with PFF; no penetration occurred when the medium was supplemented with FCS. It has been reported that no oocytes cultured in modified Krebs-Ringer bicarbonate solution containing 0.4% BSA were penetrated by fresh ejaculated spermatozoa [29], while those cultured in TCM-199 containing BSA were penetrable by fresh ejaculated spermatozoa [8]. On the other hand, Yoshida *et al.* [30] reported that most oocytes cultured in modified TLP-PVA containing 10% FCS with or without 10% PFF were penetrated by fresh ejaculated spermatozoa. Therefore, it would appear to be difficult for frozen-thawed boar spermatozoa to penetrate oocytes cultured in simple media containing FCS, but further studies are required to clarify the relationships among media, supplementation with FCS or PFF, and the type of spermatozoa used.

The results of previous studies indicate that PFF added to maturation medium has a beneficial effect on male pronuclear formation in pig oocytes, as compared to the effects of serum supplements [9, 10]. Similar results were obtained in the present study: male pronuclear formation in oocytes matured in TCM-199B was significantly higher in the presence of PFF than in the presence of FCS, although the proportions of penetration and polyspermy and the mean number of spermatozoa in penetrated oocytes were similar in all the groups.

To obtain more information on *in vitro* maturation and fertilization and eventually to improve the low incidence of monospermy and low rate of male pronuclear formation in pig oocytes, it is helpful to use simple chemically defined media. The mWM used in this study was originally developed as a simple medium for the cultivation of preimplantation mouse embryos [20], but it has also been used for the culture of pig oocytes [16, 17] and embryos [31, 32]. Although mWM containing PFF is not a completely chemically defined medium because of the heterogeneity of PFF, the results of the present study indicate that this medium should be very helpful for the study of *in vitro* maturation and fertilization of pig oocytes by analysing the importance of its individual components.

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