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# In Vitro Maturation of Porcine Follicular Oocytes Using Medium-II

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## Summary

The present study was conducted to investigate in vitro maturation of porcine follicular oocytes cultured in Medium-II. The Medium-II was prepared by modification of Kreb's-Ringer Bicarbonate Solution (KRB) based on a composition of inorganic salts of porcine follicular fluid (1). The maturation rate of the oocytes cultured in Medium-II supplemented with either FCS or BSA was 60.0% (18/30) and 66.1% (37/56), respectively. There was no significant difference in the rate compared to the control medium (mTCM199). After in vitro fertilization, on the other hand, the rate of both pronucleus formation and early development of the oocytes cultured in Medium-II was significantly lower than those of the oocytes cultured in mTCM199 ($P<0.05$). These results suggest that Medium-II could be useful as a chemical defined medium for in vitro maturation of porcine follicular oocytes and that this medium would be contributed for further study to demonstrate a factor (s) for cytoplasmic maturation.

In the last decade, a technique for in vitro maturation (IVM) of ovarian follicular oocytes, which are obtainable from an abattoir, followed by in vitro fertilization (IVF) and subsequently in vitro culture (IVC) of cleaved eggs produced has been developed in farm animals including cattle (2), sheep (3) and pigs (4). For these procedures, several media can be used for culture: e.g. DMEM, Ham's F10 and TCM199 (5). Each of the media, however, requires fetal calf serum (FCS) to support maturation of oocytes and early embryogenesis. It is also general that some somatic cells are needed as a feeder cell to overcome the so-called "cell-block" phenomenon in in vitro culture. Recently, some efforts to develop a simple synthetic medium without FCS have been carried out to analize a factor(s) needed for early development of the embryo (6). In contrast, there is little report to cultivate the oocytes for in vitro maturation in a chemical defined medium.

In this study, we demonstrated whether Medium-II could be used for in vitro maturation of porcine follicular oocytes. We modified Kreb's-Ringer Bicarbon-

ate Solution (KRB) by addition of some inorganic salts based on the composition of porcine follicular fluid, which is designated as Medium-II.

## Materials and Methods

*Collection of oocytes*

Ovaries of prepubertal gilts were collected at a local slaughterhouse and transferred to the laboratory within 30 minutes under a warm condition. Follicles within 2-5 mm in diameter were aspirated by a 10 ml syringe with an 18-gauge needle and the oocyte-cumulus complexes were collected. Only the oocytes with some layers of unexpanded cumulus cells were selected, washed 3 times with Dulbecco's phosphate buffered saline (PBS, Nissui, JAPAN) and then washed with the maturation medium.

*Sperm preparation*

A sperm rich portion of ejaculated semen was collected from a Meishan boar by a glove-hand method and filtrated with a double of gauze to remove the gel fraction. After dilution of the sperm fraction with 2-3 times volume of Kiev's solution (glucose 60 g/l, Na citrate 3.7 g/l, $NaHCO_3$ 1.2 g/l and EDTA 3.7 g/l), the sperm suspension was kept for 10-20 h at 15°C before use. Immediatly before in vitro fertilization, the sperm suspension was washed 3 times with 0.9% NaCl containing 0.1% BSA and dibekacin sulfate (100 $\mu$g/ml, Meiji-Seika, JAPAN) by centrifugation for 5 min at 1,000 $\times$ g. The resulting sperm pellet was resuspended and diluted to $2 \times 10^8$ cells/ml in the sperm preincubation medium and incubated for 2 h at 37°C.

*In vitro fertilization*

After culture for maturation, the oocytes were transferred to drops of the fertilization medium covered with mineral oil (SQUIBB, USA), and inseminated with the preincubated sperm at a concentration of $2 \times 10^6$ cells/ml. Eight hours after insemination, the oocytes were washed 3 times with the embryo culture medium and then each of 20-30 oocytes was transferred to a 100 $\mu$l-droplet of the culture medium under the oil.

*Culture medium and culture condition*

For in vitro culture of the oocytes, modified TCM199 (mTCM199) with Earle's salts (Nissui, JAPAN) and Medium-II were used. mTCM199 was supplemented with 0.91 mM sodium pyruvate, 3.05 mM glucose and 2.92 mM calcium lactate (Wako, JAPAN) as same as a recipe described by Yoshida *et al.* (7). Medium-II (Table 1) was made up based on a composition of inorganic salts of porcine follicular fluid (1). In both media, PMSG (10 IU/ml, Peamex, Sankyou,

TABLE 1. *A composition of Medium-II**

| Component | mM | mg/100 ml |
|---|---|---|
| NaCl | 97.02 | 567.0 |
| KCl | 14.71 | 109.7 |
| $KH_2PO_4$ | 1.19 | 16.2 |
| $CaCl_2 \cdot 2H_2O$ | 2.34 | 34.4 |
| $MgSO_4 \cdot 7H_2O$ | 1.19 | 29.3 |
| $NaHCO_3$ | 25.07 | 210.6 |
| D-Glucose | 5.55 | 100.0 |
| Na-pyruvate | 0.50 | 5.5 |
| Ca-lactate | 1.43 | 44.1 |
| Phenol red | | 1.0 |

* The pH was adjusted to 7.4 with 0.2N NaOH.

JAPAN), hCG (10 IU/ml, gifted from NIAS), estradiol-17$\beta$ (1 $\mu$g/ml, Sigma, USA) and dibekacin sulphate (100 $\mu$g/ml) were added.

For sperm preincubation, the pH of mTCM199 was adjusted to 7.8 with 0.2 N NaOH. The fertilization medium consisted of 10% FCS (Flow Lab., USA)-mTCM199 supplemented with 2 mM caffeine (Wako, JAPAN). For embryo culture, mBMOC-3 (8) was used. The culture condition throughout the experiment was performed at 39°C in an atmosphere of 5% $CO_2$ in air under high humidity.

*Experiment 1*

To investigate in vitro maturation of porcine oocytes in Medium-II, the oocytes were cultured up to 48 h in the medium supplemented with either FCS or BSA (5 mg/ml, Sigma, USA). After culture, the oocytes were treated with 0.25% trypsin to remove the cumulus mass, fixed with aceto-ethanol (acetic acid : ethanol = 1 : 3, v/v) and then stained with 1% aceto-orcein. The oocytes having Metaphase-2 with a polar body was recognized as completely matured one.

*Experiment 2*

To test a capacity of the oocytes matured to fetilize and cleave, the oocytes were cultured for 42 h and inseminated as described above. After in vitro fetilization, some oocytes were fixed and stained to examine a formation of pronucleus. The others were allowed to further develop. Forty-eight hours after insemination, the number of cleaved eggs to 2-4 cell stage was scored.

In both experiments, the data was accumulated from at least 2 trials. A statical significance of the results was evaluated by $\chi^2$ test.

## Results

In the another experiment (9), we demonstrated a time-course of nuclear maturation of porcine oocytes. The results showed that at 12 h after culture all of the oocytes still had a GV-phase nucleus, at 24 h many oocytes had metaphase 1, at 30 h the oocytes with Metaphase 2 first appeared and then the ratio of those oocytes gradually increased and at 42 h reached to peak.

*Experiment 1.*

Table 2 shows the results obtained in Experiment 1. The oocytes that had completely nuclear maturation were found 42 h after culture at a high ratio in either mTCM199 or Medium-II. There was no significant difference on nuclear maturation rate among any combined media. Nuclear maturation rate of the oocytes 48 h after culture was similar to that 42 h after culture in both media (data not shown).

*Experiment 2.*

The rate of both male and female pronucleus formation was significantly

TABLE 2. *Meiotic maturation of porcine oocytes cultured in different media**

| Culture medium | Additive | No. of oocytes examined | No. of oocytes matured (%) |
|---|---|---|---|
| mTCM199 | FCS | 48 | 37 (77.1)[a] |
| | BSA | 50 | 37 (74.0)[a] |
| Medium-II | FCS | 30 | 18 (60.0)[a] |
| | BSA | 56 | 37 (66.1)[a] |

* Date was accumulated from at least 2 experiments.
[a] Values with a common superscript are not significantly different ($P>0.05$).

TABLE 3. In vitro fertilization of porcine oocytes matured in either Medium-II or mTCM199

| Culture medium | No. of oocytes examined | No. of oocytes matured[1] (%) | No. of oocytes with | |
|---|---|---|---|---|
| | | | F.P.[2] formation (%) | M.P.[3] formation (%) |
| Medium-II | 85 | 76 (89.4)[a] | 49 (57.7)[a] | 10 (11.8)[a] |
| mTCM199 | 65 | 61 (93.9)[a] | 56 (86.2)[b] | 22 (33.9)[b] |

[a,b] Values without a common superscript are significantly different ($P<0.05$).
[1] They were matured for 42h and inseminated for 8h.
[2] F.P.; Female pronucleus
[3] M.P.; Male pronucleus

TABLE 4. *Cleavage of porcine oocytes matured in either Medium-II or mTCM199, followed by in vitro fertilization*

| Culture medium | No. of oocytes examined | No. of oocytes developing to | | Total no. of oocytes showing cleavage (%) |
|---|---|---|---|---|
| | | 2cell | 4cell | |
| Medium-II | 180 | 18 | 14 | 32(17.8)[a] |
| mTCM199 | 183 | 29 | 21 | 50(27.3)[b] |

[a,b] Values without a common superscript are significantly different ($P<0.05$).

higher ($P<0.05$) in the oocytes cultured in mTCM199 than that in those cultured in Medium-II (Table 3). Although the maturation rate was similar in both media, the rate of early development 48 h after insemination was significantly higher ($P<0.05$) in the oocytes cultured in mTCM 199 (Table 4).

## Discussion

In the present study, we demonstrated a defined culture medium to mature porcine follicular oocytes in vitro. We prepared a modified defined medium, Medium-II, which composition is based on that of inorganic salts of porcine follicular fluid (1). The results showed that Medium-II would sufficiently support in vitro nuclear maturation of the oocytes. McGaughey (10) cultured porcine follicular oocytes in BMOC-3, which is originally used for culture of murine embryo (11), and found that the completion of nuclear maturation occurred in vitro. Although Iritani et al. (12) also used an another defined medium, mKRB for culture of porcine oocytes and showed that 60.6% of the oocytes cultured had complete nuclear maturation, the use of both defined media was limited especially when IVF and IVC were employed. Instead, TCM199 has been mainly used for IVM of porcine follicular oocytes (5). The results in this study revealed that Medium-II would be a simple chemical defined medium to develop a serum-free culture medium since the addition of BSA instead of FCS did not have any detrimental effect on nuclear maturation.

Although the completion of nuclear maturation of the oocytes was found in Medium-II, the rate of both pronucleus formation and early development after IVF was significantly lower than that in the oocytes cultured in mTCM199. This result indicates that a cytoplasmic maturation may occur incompletely in the oocytes cultured in Medium-II. Since a mechanism of the cytoplasmic maturation is still unknown, it is necessary to elucidate a factor(s) essential for cytoplasmic maturation in order to establish an IVM-IVF system in pigs. FCS is usually required to support in vitro maturation of the oocyte. To analyze a component of FCS effective for in vitro maturation, Medium-II would be contributed for

further study.

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