Effects of incubation temperature and bicarbonate on maturation of pig oocytes *in vitro*

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Summary. Pig oocytes cultured at 39°C had a higher percentage of polar body formation than did those cultured at 37°C. A culture medium based on Medium 199 with Earle's salts and supplemented with 15% serum from a young castrated boar was just as good as the same formulation containing additional pyruvate, lactate and insulin, and superior to a modified Krebs–Ringer bicarbonate medium.

When the bicarbonate buffer system (Earle's salts) of Medium 199 was replaced with a phosphate buffer system (Hank's salts), the rate of polar body formation was decreased. When the Hank's based medium was supplemented with a bicarbonate buffer system, polar body formation was restored to the level in Earle's based medium. This suggests that CO₂/bicarbonate may be important for the normal maturation of pig oocytes.

Introduction

The successful development of mammalian fertilized ova and preimplantation embryos *in vitro* requires the presence of bicarbonate in the culture medium (Quinn & Wales, 1973; Kane, 1975). The role of bicarbonate may be more than acting simply as a buffer system. Quinn & Wales (1971, 1974) found that ¹⁴CO₂ was incorporated mainly into amino acids and protein, probably by the pyruvate carboxylase mediated condensation of carbon dioxide with pyruvate to form oxaloacetate. Radiolabel was found also in acetate, malate, ketoglutarate, and citrate (Wales, Quinn & Murdoch, 1969) in mouse and rabbit embryos, and in lactate and pyruvate in rabbit embryos. Kane (1975) reported that 1-cell rabbit zygotes develop to morula in medium buffered by *N*-2-hydroxyethyl piperazine-*N'*-2 ethanesulphonic acid (Hepes), but that bicarbonate was necessary for development to the blastocyst stage.

The maturation *in vitro* of oocytes aspirated from preovulatory follicles has been described for many mammalian species. Most investigations utilized a bicarbonate buffering system that was maintained by incubation in an atmosphere of 5% CO₂ in air, although some (Peluso & Hutz, 1980; Dekel, Aberdam & Sherizly, 1984; Racowsky & Satterlie, 1985) have not done so. Nearly all previous studies have maintained cultures at 37°C. The normal body temperature of pigs is 39°C. The present study was undertaken to determine the effects of temperature and of bicarbonate versus phosphate buffering systems on the maturation of pig oocytes *in vitro*.

Materials and Methods

Culture media. Pig serum was collected from castrated boars at 10–11 weeks of age, and stored at –76°C in 500 ml samples. Before use, the serum was heat-treated at 56°C for 1 h, centrifuged at 47 800 *g* for 20 min at 4°C, and passed through 5.0 µm, 0.45 µm, and 0.22 µm membrane filters

under pressure. The serum was then added to sterile medium for immediate use, or stored at -20°C for later use. Serum steroid hormones were assayed by radioimmunoassay (RIA; Jaffe & Behrman, 1974). Concentrations of oestradiol, testosterone, and dihydrotestosterone were <0.5 ng/ml, while progesterone was measured at 0.51 ng/ml.

Several culture media were utilized in these experiments. Most of the Medium 199 was purchased from GIBCO Laboratories, Grand Island, New York. Some was obtained from KC Biological, Inc., Lenexa, Kansas. TC-199H consisted of Medium 199 with Hank's salts, 1 mM-glutamine, 10 mM-Hepes, 15% pig serum, and 50 μg gentamycin sulphate/ml. TC-199E was the same as TC-199H, but with Earle's salts. TC-199MH was the same as TC-199H, but with the same amount of sodium bicarbonate as TC-199E (2.2 g/l). TC-199TC was the same as TC-199E, but further supplemented with 0.03 mM-pyruvate, 2.5 mM-lactate and 12.5 mU insulin/ml (Iletin I, bovine and porcine mixture, 100 i.u./ml Eli Lilly & Co., Indianapolis, IN), and was essentially identical to a formulation described by Tsafri & Channing (1975) to be optimal for maturation of pig oocytes. A modified Krebs-Ringer bicarbonate medium was prepared according to Toyoda & Chang (1974), substituting 50 μg gentamycin/ml for the original antibiotics.

Pyruvate and lactate concentrations in culture media and components were determined with enzyme assay kits purchased from Sigma Chemical Company, St Louis, MO (Pyruvate Kit 726; Lactate Kit 826).

Collection of oocytes. Ovaries were obtained at a local abattoir. Pigs were rendered unconscious by a blow to the head with a captive bolt and killed by exsanguination. The carcasses were skinned and the ovaries removed within 5 min. The ovaries were pooled until approximately 50 had been collected (2 h). During this time, the ovaries were maintained at a temperature of 25 – 27°C . Medium-sized follicles (3 – 7 mm diameter) were then gently aspirated with a 3 ml syringe equipped with a 23 -gauge short-bevel needle. The pooled follicular contents were centrifuged at $57g$ for 15 sec and the supernatant fluid was removed. The pelleted cumulus-oocyte complexes were resuspended (60 – 80 oocytes/ 12 ml tube) in TC-199H and maintained at 39°C during the 30 min journey to the laboratory.

The collected oocytes were rinsed twice in TC-199H and then distributed to various media as described below for culture. Only oocytes with tightly adherent cumulus cells were cultured. Oocytes without cumulus cells were discarded.

Culture procedures. Cultures were routinely performed in 35×10 mm Lux Contur dishes (Miles Scientific, Naperville, Illinois) with 30 – 50 cumulus-oocyte masses in a volume of 2 ml culture medium. Except as noted below, the dishes were incubated at 39°C in an humidified atmosphere of 5% CO_2 in air. After 46 – 48 h incubation, the oocytes were removed for examination. At this time it was noted that many of the cumulus cells had separated from the cumulus-oocyte complexes and were firmly adherent to the dish.

Maturation scoring. The criterion for maturation was presence of a polar body. Most of the still-adhering cumulus cells were removed by repeated pipetting with a fine-bore pipette. Oocytes were then aspirated into a 0.3 mm $\times$ 0.3 mm square capillary (Microcell 8330, Vitro Dynamics, Rockaway, NJ) and examined at $\times 200$ magnification with a Nikon Labophot equipped with Nomarski differential interference optics. The contents of each capillary were examined four times, rotating 90° around the long axis after each examination until each oocyte had been viewed from 4 sides. The presence or absence of a polar body was noted for each oocyte.

Statistical analysis. Each experiment consisted of a comparison between two treatments. In each experiment, the oocytes were allocated equally between the treatments. The ratios of eggs with polar bodies/total eggs cultured were compared by a test for two proportions based on the large sample normal approximation to the distribution of the difference in sample proportion (Bailey, 1981).

Results

The effects of culture media and incubation conditions on polar body formation are summarized in Table 1.

Table 1. The effects of various incubation conditions on polar body formation during maturation of pig oocytes *in vitro*

Exp.	Treatments PBF/eggs (% PBF)		No. of replicates	Significance
1	TC-199E 37°C 45/122 (37)	TC-199E 39°C 92/129 (71)	6	$P < 0.001$
2	TC-199E 191/454 (42)	KRB 117/452 (26)	15	$P < 0.001$
3	TC-199E 137/288 (48)	TC-199TC 128/301 (43)	7	$P < 0.2$
4	TC-199H* (GIBCO) 51/151 (34) (KCB) 16/74 (22)	TC-199E (GIBCO) 109/146 (75) (KCB) 42/75 (56)	5 2	$P < 0.001$ $P < 0.001$
5	TC-199E (KCB) 68/173 (39)	TC-199E (GIBCO) 134/191 (70)	4	$P < 0.001$
6	TC-199E 67/161 (42)	TC-199MH 64/164 (39)	3	$P < 0.6$

* Incubated in air atmosphere.

KRB, modified Krebs–Ringer–bicarbonate; PBF, polar body formation; KCB, KC Biological, Inc.

Experiment 1

Incubation in TC-199E for 48 h at 39°C resulted in a significantly greater (71%) rate of polar body formation than at 37°C (37%).

Experiment 2

TC-199E was compared to a modified Krebs–Ringer–bicarbonate solution containing glucose, pyruvate, lactate, bovine serum albumin, and antibiotics (Toyoda & Chang, 1974). This medium has been used successfully to mature cumulus-intact pig oocytes *in vitro* (Iritani, Niwa & Imai, 1978; Nagai, Niwa & Iritani, 1984). As can be seen in Table 1, oocytes cultured in TC-199E formed polar bodies at a greater rate (42%) than did those cultured in the Krebs–Ringer–bicarbonate medium (25%).

Experiment 3

There was no difference in the ability of Media TC-199TC and TC-199E to support polar body formation.

Experiment 4

Oocytes cultured in Medium TC-199E under 5% CO₂ produced polar bodies twice as often as did oocytes cultured in Medium TC-19H in air. This difference was observed for the media obtained from the two different suppliers.

Experiment 5

The results of Exp. 4 suggested that there was an effect of source of the culture medium on polar body formation. The results of Exp. 5 confirmed that a higher proportion of polar bodies was found in the oocytes incubated in the GIBCO TC-199E medium.

Experiment 6

The results of Exp. 6 indicate that the addition of increased bicarbonate to a Hank's based medium (TC-199MH) and subsequent incubation under 5% CO₂ in air allows polar body formation at a rate equal to that observed in an Earle's based medium (TC-199E).

Pig serum and TC-199E from cultures at 0, 24 and 48 h incubation times with egg-cumulus cell complexes were assayed for the presence of pyruvate and lactate. The results are shown in Table 2.

Table 2. Pyruvate concentrations (mm, mean $\pm$ s.e.m.) in culture medium after incubation with egg-cumulus masses

Pig serum	0.207 $\pm$ 0.002
TC-199E without serum	0.007 $\pm$ 0.001
TC-199E after 0 h	0.035 $\pm$ 0.003
TC-199E after 24 h	0.042 $\pm$ 0.002
TC-199E after 48 h	0.060 $\pm$ 0.009

Pig serum contained 0.207 $\pm$ 0.002 mm-pyruvate, while TC-199E without serum contained 0.007 $\pm$ 0.001 mm-pyruvate. TC-199E after 0, 24 and 48 h incubation with egg-cumulus complexes contained 0.035 $\pm$ 0.003 mm, 0.042 $\pm$ 0.002 mm, and 0.60 $\pm$ 0.009 mm-pyruvate respectively. None of the media or its components contained detectable levels of lactate.

Discussion

In the present study, several culture media were compared for their ability to promote polar body formation in immature oocytes. Kuehl & Dukelow (1979) reported that the inclusion of insulin in a medium based on TC-199 with Earle's salts and L-glutamine did not increase in-vitro maturation or fertilization of monkey ova, although fibroblast monolayers were suppressed. Tsafirri & Channing (1975) had reported that Medium TC-199 containing added pyruvate, lactate and insulin gave higher rates of germinal vesicle breakdown (GVBD) and polar body formation than did unsupplemented media. In the present study the complex medium of Tsafirri & Channing (1975) was shown to have no advantage over the simpler formulation lacking these additions.

The difference between the current study and that of Tsafirri & Channing (1975) may be due to the difference in incubation temperatures used, since Exp. 1 showed that maintaining the cultures at 39°C was advantageous to incubation at 37°C. Lenz, Ball, Liebfried, Ax & First (1983) reported that fertilization of cow oocytes *in vitro* was greatest when oocytes were maintained in culture at the core body temperature of the species (39°C) during maturation as well as during sperm penetration. It seems clear that attention should be paid to the normal body temperature of the species under investigation in future studies of gamete physiology.

Another difference may be related to the source of pig serum. Tsafri & Channing (1975) used serum with low levels of sex hormones. We chose young castrated boars as a serum source likely to have very low levels of sex hormones and this was confirmed by RIA. The serum from many animals was pooled to produce a large single lot, and frozen as 500 ml samples, resulting in a consistent source of pig serum uniformly low in sex steroids.

One major difference between the Media TC-199E and TC-199H is in the buffer system employed. Media based on Earle's salts contain 6–7 times the amount of bicarbonate that is found in media based on Hank's salts. Cultures in Earle's media are usually maintained under an atmosphere of 5% CO₂ in air to maintain the appropriate pH. Media with lower bicarbonate levels (i.e. based on Hank's salts) do not require an atmosphere enriched in CO₂.

The vast majority of investigators utilize an atmosphere of 5% CO₂ in air in which to mature or fertilize mammalian oocytes *in vitro*, although some reports relate to cultures in an atmosphere of air only (Peluso & Hutz, 1980; De Felici & Siracusa, 1982; Dekel *et al.*, 1984). In the present study, Medium TC-199 based on a predominately bicarbonate buffer system (Medium TC-199E; Earle's salts) and incubated in 5% CO₂ yielded twice the percentage of polar body formation as the same medium based on a predominately phosphate buffer system (Medium TC-199H; Hank's salts) and incubated in air. When the Hank's based medium was supplemented with extra bicarbonate, however, the percentage of polar body formation was increased to that of the Earle's based medium, demonstrating that bicarbonate ion is required for normal polar body formation. This extends earlier observations (Biggers, Whittingham & Donahue, 1967; Wales *et al.*, 1969; Quinn & Wales, 1971, 1973) on the importance of CO₂ fixation for the development of preimplantation embryos in culture. Quinn & Wales (1973) further found that embryos maintained in phosphate-buffered medium had reduced incorporation of low molecular weight precursors into macromolecules.

Oviducal fluid is a rich source of bicarbonate ion (Vishwakarma, 1962; Hamner, 1973; Maas, Storey & Mastroianni, 1977). Biggers *et al.* (1967) suggested that carbon dioxide could be fixed via pyruvate carboxylase to form oxaloacetate in the mouse oocyte and zygote. This was strengthened by further reports that the primary energy source in cumulus-free maturing mouse oocytes is pyruvate (Eppig, 1976; De Felici, Vivarelli, Giustiniani & Siracusa, 1982). Kuehl & Dukelow (1979) found that pyruvate was beneficial to the *in-vitro* maturation and fertilization of monkey ova. The ability of cumulus-intact pig oocytes in the present investigation to mature in the absence of exogenous pyruvate may be due to the presence of sufficient pyruvate or equivalent substrate in the supplementing serum. Alternatively, there may be production of pyruvate from glucose via glycolysis in the cumulus cells (Donahue & Stern, 1968; Leese & Barton, 1985). In the present report, TC-199E was shown to contain as much pyruvate as was added by Tsafri & Channing (1975). Further, there was an increase in pyruvate concentration after egg–cumulus cell complexes had been incubated for 24 and 48 h. It appears therefore that the egg–cumulus cell complexes are capable of producing pyruvate to meet metabolic needs.

We used polar body formation rather than germinal vesicle breakdown as an endpoint for oocyte maturation for two reasons. Firstly, it is our experience that the accurate determination of meiotic stage in fixed and stained whole-mount pig oocytes is problematic, due to the characteristically great amount of yolk in this species. In this regard, we are in agreement with Racowsky & McGaughey (1982) who resorted to analysis of chromosome spreads. Secondly, we have fixed, embedded in paraffin wax, and serially sectioned a large number of pig oocytes. Germinal vesicle breakdown was observed in >99.5% of oocytes which had been cultured, whereas >95% of uncultured oocytes had intact germinal vesicles (L. A. Eng, E. T. Kornegay & J. Huntington, unpublished data). However, the highest rate of polar body formation in the current report was only 75%. These figures are in close agreement with those reported by Motlik, Crozet & Fulka (1984). The observation of polar body formation is therefore a more stringent criterion of normal physiological oocyte maturation than is germinal vesicle breakdown.

The apparent superiority of media from one supplier over that of another in Exp. 4 and Exp. 5

should be viewed with caution: only single lots of both Hank's and Earle's based media from each supplier were tested and it is equally likely that differences were due to lot variations.

In summary, the culture of immature pig oocytes at 39°C in Medium TC-199E in an atmosphere of 5% CO₂ in air was shown to be superior to other culture regimens tested in promoting polar body formation. Bicarbonate, but not pyruvate, lactate or insulin, was shown to be a necessary component of the culture medium. The requirement for bicarbonate may be related to the metabolic needs of the maturing oocyte.

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