Zygote **9** (November), pp 331–337. © 2001 Cambridge University Press Printed in the United Kingdom

Effect of incubation temperature on *in vitro* maturation of porcine oocytes: nuclear maturation, fertilisation and developmental competence

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Date submitted: 12.4.01. Date accepted: 14.5.01

Summary

The present study examined the effect of low culture temperature during *in vitro* maturation (IVM) of pig oocytes on their nuclear maturation, fertilisation and subsequent embryo development. In experiment 1, oocytes were cultured at 35 or 39 °C for 44 h in modified tissue culture medium 199 supplemented with 10 ng/ml epidermal growth factor, 0.57 mM cysteine, 75 µg/ml potassium penicillin G, 50 µg/ml streptomycin sulphate, 0.5 µg/ml LH and 0.5 µg/ml FSH to examine the nuclear maturation status. In experiment 2, oocytes were cultured at 35 °C for 44 or 68 h and nuclear maturation was examined. In experiment 3, oocytes matured for 44 or 68 h at 39 °C and for 68 h at 35 °C were co-incubated with frozen-thawed spermatozoa for 5-6 h. Putative embryos were transferred into North Carolina State University (NCSU) 23 medium containing 0.4% bovine serum albumin. At 12 h after insemination, some oocytes were fixed to examine the fertilisation rate and the remaining embryos were examined at 48 and 144 h for cleavage and blastocyst formation rate, respectively. Compared with 39 °C, culture of oocytes at 35 °C for 44 h significantly ($p < 0.05$) reduced the metaphase II (M II) rate (79% vs 12%). However, extension of culture time to 68 h at 35 °C significantly increased ($p < 0.05$) the M II rate (7% vs 58%). In experiment 3, compared with other groups, fewer ($p < 0.05$) oocytes reached M II when cultured at 35 °C for 68 h (69-81% vs 49%). Extension of culture duration to 68 h at 39 °C stimulated spontaneous activation (28%) of oocytes. No difference in cleavage rates was observed among different groups. Compared with oocytes matured for 44 h at 39 °C (31%), the proportion of blastocysts obtained was low ($p < 0.05$) for oocytes matured at 35 °C (13%) or 39 °C (3%) for 68 h. The results indicate that lower culture temperature can delay nuclear maturation of pig oocytes. However, extension of culture time can stimulate nuclear maturation and these oocytes are capable of fertilisation and development to the blastocyst stage at moderate rates.

Keywords: Culture temperature, Embryo development, *In vitro* maturation, Pig oocytes

Introduction

In vitro maturation (IVM) of oocytes aspirated from ovarian follicles has been described for a variety of mammalian species including the pig. Successful nuclear maturation of pig oocytes to the metaphase II stage can be achieved in a variety of media, ranging from complex to defined (Naito *et al.*, 1988; Funahashi & Day, 1993*a*, *b*; Yoshida, 1993; Wang & Niwa, 1995*a*, *b*; Wang *et al.*, 1997; Abeydeera, 2000). However, developmental competence of these oocytes depends on the composition of the culture medium (Wang *et al.*, 1997). In our laboratory, routine IVM is carried out in a defined medium that results in matured oocytes capable of development to the blastocyst stage following fertilisation (Abeydeera, 2000). Furthermore, transfer of morula-stage embryos to recipient gilts has resulted in successful pregnancies and the birth of live piglets. All the above-mentioned studies used a culture temperature of 37 °C (Naito *et al.*, 1988), 38.5 °C (Yoshida, 1993) or 39 °C (Funahashi & Day, 1993*a*, *b*; Wang & Niwa, 1995*a*, *b*; Wang *et al.*, 1997; Abeydeera, 2000). Recent experiments conducted by Hunter *et al.* (1997) revealed that under *in vivo* conditions, the mature ovarian follicles (7–10 mm in diameter) in pigs are always cooler

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https://doi.org/10.1017/S0967199401001381 Published online by Cambridge University Press

than the ovarian stroma (35.6 vs 37.3 °C) and deep rectal temperature (35.6 vs 38 °C). Similarly in rabbits, the mean temperature of antral follicles is about 2.8 °C lower than the core body temperature (Grinsted *et al.*, 1980). Conventional IVM culture of pig oocytes is performed at a much higher temperature (38.5–39 °C) than for those oocytes matured within the ovarian follicular environment *in vivo*. Hunter *et al.* (1997) concluded that follicular temperature could influence the meiotic progression and cytoplasmic maturation of oocytes. However, there is little or no information available on the relationship between oocyte maturation temperature and the subsequent developmental competence.

Although successful culture techniques are available for pig oocyte maturation and fertilisation, polyspermy remains a major problem (Niwa, 1993; Nagai, 1994; Niwa & Funahashi, 1999; Day, 2000). In general, initial sperm penetration triggers the release of cortical granule contents and establishes the block to polyspermy at the level of the zona pellucida (Kim *et al.*, 1996*b*). Microfilaments are involved in the movement and stabilisation of cortical granules beneath the plasma membrane and cortical granule exocytosis (Kim *et al.*, 1996*a*; DiMaggio *et al.*, 1997). It has been shown that microtubules are sensitive to higher temperatures (Fiorenza & Mangia, 1992). Similarly, culture of pig oocytes at a higher temperature compared with its *in vivo* thermal conditions may interfere with microfilaments and could subsequently affect the normal maturation kinetics leading to defective nuclear and/or cytoplasmic maturation.

It was hypothesised that IVM at a temperature that mimics the thermal environment of the porcine preovulatory follicles *in vivo* may provide a better culture condition for nuclear and/or cytoplasmic maturation of pig oocytes. Therefore, this study was designed to examine the effects of culture temperature (35 vs 39 °C) on nuclear maturation, fertilisation and subsequent embryo development of pig oocytes.

Materials and methods

Culture media

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co., St Louis, MO. The basic medium used for *in vitro* oocyte maturation (IVM) was a modified (m) Tissue Culture Medium 199 (TCM; Gibco, Grand Island, NY) supplemented with 0.1% polyvinylalcohol, 75 µg/ml potassium penicillin G, 50 µg/ml streptomycin sulphate and with additional supplementation of 3.05 mM D-glucose and 0.91 mM sodium pyruvate (mTCM; Abeydeera, 2000). The complete IVM medium was prepared by adding 10 ng/ml epidermal growth factor (E 4127), 0.57 mM cysteine, 0.5 µg/ml LH (L 5269) and 0.5 µg/ml FSH (F 2293) to mTCM. Basic *in vitro* fertilisation (IVF) medium, designated modified Tris-buffered medium (mTBM), was essentially the same as that described by Abeydeera & Day (1997). The complete IVF medium contained 0.1% bovine serum albumin (BSA) (Sigma; A 7888) and 1 mM caffeine. Culture medium for embryo development was North Carolina State University (NCSU) 23 (Petters & Wells, 1993) containing 0.4% BSA (A 8022), 75 µg/ml potassium penicillin G and 50 µg/ml streptomycin sulphate.

In vitro maturation of oocytes

Ovaries recovered from prepubertal gilts at a local slaughterhouse were transported to the laboratory in 0.9% NaCl (w/v) containing 75 µg/ml potassium penicillin G and 50 µg/ml streptomycin sulphate maintained at 25–28 °C. Oocytes were aspirated from medium-sized follicles (3–6 mm diameter) with an 18 gauge needle fixed to a 10 ml syringe. Cumulus-oocyte complexes (COC) surrounded by ≥ 3 cumulus cell layers with evenly granulated cytoplasm were selected under a stereomicroscope and washed three times in IVM medium. Then, 40–50 oocytes were transferred into a 500 µl of medium in a Nunc 4-well multidish (Nunc, Roskilde, Denmark) which had previously been covered with paraffin oil (light mineral oil, Fisher Scientific, Fair Lawn, NJ) and equilibrated in a 5% CO_2 incubator.

In vitro fertilisation

After the completion of maturation culture, cumulus cells were removed with 0.1% hyaluronidase in mTCM. Denuded oocytes were washed three times with IVF medium and 30-35 oocytes each were placed in 50 µl drops of the same medium covered with paraffin oil in a 35 × 10 mm^2 polystyrene culture dish. The dishes were kept in the incubator for about 45 min until spermatozoa were added for fertilisation. A frozen semen pellet was thawed and washed three times by centrifugation at 1900 *g* for 4 min in Dulbecco's phosphate-buffered saline (DPBS; Gibco) supplemented with 0.1% BSA (A 8022), 75 µg/ml potassium penicillin G and 50 µg/ml streptomycin sulphate (pH 7.2, 39 °C). At the end of the washing procedure, the sperm pellet was resuspended in 100 µl of IVF medium and sperm concentration was determined using a haemacytometer. After appropriate dilution, 50 µl of this suspension was added to 50 µl drops of IVF medium that contained oocytes to give a final concentration of 1.5×10^5 cells/ml. Oocytes were co-incubated with spermatozoa for 5–6 h at 39 °C in an atmosphere of 5% CO_2 in air.

https://doi.org/10.1017/S0967199401001381 Published online by Cambridge University Press

Assessment of nuclear maturation and fertilisation parameters

After IVM culture or 12 h after IVF, oocytes were mounted, and fixed for 72 h in 25% (v/v) acetic acid in ethanol at room temperature. Oocytes were stained with 1% (w/v) orcein in 45% acetic acid (v/v) and examined under a phase-contrast microscope at ×200 and ×400 magnification. Nuclear maturation stage of oocytes was assessed according to Hunter & Polge (1966). Sperm penetration of oocytes was considered normal when they had a female pronucleus (FPN), two polar bodies (PB), and one or more swollen sperm head(s) and/or male pronucleus(i). Other forms of oocyte activation (such as one female FPN with one PB or two FPN with or without PB) associated or not with sperm penetration were considered as abnormal and spontaneous oocyte activation, respectively.

Embryo culture

After sperm-oocyte co-incubation, presumptive zygotes were washed three times in embryo culture medium (NCSU 23 + 0.4% BSA) and transferred to a 4-well multidish containing 500 µl of the same medium and incubated at 39 °C, 5% CO_2 in air. At 48 and 144 h after IVF, cleavage rate and blastocyst formation, respectively, was evaluated under a stereomicroscope.

Experimental design

Experiment 1
Porcine oocytes were matured at 35 and 39 °C for 44 h and then fixed in order to examine the status of nuclear maturation.

Experiment 2
The nuclear status of oocytes matured for 44 and 68 h at 35 °C was examined.

Experiment 3
Oocytes were matured for 44 and 68 h at 35 °C. In addition, some oocytes obtained on the same day as the experiment and the following day were cultured for 44 h at 39 °C. Oocytes obtained on the same day (D 1) served as the positive control to examine the normality of oocytes and oocytes from the following day (D 2) served as the positive control for the IVF. After IVM, a proportion of oocytes from each group was fixed to examine the nuclear maturation. Remaining oocytes were subjected to IVF as described above. At 5–6 h after IVF, presumptive zygotes were cultured for 144 h in embryo culture medium and a proportion of presumptive zygotes were fixed (12 h after IVF) to examine the fertilisation parameters.

Statistical analysis

Data were analysed by ANOVA and Fisher's protected least significant difference test using the STATVIEW program (Abacus Concepts, Berkeley, CA). All percentage data were subjected to arscine transformation before statistical analysis. Data are expressed as mean + SEM. A probability of $p < 0.05$ was considered to be statistically significant.

Results

Compared with those pig oocytes matured at 39 °C for 44 h, nuclear maturation to metaphase II (M II) stage was either significantly ($p < 0.05$) inhibited or delayed in oocytes cultured at 35 °C (80% vs 12%; Table 1). However, more than half the oocytes cultured at 35 °C could initiate maturation as evidenced by germinal vesicle breakdown (GVBD) and 32% reached the metaphase I (M I) stage. Extension of culture time from 44 to 68 h at 35 °C significantly increased ($p < 0.001$) the proportion of oocytes completing nuclear maturation to M II stage (58% vs 7%; Table 2).

When oocytes were cultured for 44 h at 39 °C, 81% (D 1) to 82% (D 2) of oocytes reached M II stage and extending the culture by 24 h resulted in 97% of oocytes reaching M II or beyond: 69% at M II and 28% showed spontaneous activation, cleavage and/or cytoplasmic fragmentation (Table 3). Compared with other groups, only 49% of oocytes reached M II stage ($p < 0.05$) when oocytes were cultured for 68 h at 35 °C.

Table 1 Nuclear maturation status of pig oocytes cultured for 44 h at 35 or 39 °C

Culture temp. (°C)	No. oocytes examined	% (± SEM) oocytes at the stage of			
		GV	GVBD	M I	M II
35	98	45.6 ± 8.1[a]	10.7 ± 6.4	31.8 ± 5.6[a]	11.9 ± 5.0[a]
39	108	4.9 ± 2.5[b]	2.8 ± 2.8	13.3 ± 2.8[b]	79.1 ± 5.5[b]

GV, germinal vesicle; GVBD, GV breakdown; M I, metaphase I; M II, metaphase II.
[a-b] $p < 0.05$ at least.

https://doi.org/10.1017/S0967199401001381 Published online by Cambridge University Press

Table 2 Nuclear maturation status of pig oocytes cultured at 35 °C for 48 or 68 h

Culture time (h)	No. of oocytes examined	% (± SWEM) oocytes at the stage of				
		GV	GVBD	M I	A I–T I	M II
48	94	45.7 ±4.3	12.1 ± 12.1	35.2 ± 9.3	0.0 ± 0.00	7.1 ± 1.5[a]
68	97	27.0 ± 8.1	0.0 ± 0.0	15.1 ± 1.6	0.0 ± 0.0	58.0 ± 9.7[b]

A I–T I, anaphase I-telophase I; other abbreviations as in Table 1.
[a,b] $p < 0.001$ at least.

Table 3 Effect of temperature and culture time on IVM of pig oocytes

IVM condition	No. examined	% (± SEM) oocytes at the stage of				
		GV	GVBD	M I	M II	> M II
44 h at 39 °C (D 1)	103	5.9 ± 1.7	2.9 ± 1.6	10.6 ± 1.8	80.6 ± 1.8[a,b]	0.0 ± 0.0
44 h at 39 °C (D 2)	93	6.6 ± 0.6	1.0 ± 1.0	10.8 ± 1.0	81.7 ± 0.5[a334]	0.0 ± 0.0
68 h at 39 °C	104	2.2 ± 1.2	0.0 ± 0.0	0.0 ± 0.0	69.0 ± 6.3[b]	28.0 ± 6.0[b]
68 h at 35 °C	106	28.0 ± 3.2	0.9 ± 0.9	22.1 ± 4.7	48.9 ± 4.1[c]	0.0 ± 0.0

D 1, oocytes aspirated on day 1 (Monday); D 2, oocytes aspirated the next day (Tuesday); > M II, includes oocytes showing spontaneous activation, cleavage and fragmentation; other abbreviations as in Table 1.
[a-c] $p < 0.05$.

Table 4 Effect of temperature and culture time during IVM on IVF parameters pig oocytes

IVM condition	No. examined	(± SEM) oocytes with				
		Normal penetration[a]	Polyspermy[b]	MPN	MNS	Abnormal oocytes[c]
44 h at 39 °C (D 1)	99	55.2 ± 11.4[de]	32.6 ± 8.6	93.9 ± 1.5[d]	1.5 ± 0.1	0.9 ± 0.9[d]
44 h at 39 °C (D 2)	95	69.4 ± 6.0[d]	42.4 ± 7.7	90.4 ± 3.7[d]	1.6 ± 0.1	2.2 ± 2.3[d]
68 h at 39 °C (D 1)	95	41.0 ± 5.1[c]	57.2 ± 15.3	92.0 ± 1.1[d]	1.6 ± 0.2	30.6 ± 4.1[e]
68 h at 35 °C (D 1)	94	49.3 ± 8.1[de]	35.0 ± 8.1	69.3 ± 6.3[e]	1.4 ± 0.1	5.2 ± 3.6[d]

[a]Oocyte with a female pronucleus, two polar bodies, and one or more swollen sperm head(s) and/or male pronucleus (i).
[b]Percentage of normally penetrated oocytes with more than one swollen sperm head or male pronucleus.
[c]Abnormal includes those oocytes showing spontaneous activation, penetrated oocytes with one female pronucleus and one polar body or two female pronuclei with one polar body, cleavage and cytoplasmic fragmentation.
[d-c] $p < 0.05$ at least.

Table 5 Effect of temperature and culture time during IVM on embryo development

IVM condition	No. examined	% (± SEM) embryo development	
		Cleavage[a]	Blastocysts[a]
44 h at 39 °C (D 1)	209	60.2 + 9.8	30.6 + 4.5[b]
44 h at 39 °C (D 2)	175	62.4 + 6.5	33.3 + 4.9[b]
68 h at 39 °C	174	49.4 + 9.4	3.4 + 2.1[c]
68 h at 35 °C	173	45.7 + 5.0	12.9 + 3.7[d]

[a]Percentage of number examined.
[b-d] $p < 0.05$ at least.

https://doi.org/10.1017/S0967199401001381 Published online by Cambridge University Press

Except for the 44 h at 39 °C (D 1) and 68 h at 39 °C treatment groups, normal fertilisation rates (as evidenced by penetrated oocytes showing one female pronucleus and two polar bodies) did not differ among experimental groups (Table 4). In comparison with other groups, male pronuclear formation was significantly ($p < 0.05$) lower in the 68 h at 35 °C group. Many (31%) penetrated and non-penetrated oocytes obtained from the 68 h at 39 °C group showed abnormal activation as described in Materials and Methods.

As shown in Table 5, no differences in cleavage rates were observed among different treatment groups. Compared with the 68 h at 39 °C (D 1) and 68 h at 35 °C (D 1) groups, a significantly ($p < 0.05$) higher proportion of embryos developed to the blastocyst stage following IVF of oocytes matured in both 44 h at 39 °C groups. However, compared with the 68 h at 39 °C group (3%), a significantly ($p < 0.05$) higher blastocyst rate (13%) was observed for oocytes matured in the 68 h at 35 °C group.

Discussion

Conventional *in vitro* maturation systems for pig oocytes are maintained at 38.5–39 °C, which closely resembles body temperature. Although successful nuclear maturation is achieved, the degree of complete cytoplasmic maturation is questionable since many oocytes show polyspermic penetration and/or poor male pronuclear (MPN) formation following IVF (Sirard *et al.*, 1993; Nagai, 1994; Day, 2000). Supplementation of IVM medium with porcine follicular fluid (Naito *et al.*, 1988), co-culture with extroverted follicles (Mattioli *et al.*, 1988), cysteine (Yoshida *et al.*, 1993), epidermal growth factor (Ding & Foxcroft, 1994; Wang & Niwa, 1995*a*, *b*) or limiting the exposure to gonadotropic hormones during culture (Funahashi & Day, 1993*a*, *b*) and NaCl concentration in culture medium (Funahashi *et al.*, 1994) have been shown to improve the cytoplasmic maturation as evidenced by higher MPN formation. Despite the improvements achieved in MPN formation, compared with other domestic animals, polyspermy in pig oocytes remains a major unresolved problem following *in vitro* fertilisation.

It is noteworthy that under *in vivo* conditions, the temperature of preovulatory follicles in rabbits (Grinsted *et al.*, 1980) and pigs (Hunter *et al.*, 1997) is lower than the body temperature and the temperature of the ovarian stroma. The measurement of mean temperature of mature Graafian follicles (7–10 mm) was 35.6 ± 0.3 °C and ranged from 34.5 to 37.2 °C (Hunter *et al.*, 1997). Hunter *et al.* (1997) suggested that one approach to the involvement of temperature in the normal progression of meiotic maturation in the oocyte would be to undertake *in vitro* studies at temperatures lower than those currently used. In this study we chose a culture temperature of 35 °C as it was the lowest temperature recorded in preovulatory follicles. It was hypothesised that culture of pig oocytes during IVM at a lower temperature (35 °C) may provide a better thermal environment for the completion of nuclear and/or cytoplasmic maturation. Only a few (7%) oocytes reached M II stage when cultured at 35 °C for 44 h (Table 1). However, about one-half (58%) of the oocyte population completed nuclear maturation to M II stage when culture duration was extended to 68 h (Table 2). Eng *et al.* (1986) found that pig oocytes cultured at 39 °C had a higher percentage of polar body formation than did those cultured at 37 °C. It seems that at lower culture temperatures oocytes require a much longer culture period to complete the maturation process. Collectively, the above findings suggest that culture of pig oocytes at lower temperatures delays nuclear and/or cytoplasmic maturation. Reduction in temperature is also known to prolong the mitotic cycle in somatic cells (Mazia, 1961). Low temperature may interfere with the normal enzymatic and/or metabolic activity of oocytes. A recent study showed that lowering the culture medium temperature caused the disappearance of microtubules (Wang *et al.*, 2000), suggesting that microtubules are temperature-sensitive. Microtubules are necessary for the formation of the metaphase plate and polar body extrusion during oocyte maturation. It is clear that low temperatures act by disassembling microtubules and that this disassembly is the result of the depolymerisation of the major structural protein of microtubules (Petzelt, 1979). Therefore, microtubule assembly in pig oocytes cultured at 35 °C may be retarded resulting in lower M II rates.

Despite the lower rate of nuclear maturation, pig oocytes cultured at 35 °C for 68 h can be fertilised (Table 4) and are capable of development to the blastocyst stage (13%) following IVF (Table 5). However, under the conventional IVM system (39 °C for 44 h), the proportion of oocytes that completed nuclear maturation to the M II stage (81–82% vs 49%; Table 3) and development to the blastocyst stage (31–33% vs 13%; Table 5) following IVF was significantly higher. Extension of the culture period by 24 h for oocytes matured at 39 °C was not beneficial because many oocytes (28%) exhibited spontaneous activation (Table 3). It is well known that ageing of oocytes either *in vivo* or *in vitro* triggers this process. Compared with 40 h, a higher proportion of pig oocytes had metaphase II plates located outside the thick microfilament domain when cultured for 50–60 h at 39 °C (Kim *et al.*, 1996*c*). These authors suggested that oocyte ageing causes a disruption of microfilaments such that atypical development resulted after parthenogenetic activation. Although spermatozoa could penetrate some of the matured

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oocytes, about half (57%) of them were polyspermic, indicating that aged oocytes may be defective in their ability to establish a block of polyspermy. However, penetration of spontaneously activated oocytes was not observed. This observation suggests that the zona pellucida of activated oocytes has undergone zona hardening. The above factors may be the underlying reasons why only a few (3%) embryos reached the blastocyst stage from oocytes matured at 39 °C for 68 h.

In rabbits, there was a significant difference between follicles (small or large) and the ovarian stroma (Grinsted *et al.*, 1980). However, no temperature difference was found between small and large follicles. On the other hand, the temperature of small (< 5–6 mm) follicles was similar to that of ovarian stroma but was cooler in large (7–10 mm) follicles in the pig (Hunter *et al.*, 1997). It seems that a gradual reduction in follicular temperature is taking place during follicular development in the pig. However, culture of pig oocytes at lower temperature for a prolonged period was not beneficial. It would be interesting to examine whether a temperature gradient, as observed during follicle development *in vivo*, during IVM is beneficial for pig oocytes. Such culture conditions may be more suitable for the completion of nuclear and cytoplasmic maturation.

As shown in Table 5, although the proportion is low, embryo development to the blastocyst stage is possible from oocytes matured at 35 °C for 68 h. We did not observe morphological differences between blastocysts derived from oocytes cultured at 35 and 39 °C. However, our previous studies indicated that embryos (morula stage) generated from oocytes cultured at 39 °C are viable since they resulted in pregnancies and live birth following embryo transfer (Abeydeera, 2000). Since similar viability tests were not carried out in the present study, it is difficult to conclude that embryos derived from oocytes matured at 35 °C are viable.

In conclusion, the results of this study indicate that pig oocytes can complete maturation when cultured at 35 °C with some degree of developmental competence to the blastocyst stage. Under certain circumstances oocyte maturation at a lower temperature could be useful to coincide with a timed IVF program.

Acknowledgements

The authors thank T. Cantley and K. Green for the transport of ovaries. This manuscript is based on work supported as part of the National Cooperative Program on Non-Human In Vitro Fertilization and Preimplantation Development and was funded by the National Institute of Child Health and Human Development through Cooperative Agreement HD 34588, and is a contribution from the Missouri Agricultural Experimental station: Journal Series Number 13,023.

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