Physiological temperature variants and culture media modify meiotic progression and developmental potential of pig oocytes *in vitro*

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Abstract

Ovarian follicles *in vivo* are cooler than surrounding abdominal and ovarian tissues. This study investigated whether typical follicular temperatures influence the maturation and developmental potential of pig oocytes *in vitro*. Oocytes were synchronised at the germinal vesicle (GV) stage and incubated at 39, 37 or 35.5 °C. When compared with 39 °C, which is often used for *in vitro* studies, lower temperatures delayed spontaneous progression to the metaphase I and II (MI and MII) stages of meiosis. The MII was delayed by about 12 h per °C. All oocytes had normal morphology. Oocytes reaching GV breakdown (GVBD) at 39 °C were subsequently unaffected by cooling, demonstrating thermal sensitivity during the pre-GVBD stage only. Simultaneous assay of maturation-controlling kinases (maturation promoting factor (MPF) and MAPK) showed that cooling delayed kinase activation, provided it was applied prior to GVBD. Activity profiles remained coupled to the stage of meiosis. Neither enzyme was directly thermally sensitive over this temperature range. Following *in vitro* fertilisation, fewer blastocysts developed from embryos derived from 35.5 or 37 °C oocytes as compared with those from 39 °C oocytes. Manipulation of fertilisation timings to allow for delayed maturation showed that over-maturing or aging at lower temperatures compromises subsequent embryo development, despite normal nuclear maturation; the GV stage was again the thermally sensitive period. Cleavage rates were improved by the culture of oocytes with follicle-stimulating hormone (FSH) at 37 but not at 35.5 °C. Inclusion of 20% follicular fluid in the oocyte medium restored the blastocyst rate to that seen at higher temperatures. Thus, FSH and follicular fluid may allow oocytes to achieve normal developmental potential at *in vivo* temperatures.

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Introduction

The testes of most mammal species descend via the inguinal canals to become suspended in a scrotal sac. This creates a temperature gradient of 3–4 °C between the abdomen and testes which is considered to be essential for spermatogenesis. In females, the ovaries are embedded in the abdomen and are thus generally assumed to function optimally at deep body temperatures. However, this might not be the case as it is reported that pre-ovulatory follicles may be 1.4 °C cooler than ovarian stroma in rabbits (Grinstead *et al.* 1980), 2.3 °C in women (Grinstead *et al.* 1985) and 1.5 °C in cattle (Greve *et al.* 1996). Hunter and colleagues carried out a series of large-scale, elegant examinations of temperatures in tissues surrounding pig ovaries using various direct observational techniques. They found that large Graafian follicles

(7–10 mm diameter) were cooler (by 0.5 °C) than smaller ones (5–7 mm diameter) and cooler (by 0.6–1.7 °C) than the ovarian stroma (37.3 °C), and that both large Graafian follicles and ovarian stroma were cooler than deep body temperature (39 °C; Hunter *et al.* 1997, 2000, 2006). Biophysical analysis of follicular fluids suggests that growing follicles of a variety of species respond endothermically to fluid uptake, and mathematical modelling indicates that this would be sufficient to account for their progressive cooling (Luck *et al.* 2001). Counter-current heat transfer between the follicular vessels and/or utero-ovarian vessels may be a means of helping to maintain such hypothermic microenvironments (Einer-Jensen & Hunter 2005). Thus, it appears that temperature gradients exist between deep body tissues, ovarian stroma and follicles in many species including the pig, although the true extent of the

heterothermy remains to be confirmed (Hunter & Einer-Jensen 2005, Hunter *et al.* 2006).

The existence of follicular cooling raises the question of whether oocytes, like sperm, develop advantageously at lower temperatures. Reduced temperature may be required for successful oogenesis or oocyte maturation, or for subsequent embryo or foetal development (Hunter *et al.* 1997, 2000, 2006).

It is currently not possible to investigate directly the effect of temperature gradients within the ovary because there is no practical way of manipulating the local thermal environment *in vivo*. It is also difficult to mimic *in vivo* conditions closely *in vitro* because of the spontaneous resumption of maturation in oocytes when released from Graafian follicles. Pig oocytes showed better maturation rates when cultured at 39 °C than at 37 °C, suggesting that closer attention should be paid to the normal body temperature of the species (Eng *et al.* 1986). As a result of these studies, supposed deep body temperatures (38.5–39 °C) have been widely adopted for *in vitro* work with oocytes and early embryos. Nonetheless, despite numerous attempts and with extensive manipulation of culture media formulations, rates of blastocyst formation by *in vitro*-matured and fertilized oocytes rarely exceed 30% (Abeydeera *et al.* 1998, Wu *et al.* 2002, Shimada *et al.* 2003). This is particularly so in the pig where *in vivo*-matured oocytes appear to have much greater developmental potential. The reason for the comparatively poor developmental potential of *in vitro*-matured oocytes is unknown.

Meiotic maturation of oocytes is a highly regulated process that is ensured by intrinsic mechanisms (Moor *et al.* 1990). MAPK and maturation promoting factor (MPF) are serine–threonine kinase enzymes which play an important role in the regulation of meiosis as in other cell cycles. MAPK has ubiquitously expressed 44 and 42 kDa isoforms, known as ERK 1 and 2, which are activated in response to growth factors and tumour promoters in cultured cells. MAPK is activated by mitogens in quiescent cells and has a vital role in signal transduction at the G0–G1 transition. Once activated, it phosphorylates and activates a ribosomal subunit, S6 kinase II, and phosphorylates microtubule-associated protein 2 and myelin basic protein (MBP; Sobajima *et al.* 1993). MAPK exists in the germinal vesicle (GV) stage porcine oocytes; it is activated in MI oocytes and maintained until MII, showing that the MAPK cascade is crucial to porcine oocyte maturation (Inoue *et al.* 1998). MPF is a heterodimer that comprises of p34^{cdc2} (catalytic) and cyclin B (regulatory) subunits and is a universal eukaryotic G2–M-phase regulator for both meiosis and mitosis (Nurse 1990). It controls important nuclear events such as cytoskeletal organization and membrane integrity. Its activity is controlled by the association of cdc2 with cyclin B, which initially forms as pre-MPF, and the phosphorylation and

dephosphorylation of the cdc2 subunit at tyrosine 15 and threonine 14 residues (Wu *et al.* 1997).

In the present study, we have examined *in vitro* the effect on oocyte maturation of culture temperatures within the observed range for ovarian follicles and tissues (35.5, 37 and 39 °C). We have done this by examining the progression of morphological nuclear maturation rather than just its final, accumulated frequency and by examining the corresponding activities of MAPK and MPF. This approach follows our earlier analysis of the links between kinase activity and nuclear maturation in pig oocytes (Ye *et al.* 2003) and conclusion that the speed of nuclear progression may influence subsequent developmental potential (Miyoshi *et al.* 2002, Ye *et al.* 2005). Since pig oocytes are meiotically heterogeneous *in vivo* prior to selection for ovulation (Guthrie & Garrett 2000) and prove to be asynchronous in conventional maturation culture systems (Gruppen *et al.* 1997), we have used a reliable synchronisation system in which oocytes are reversibly blocked at the GV stage by pre-treatment with cycloheximide (CHX; Ye *et al.* 2002). Such oocytes retain and even improve their developmental competence (Ye *et al.* 2005). We have then evaluated the postfertilisation developmental potential of oocytes matured at the three temperatures. We have also considered whether there is an interaction between temperature and components of the culture medium including follicle-stimulating hormone (FSH), which has a distinct beneficial effect on oocyte developmental potential (Ye *et al.* 2005), and follicular fluid from maturing follicles.

Materials and Methods

Culture media

All chemicals and reagents were from Sigma–Aldrich unless otherwise stated. The *in vitro* maturation (IVM) media were basic defined medium (DM; M199 containing Earle's salts, 25 mmol HEPES and sodium bicarbonate/l, 3 mmol L-glutamine/l, 0.1% (w/v) BSA, 0.57 mmol cysteine/l, 10 ng epidermal growth factor/ml (human recombinant), 0.2 µg porcine luteinizing hormone/ml (NIDDK, Bethesda, MD, USA), 100 IU penicillin/ml and 0.1 mg streptomycin/ml), DM supplemented with 50 ng porcine FSH/ml (NIDDK; designated FDM) and FDM supplemented with 20% (v/v) porcine follicular fluid (designated FFDm). The follicular fluid was the pooled aspirate of healthy follicles, 6–8 mm diameter, taken to represent the antral environment of growing follicles at the stage immediately following that of the follicles from which oocytes were derived for culture (discussed below). The pooled fluid was centrifuged at 2500 g for 20 min to remove cell debris, filtered through 0.22 µm membrane filters and stored at –20 °C for the duration of the experimental sequence. The *in vitro* fertilisation (IVF) medium was a modified

Tris-buffered medium (pH 9.9 at 4 °C, balanced to pH 7.2 at 39 °C in 5% CO₂ for 12–24 h, containing 113 mmol NaCl/l, 3 mmol KCl/l, 7.5 mmol CaCl₂·2H₂O/l, 20 mmol Tris/l as crystallised free base, 11 mmol glucose/l and 5 mmol sodium pyruvate/l), supplemented with 0.1% (w/v) BSA, 20 µmol freshly prepared adenosine/l and 0.2 mmol freshly prepared reduced glutathione/l, but free of antibiotics. The basic embryo *in vitro* culture (IVC) medium was NCSU23 (pH 8.3 at 4 °C, balanced to pH 7.4 at 39 °C in 5% CO₂ for 12–24 h, containing 108.7 mmol NaCl/l, 4.8 mmol KCl/l, 1.7 mmol CaCl₂·2H₂O/l, 1.2 mmol KH₂PO₄/l, 1.2 mmol MgSO₄·7H₂O/l, 25.1 mmol NaHCO₃/l, 5.6 mmol glucose/l, 1.0 mmol glutamine/l, 7.0 mmol freshly prepared taurine/l, 5.0 mmol freshly prepared hypotaurine/l, 0.4% w/v BSA), supplemented with 0.2 mmol reduced glutathione/l (freshly prepared), 50 IU penicillin/ml and 50 µg streptomycin/ml. During the first 48 h of culture, glucose in the basic IVC medium was replaced with 4.5 mmol sodium lactate (DL-lactic acid) /l and 0.3 mmol sodium pyruvate/l.

IVM of oocytes and assessment of meiotic stage

Pre-pubertal pig ovaries were collected from a local commercial abattoir and transported to the laboratory in a warm flask in PBS (~30 °C) within 3 h of slaughter. Selected ovaries were washed thrice in sterile PBS. Follicles of 3–5 mm diameter with a translucent appearance and extensive vascularisation were aspirated using a 21-gauge needle attached to a 10 ml syringe primed with 0.5 ml Dulbecco's PBS (DPBS, Ca²⁺-free). The fluid was expelled into 5 cm diameter, sterile Petri dishes and held at 37 °C whilst being inspected for oocytes. Cumulus oocyte complexes (COCs) with more than three intact and compact cumulus layers were selected for culture, after washing in DPBS supplemented with 0.025% polyvinyl alcohol (PVA) and in maturation medium. Groups of 25–40 COCs were cultured in single 35 mm diameter dishes (Nunc, Roskilde, Denmark) at a ratio of one oocyte per 10 µl medium. To synchronise meiotic maturation, COCs were pre-incubated in maturation medium supplemented with 5 µg CHX/ml for 16 h, essentially as described by Ye *et al.* (2002). The COCs were then thoroughly washed (thrice in DPBS with 0.025% PVA and thrice in maturation medium without CHX) and further cultured without CHX for various periods as described below for each experiment. All culture drops containing oocytes were covered with a thin layer of mineral oil pre-equilibrated with medium M199 and incubated in 5% CO₂ humidified air at selected temperatures (39, 37 or 35.5 °C). Incubator temperatures were carefully adjusted at the start of each experiment and monitored continuously at the sample location by means of thermistors immersed in adjacent wells of medium. Incubation temperatures remained constant to ±0.2 °C

of their expected values except for short periods when doors were opened for sample servicing.

To assess meiotic progression, single dishes of COCs were withdrawn from the incubator and the cumulus cells removed by vortexing for 2–3 min in medium containing 0.1% (w/v) hyaluronidase (type IV), 2 mmol EDTA/l, 125 mmol NaCl/l, 3 mmol sodium citrate/l and 10 mmol Na₂HPO₄/l. Oocytes were mounted under cover slips on slides and fixed for 48 h with acetic acid:ethanol (1:3 v/v). After staining with 1% lacmoid in 45% acetic acid and destaining with acetoglycerol (20% acetic acid, 20% glycerol), the oocytes were examined under a phase contrast microscope at 400× magnification. Meiotic stages were classified as GV, GV breakdown (GVBD: diakinesis and prometaphase I), metaphase I (MI), anaphase I/telophase I (AI/II) and metaphase II (MII) as previously described (Ye *et al.* 2002).

In vitro double kinase assay

To assess the activities of MAPK and MPF, denuded oocytes were washed twice using DPBS containing 0.1% PVA and batches of 10 oocytes were transferred, in minimal washing medium using a fine pipette, into 5 µl ice-cold lysis buffer containing 45 mmol β-glycerophosphate/l (pH 7.3), 12 mmol *p*-nitrophenylphosphate/l, 20 mmol 3-(*N*-morpholino)-propanesulfonic acid (MOPS) /l, 12 mmol MgCl₂/l, 12 mmol EGTA/l, 1.4 mmol EDTA/l, 20 mmol Na₃VO₄/l, 10 mmol NaF/l, 2 mmol dithiothreitol (DTT) /l, 2 mmol phenylmethylsulphonyl fluoride/l, 2 mmol benzamidine/l, 20 µg leupeptin/ml, 20 µg pepstatin A/ml and 20 µg aprotinin/ml. Oocytes were lysed by freeze-thawing in liquid nitrogen. The lysate was then centrifuged at 13 000 *g* for 5 s and stored at –70 °C.

The activities of MAPK and MPF were measured using MBP and histone H1 as their substrates respectively, as previously described (Ye *et al.* 2003). The oocyte lysate was thawed on ice, briefly vortexed and added to 5 µl of kinase assay buffer containing 45 mmol glycerophosphate/l (pH 7.3), 12 mmol *p*-nitrophenylphosphate/l, 20 mmol MOPS/l, 12 mmol MgCl₂/l, 12 mmol EGTA/l, 0.1 mmol EDTA/l, 2 mmol Na₃VO₄/l, 10 mmol NaF/l, 2 mg histone H1/ml 3 mg MBP/ml, 4 µmol protein kinase A/l inhibiting peptide (Santa Cruz Biotechnology; Autogen Bioclear, Calne, UK), 4 µmol protein kinase C/l inhibiting peptide (Promega) and 0.5 µCi (34 µmol/l) [γ-32P]ATP (Amersham Pharmacia Biotech). The reaction mixture was incubated for 30 min at 37 °C with gentle shaking. The reaction was stopped with the addition of 10 µl ice cold 2× SDS sample buffer (125 mmol Tris-HCl/l (pH 6.8), 200 mmol DTT/l, 4% (w/v) SDS, 0.1% (w/v) bromophenol blue and 20% (w/v) glycerol). After boiling for 4–5 min, the substrates were separated by PAGE (SDS-PAGE, 15% gels) using Mini-Protean II dual slab gel (Bio-rad) under 140 V for 1.5 h. The gels were dried on 3 mm filters and exposed to phosphor screens (Kodak). Captured phosphor images were

quantified using an FX phosphor image analysis system (Bio-rad). Freshly isolated GV oocytes were analysed with oocyte samples throughout the assays as a negative control.

IVF and embryo culture

Fresh extended pig semen (PIC Kingston Bagpuize, Oxfordshire, UK; stored up to 5 day at 21 °C) was washed twice by centrifugation (5 min, 500 g) in DPBS (Ca²⁺ and Mg²⁺-free) supplemented with 0.1% BSA, 100 IU penicillin/ml and 100 µg streptomycin/ml. The sperm pellet was resuspended in IVF medium and sperm concentration determined by haemocytometer after dilution in 18% (w/v) NaCl saline. The sperm suspension (1 × 10⁶ sperm cells/ml) was pre-incubated for a short period (10 min) at 39 °C before co-incubation with oocytes. At the end of maturation, culture dishes of oocytes were denuded of cumulus cells by brief vortexing (1–2 min) in warm maturation medium (balanced at 39 °C in 5% CO₂), washed in the same medium and transferred to IVF medium droplets which were covered with mineral oil and pre-incubated for 1–2 h. Before insemination, oocytes were set in IVF medium droplets (5–10 oocytes in 35 µl IVF medium; 4–7 drops per 5 cm diameter dish) in the incubator for 20–30 min. An equal volume of sperm suspension (35 µl) was introduced into each droplet, producing a final sperm concentration of 5 × 10⁵ cells/ml. Oocyte–sperm co-incubation was carried out for 9 h at 39 °C under 5% CO₂ in humidified air. After a brief wash in IVC medium, oocytes/putative zygotes were cultured (30–50 in 600 µl IVC medium) for 2 days in 4-well plates (Nunc). Cleaved embryos were then transferred into fresh IVC medium and cultured for a further 4 days. Putative zygotes and embryos were cultured at 39 °C in humidified 5% CO₂, 5% O₂ and 90% N₂.

Experiment 1

To determine the effect of culture temperature on oocyte meiotic progression, freshly aspirated GV stage COCs were cultured under two temperature protocols: a) COCs were synchronised with CHX for 16 h at 39, 37 and 35.5 °C, and then cultured without CHX at the same temperatures up to a further 72 h ('constant temperature' protocol); oocytes were sampled for assessment at 24, 36, 48, 60 or 72 h after removal from CHX and b) COCs were synchronised with CHX for 16 h at 39 °C, cultured without CHX for 8 h at 39 °C, and then cultured at 39, 37 or 35.5 °C ('temperature shift' protocol); oocytes were sampled for assessment at 16 h after the temperature shift. The basic maturation medium (DM) was used in this experiment and in experiments 2 and 3 because meiotic progression is highly synchronised in oocytes cultured in this medium at 39 °C (Ye *et al.* 2005). Five

oocyte batches were studied per treatment and dishes of 25–40 oocytes were assessed at each time point, with a total of 3997 oocytes examined for the constant temperature protocol and a total of 503 oocytes examined for the temperature shift protocol.

Experiment 2

To determine the effect of culture temperature on kinase activity, freshly aspirated GV stage COCs were cultured in DM under the constant temperature and temperature shift protocols as used in Experiment 1. During each protocol, 10 oocytes were removed at 2, 4, 6, 8, 12, 16, 20 and 24 h (after removal from CHX or the temperature shift respectively) and stored at –70 °C to be used in the double kinase assay to determine MAPK and MPF activities. Four oocyte batches were studied per treatment, with a total of 960 oocytes examined under each protocol.

Experiment 3

To determine the possible temperature sensitivity of the double kinase assay, COCs were synchronised with CHX for 16 h, cultured without CHX for 0 (GV), 12 (MI) and 24 h (MII), all at 39 °C, and stored in lysis buffer at –70 °C to be used in the double kinase assay. The incubation temperature during the kinase assay was set at 35.5, 37 or 39 °C and the activities of MAPK and MPF were compared between the assay temperatures. Four batches of oocytes were studied per treatment, with a total of 360 oocytes examined.

Experiment 4

To determine the possible interaction between temperature and medium on oocyte developmental potential after fertilisation, the following combinations of variables were used: (a) COCs were cultured as in Experiment 1 in DM either with the constant temperature protocol, for which oocytes were fertilised *in vitro* after maturation culture for 24–36, 24–48 and 36–60 h at 39, 37 and 35.5 °C respectively, or with the temperature shift protocol, for which oocytes were fertilised *in vitro* after maturation culture for a total of 24 h; (b) COCs were cultured in FDM for 36, 36 and 36–60 h at 39, 37 and 35.5 °C respectively, after pre-incubation with CHX for 16 h at the same temperatures, and oocytes were then fertilised *in vitro* and c) COCs were cultured in FFDM for 36–48 h at 35.5 °C after pre-incubation with CHX for 16 h at the same temperature, and oocytes were then fertilised *in vitro*. Culture timings were coordinated so that sets of oocytes cultured at different temperatures and media were harvested simultaneously for IVF with the same sperm preparations. The proportions of cleaved oocytes (zygotes) present at 2 days after IVF were recorded. The rates of blastocyst formation, recorded

after culture of cleaved embryos for a further 4 days, were calculated as the proportions of blastocysts relative to the total number of cultured oocytes. Three replicates were performed for each treatment, with a total of 3506 oocytes cultured and fertilised.

Statistical analysis

The proportions of meiotically responding (maturing or matured) oocytes and developing embryos out of the known total numbers of oocytes cultured in single dishes/droplets were analysed by fitting a Generalised Linear Model assuming binomial errors. The significances of the factors of temperatures (39, 37 and 35.5 °C) and media (supplements of FSH and follicular fluid) and their interactions were tested using an analysis of deviance table (GenStat 6.1, VSN International, Hemel Hempstead, Herts, UK). Analysed data are presented as mean proportions and approximate standard errors predicted from the fitted model. Enzyme activity curves are shown with an estimated standard error of the difference between means (S.E.D). Differences in kinase activities at different time points at each temperature were tested using ANOVA. A probability of $P < 0.05$ was considered statistically significant.

Results

Experiment 1

In the pig, MI is a relatively long component of the meiotic transition from GV to MII, compared with other phases (the diakinesis of prophase I, prometaphase I, AI and TI; Ye *et al.* 2002). Therefore, GV, MI and MII were used as representative stages to indicate meiotic progression (Ye *et al.* 2005). As shown in Fig. 1a, GVBD first appeared at 4, 4 and 6 h after pre-treatment with CHX, when oocytes were cultured at constant temperatures of 39, 37 and 35.5 °C respectively. Accordingly, the proportions of oocytes remaining at GV showed the first significant decline ($P < 0.05$) at 4 ($75.2 \pm 3.4\%$), 6 ($73.8 \pm 3.6\%$) and 8 h ($79.0 \pm 3.5\%$) respectively. The proportions of GV oocytes continued to decline but they took substantially different amounts of time (24, 36 and 60 h respectively) to reach basal levels ($< 10\%$). At 39 °C, the proportion of oocytes progressing to MI had significantly increased at 8 h (22.4%) and reached its peak level at 12 h (82.6%); at 37 and 35.5 °C, oocytes did not progress to MI until at least 12 h, and $< 20\%$ had reached MI by the end of the culture (Fig. 1b). After 24 h of culture, almost all ($> 80\%$) oocytes had matured to MII, but only 51.7% and 22.1% at 37 and 35.5 °C had done so (Fig. 1c). With oocytes continuously catching up to mature at 37 and 35.5 °C, however, similar proportions of oocytes ($77.6 \pm 2.6\%$ and $82.0 \pm 2.2\%$, $P < 0.05$) matured eventually to MII after 36–48 and 60 h of culture, when compared with

Figure 1 The proportion of oocytes (a) remaining at GV, or at (b) MI or (c) MII, after varying periods of culture at 39 (squares), 37 (circles) and 35.5 °C (triangles). COCs were synchronised with CHX for 16 h and then cultured without CHX at the same temperatures in DM. In (a) the slopes of the trend lines under each temperature were not significantly different (no interaction between temperature and time), but the first significant decrease was delayed with each step of cooling. At MI (b), there was no significant change in the proportions after 12 h at 37 and 35.5 °C. A total of 3997 oocytes, with an average of 167 at each time-temperature point, were examined.

those at 39 °C after 24 h of culture ($82.1 \pm 2.3\%$). All mature oocytes showed normal morphology.

The additional times taken at lower temperatures for the proportions of GV oocytes to reach basal levels were similar to those taken for the proportions of MII to reach the maximal levels, when compared with the culture at

39 °C; this implied that the transition from GV to GVBD might be the stage of nuclear maturation most affected by temperature. Culture for 8 h at 39 °C resulted in the majority of oocytes undergoing GVBD (80%) with 22% progressing to MI. As shown in Fig. 2, no matter whether the culture temperature was set constantly at 39 or shifted to 37 and 35.5 °C after the initial 8 h at 39 °C, similar maximal proportions of oocytes matured to MII by 24 h of culture ($82.1 \pm 2.6\%$, $86.0 \pm 2.0\%$ and $82.7 \pm 2.3\%$ respectively; $P < 0.05$).

Experiment 2

Experiment 2 determined the activities of MAPK and MPF in oocytes matured at 39, 37 or 35.5 °C, either constantly for 24 h or after a temperature shift, i.e. after 8 h at 39 °C. Conditions were as in Experiment 1, so as to obtain reliably predictable stages of nuclear maturation, but with shorter intervals of sampling. As shown in Fig. 3a, when matured at 39 °C, MPF activity peaked at ~12 h MI, followed by a decrease between 16 and 20 h and a rise at 24 h MII. The activity of MAPK (Fig. 3b) also peaked after 12 h at 39 °C and remained at this level for the remainder of the 24 h culture period. These profiles are consistent with those observed previously (Ye *et al.* 2003). At 37 °C the pattern was similar to that at 39 °C, with a peak in both activities after 12 h, although MPF activity levels began to increase slightly later (6 h rather than 4 h). At 35.5 °C the increase in MPF activity was delayed until 12 h, peaking at 16 h, and decreasing at 20 h. MPF activities did not increase again during the culture period. The onset of MAPK activity was also delayed, beginning at 12 h and peaking at 16 h.

Figure 4 shows the activities of MPF and MAPK in oocytes matured under the temperature-shift protocol.

Figure 2 The proportions of oocytes at GV (hatched area), MI (open area) and MII (closed area) after culture under the temperature shift protocol (see text). COCs were synchronised with CHX for 16 h at 39 °C, cultured without CHX for 8 h at 39 °C, and then cultured at 39, 37 and 35.5 °C for a further 16 h in DM. There was no significant difference in the proportion of oocytes at MII between the temperature regimes. A total of 503 oocytes, with an average of 168 in each regime, were examined.

Figure 3 Effects of maturation temperature on activities of (a) MPF and (b) MAPK in porcine oocytes. COCs were cultured at 39, 37 or 35.5 °C for various times up to 24 h, following pre-treatment with CHX for 16 h. The activities of the enzymes were assessed using an *in vitro* double kinase assay with histone H1 and MBP as the substrate. Vertical bars show ± 1 S.E.D. (ANOVA).

When matured at 39 °C for 24 h, MPF activity began low, peaked at ~12 h (MI), decreased between 16 and 20 h and rose at 24 h (MII). The activity of MAPK also peaked after 12 h at 39 °C and remained at this level for the remainder of the 24 h culture period. When maturation temperature was lowered from 39 °C to 37 or 35.5 °C, MPF activity began low, peaked at 12–16 h (MI) and again at 24 h (MII), as it did at 39 °C. MAPK activity also began low, peaked at 12–16 h (MI) and remained high for the remaining culture period, as at 39 °C. With both enzymes, differences between the proportions of oocytes showing peaks of activity at each stage under the different regimes were not significant. In summary, when the maturation temperature was decreased after 8 h at 39 °C the activities of MAPK and MPF were similar to those in oocytes matured at 39 °C for the full 24 h period.

Experiment 3

This experiment investigated the effect of temperature on the assay of MAPK and MPF activities. The incubation temperature of the double *in vitro* kinase assay had no significant effect on the activities of MAPK or MPF at any of the three stages of nuclear maturation (Table 1).

Figure 4 Activities of (a) MPF and (b) MAPK in oocytes at GV, MI and MII after culture under the temperature shift protocol (see text). COCs were cultured at 39 °C for the first 8 h of culture followed by a decrease in temperature to either 37 °C or 35.5 °C for the remaining 24 h of culture. The activities of MPF and MAPK were assessed using an *in vitro* double kinase assay with histone H1 and MBP as the substrates. Vertical bars show ± 1 S.E.D. (ANOVA).

Experiment 4

Given that oocytes cultured at three different temperatures eventually achieve similar maximal accumulated maturation rates with normal morphology (Experiment 1), this experiment compared rates of embryo development

Table 1 Effect of assay incubation temperature on the activity of maturation promoting factor (MPF) and MAPK in porcine oocytes at three stages of nuclear maturation. See text for details of sample preparation and assay procedure. S.E.M.s are 0.148 and 0.133 for MPF and MAPK respectively at all maturation stages.

Enzyme	Incubation temperature (°C)	Enzyme activity (log ₁₀ cpm/mm ²)		
		GV	MI	III
MPF	35.5	4.643	4.950	4.803
	37	4.784	4.986	4.999
	39	4.773	4.654	4.923
	Effect of temperature	NS	NS	NS
MAPK	35.5	5.003	5.218	5.066
	37	4.980	5.254	5.328
	39	5.080	5.104	5.380
	Effect of temperature	NS	NS	NS

NS, not significant at $P < 0.05$.

following IVF of oocytes cultured at 39, 37 and 35.5 °C for 24, 36–48 and 60 h respectively. As shown in Fig. 5a, there was no difference in oocyte cleavage rates between temperature treatments (54–66%). However, few cleaved embryos developed to the blastocyst stage from oocytes cultured at 35.5 (2.4%) and 37 °C (2.7–3.8%), compared with the high proportion forming from those cultured at 39 °C (19.8%; $P < 0.001$). When oocytes cultured at 39 °C were fertilised 12 h later than normal, the blastocyst formation rate significantly declined (3.7%; $P < 0.001$), whereas when those cultured at 37 °C were fertilised 12 h earlier than normal, the blastocyst formation rate significantly increased (12.6%; $P < 0.05$). This indicated that over-maturing or aging of oocytes might compromise subsequent embryo development, despite morphologically normal nuclear maturation. Nevertheless, the cleavage rate significantly declined (22.2%, $P < 0.05$), without any improvement in blastocyst development, when oocytes cultured at 35.5 °C were fertilised at 36 h, when compared with the development rates at 60 h. However, with the temperature shift protocol, involving initial culture for 8 h at 39 °C, oocytes cultured at 35.5 and 37 °C provided similar rates of cleavage (61.1 and 65.7%) and blastocyst formation (23.7 and 18.3%) at 16 h (a total of 24 h) to those from oocytes cultured at 39 °C for 24 h.

We previously found that FSH supplementation of pig oocyte culture medium delays nuclear maturation by 12 h (Ye *et al.* 2005). Since oocytes could be fertilised and achieve optimal embryo development rates when cultured at 39 and 37 °C in the basic DM for 24 h, oocytes were also cultured in FDM for either 36 h at 39 or 37 °C or for 36, 48 or 60 h at 35.5 °C, and then fertilised. As shown in Fig. 5b, culture in FDM significantly increased both cleavage and blastocyst rates of oocytes cultured at 39 °C, when compared with those cultured in DM (74.6% vs 55.0% and 28.3% vs 19.8%; $P < 0.05$). There was a trend for improvement in both cleavage and blastocyst formation rates in FDM (compared with DM) when oocytes were cultured at 37 °C, but no difference for those cultured at 35.5 °C. Culture in FFDM (containing 20% follicular fluid) significantly increased both cleavage and blastocyst rates when oocytes were cultured for 36 h at 35.5 °C (50.7% and 13.9%; $P < 0.05$) as compared with culture in FDM (21.9% and 5.5%) and DM (22.2% and 2.6%; Fig. 5a).

Discussion

Temperature is a fundamental variable in the cellular environment. Regarding germ cells, spermatogenesis is well known to be sensitive to ambient temperatures but oocyte quality is also known to be susceptible to temperature stress. An increase in body temperature can lead to reduced female fertility in many tropical and

Figure 5 The cleavage (open bars) and blastocyst formation (black bars) rates of oocytes cultured in (a) DM and (b) FDM or FFDM and fertilised *in vitro*. For temperature treatments, (a) COCs were cultured under either the constant temperature protocol (39, 37 and 35.5 °C) for varied periods (24–60 h) or the temperature shift protocol (39–39, 39–35.5 and 39–37 °C) for a total of 24 h only, as described in the text. Alternatively, (b) COCs were cultured under the constant temperature protocol in FFDM for 36 h at 35.5 °C (first pair of bars marked with FFDM) or in FDM (all others) for 36–60 h at 35.5 °C, and 36 h at 37 or 39 °C (see text for comparisons). A total of 3506 oocytes, with an average of 234 in each time–medium treatment, were examined.

subtropical species (Zeron *et al.* 2001). Consistent with this, elevated culture temperatures (>41 °C) are disastrous for oocyte maturation and subsequent embryo development *in vitro* (Edwards *et al.* 2005). Oocytes, especially porcine, are sensitive to transient temperature shock brought about by heating *in vivo* during slaughter (Tong *et al.* 2004) or by cooling *in vitro* of slaughterhouse-derived tissue (Yuge *et al.* 2003, Wongsrikeao *et al.* 2005). These stresses result in poor maturation and subsequent embryo development *in vitro*.

Accumulating evidence suggests that physiological temperature gradients exist within ovaries and their surroundings *in vivo* (for review, see Hunter & Einer-Jensen 2005), but this has not attracted adequate attention. Very few studies have evaluated the significance of physiological temperature variants in female fertility or their possible application in *in vitro* procedures. Previous studies have suggested that the rate of bovine oocyte maturation *in vitro* and their subsequent development were either independent of

culture temperatures over the physiological range (35–39 °C; Katska & Smorag 1985, Shi *et al.* 1998) or that the rate of fertilisation was improved at 39 °C compared with 37 °C (Lenz *et al.* 1983). Unsynchronised porcine oocytes showed delayed nuclear maturation at the lower temperature (Abeydeera *et al.* 2001), but interactions with culture media were not evaluated.

Using synchronised oocytes and three physiologically relevant temperatures, the present study has demonstrated, firstly, that low temperature significantly slows the progress of porcine nuclear maturation by ~12 h per °C, and secondly, that the delay is solely attributable to effects on the transition from GV to GVBD. These effects were also seen in the meiosis-regulating kinases. Thus, there appears to be a thermally sensitive time window during this early stage of the resumption of meiosis. It has been observed that although the natural ovarian population of pig oocytes may be asynchronous in terms of nuclear maturation, potentially ovulable oocytes seem to achieve synchronisation at a stage

before GVBD (Guthrie & Garrett 2000). Many factors control or block the resumption of meiosis of oocytes in pre-ovulatory follicles but all of them can be overcome by the surge of gonadotrophins. Since the extent of cooling increases as follicles grow (Hunter & Einer-Jensen 2005), the gonadotrophin surge presumably takes place in the already-cooled follicles. Thus, the reduced temperature, acting prior to GVBD, may exert a restraining influence on the initiation of meiosis, thereby helping to determine the rate of meiotic resumption and synchronise eventual nuclear maturation.

The kinase activity data show that as the maturation temperature decreased, rises in both MAPK and MPF were delayed, although peaks in activity still corresponded to the appropriate stages of maturation at each temperature. The relatively low peak levels of both kinase activities in oocytes cultured at 35.5 °C may be due to the high proportions of oocytes remaining at the GV stage during most periods of culture. When the temperature was decreased after GVBD, MI was reached following 12–16 h in culture and MII was reached following 24 h in culture, which corresponds with the times at which MI and MII occur when oocytes are matured at 39 °C for 24 h. The peaks in activity of MAPK and MPF still occurred at MI and MII, at 12–16 and 24 h respectively.

Whilst the mechanism by which temperature influences oocytes and their kinases is currently unknown, it appeared possible that lower temperatures delayed the activation of MAPK and MPF and/or decreased their biochemical activities, as opposed to altering the overall cellular response. As the temperature sensitivity of the MPF and MAPK enzyme systems appears not to have been reported, Experiment 4 was designed to investigate whether the temperatures being tested here might influence kinase activity directly. The results show that they do not. We therefore conclude that the effect of temperature on kinase activity profiles is not simply a biochemical enzymic artefact but reflects a more fundamental cellular response, probably related to the control of kinase activation through phosphorylation and dephosphorylation.

Our results show that reduced temperature not only affects the progression of oocyte nuclear maturation but also influences their cytoplasmic maturation as expressed in subsequent embryo development. Although the rate of blastocyst formation from oocytes cultured in the basic DM at 37 °C for more than 36 h was as poor as that at 35.5 °C, the developmental potential of those oocytes appeared not to be affected by the temperature treatment itself but rather by an over-maturity or aging in the case of 37 °C. This was shown by the significant improvement in blastocyst formation rate when fertilisation took place earlier. In fact, the rate of blastocyst formation from oocytes cultured in DM at 37 °C for 24 h was comparable with that at 39 °C for 24 h, if the calculation was adjusted for the actual proportions of mature oocytes available for fertilisation (at 37 °C, 12.2% compared with 50%; at 39 °C, 19.6%

compared with 81%; Figs 1c and 5a). A similar situation existed with the cultures in FDM for the comparable developmental rates between 37 °C and 39 °C oocytes (data for nuclear maturation not shown; Fig. 5b; Ye *et al.* 2005). However, the 35.5 °C treatment severely compromised the developmental potential of oocytes cultured in DM and FDM, since similar poor blastocyst formation rates from these oocytes were observed for all culture periods tested including 36 and 72 h (data not shown), and were significantly lower than some of those at 37 and 39 °C (Fig. 5a).

Interestingly, the detrimental effect of 35.5 °C on developmental potential was not observed in oocytes which had progressed through GVBD at 39 °C. This indicated that oocytes cultured at low temperatures, particularly 35.5 °C, might suffer cytoplasmic defects during the prolonged transition from GV to GVBD but not during later maturation stages. Thus, it was again the transition from GV to GVBD that was thermally sensitive and crucial in achieving maximum developmental potential. The analysis of kinase activities under this protocol confirmed the close link between the kinases and the stage of nuclear maturation. The deeper mechanistic details of this have yet to be investigated, although other studies have shown that lowering culture temperature causes the disappearance of microtubules (Wang *et al.* 2000, 2001). As in our previous study (Ye *et al.* 2005), supplementation of the culture medium with FSH improved subsequent embryo development of oocytes cultured at 39 °C. However, this beneficial effect was lost in oocytes cultured at lower temperatures. Addition of follicular fluid to the medium significantly improved the subsequent development rate of oocytes cultured at 35.5 °C, without significantly altering the speed of nuclear maturation (data not shown). This suggests that follicular fluid can protect oocytes from potential damage by low temperatures and may exert this role *in vivo*. It therefore remains possible that low *in vivo* temperatures are either beneficial for oocyte development or not detrimental, within the follicular microenvironment.

In conclusion, low physiological culture temperatures affect both nuclear and cytoplasmic maturation of pig oocytes *in vitro*. The effect of cooling is exerted on meiotic resumption (GV to GVBD) rather than on the later stages of maturation. The activation of MPF and MAPK is influenced by temperature, but enzyme activity remains closely coupled to the stage of nuclear maturation. Culture at 35.5 °C in defined media compromises subsequent oocyte developmental potential, but this can be prevented by supplementation of media with follicular fluid. Results suggest that local cooling within the ovary slows the rate of meiotic resumption and maturation of oocytes in the hours preceding ovulation and that follicular fluid may support the achievement of oocyte developmental competence *in vivo*.

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