REPRODUCTION
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Mitochondria directly influence fertilisation outcome in the pig

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Abstract

The mitochondrion is explicitly involved in cytoplasmic regulation and is the cell's major generator of ATP. Our aim was to determine whether mitochondria alone could influence fertilisation outcome. *In vitro*, oocyte competence can be assessed through the presence of glucose-6-phosphate dehydrogenase (G6PD) as indicated by the dye, brilliant cresyl blue (BCB). Using porcine *in vitro* fertilisation (IVF), we have assessed oocyte maturation, cytoplasmic volume, fertilisation outcome, mitochondrial number as determined by mtDNA copy number, and whether mitochondria are uniformly distributed between blastomeres of each embryo. After staining with BCB, we observed a significant difference in cytoplasmic volume between BCB positive (BCB^{+}) and BCB negative (BCB^{-}) oocytes. There was also a significant difference in mtDNA copy number between fertilised and unfertilised oocytes and unequal mitochondrial segregation between blastomeres during early cleavage stages. Furthermore, we have supplemented BCB^{-} oocytes with mitochondria from maternal relatives and observed a significant difference in fertilisation outcomes following both IVF and intracytoplasmic sperm injection (ICSI) between supplemented, sham-injected and non-treated BCB^{-} oocytes. We have therefore demonstrated a relationship between oocyte maturity, cytoplasmic volume, and fertilisation outcome and mitochondrial content. These data suggest that mitochondrial number is important for fertilisation outcome and embryonic development. Furthermore, a mitochondrial pre-fertilisation threshold may ensure that, as mitochondria are diluted out during post-fertilisation cleavage, there are sufficient copies of mtDNA per blastomere to allow transmission of mtDNA to each cell of the post-implantation embryo after the initiation of mtDNA replication during the early postimplantation stages.

Reproduction (2006) **131** 233–245

Introduction

Through its various biochemical pathways, the mitochondrion generates ATP, which is necessary for cellular homeostasis and function (Moyes *et al.* 1998). The inner membrane of the mitochondrion houses the mitochondrial genome (mtDNA). In the pig, this extranuclear genome is approximately 16.7 kb in size (Ursing & Arnason 1998). mtDNA encodes 13 of the subunits of the electron transfer chain (ETC) complexes, associated with the process of oxidative phosphorylation (OXPHOS), along with 22 tRNAs and 2 rRNAs that are necessary for mRNA expression (Anderson *et al.* 1981). Expression of these mitochondrial genes is vital for cellular function, especially as the ETC is the cell's major generator of ATP (Moyes *et al.* 1998) whilst mutation or deletion can result in severe cellular impairment (Wallace 1999).

Although little is known about porcine oocyte mtDNA copy number, there is considerable intra-ovarian variability in copy number amongst other species. Mature murine oocytes contain mean mtDNA copy number values between 119 000 (Piko & Taylor 1987) and 159 000 (Steuerwald *et al.* 2000), whilst bovine oocytes possess a mean of 260 000 copies (Michaels *et al.* 1982). However, in the human, the variability ranges between 20 000 and 598 000 copies, with a mean of 193 000 (Reynier *et al.* 2001). Other reports in the human suggest mean values of 256 000 (May-Panloup *et al.* 2005), 314 000 (Steuerwald *et al.* 2000) and 795 000 (Barritt *et al.* 2002) copies. This variability in the human most likely reflects the vast range of patients analysed. For example, those oocytes failing to fertilise due to poor sperm quality and deemed to be indicative of good oocyte quality had a significantly higher mtDNA copy number than poor quality oocytes which were fertilised by good quality sperm (mean 255 000 versus 152 000 copies; Reynier *et al.* 2001). Furthermore, poor quality oocytes obtained from

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ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/rep.1.00551
Online version via www.reproduction-online.org

patients with ovarian insufficiency contained an average of only 100 000 mtDNA copies compared with an average of 256 000 copies in control oocytes (May-Panloup *et al.* 2005). In all, this high variability in mtDNA copy and the association with various forms of infertility could reflect a critical threshold of mtDNA copy number that is required for fertilisation to ensue and for subsequent embryonic development. In the mouse, this threshold level has been predicted to be approximately 100 000 copies per oocyte (Piko & Taylor 1987).

In the mouse, mtDNA replication is thought to be absent during preimplantation embryo development (Piko & Taylor 1987). Consequently, all mitochondrially derived ATP-requiring activities of the early embryo, up to compaction, will be dependent on a predetermined number of mitochondria present in the oocyte at metaphase II (MII). At compaction, there tends to be a switch in various mammalian species, including the pig (Machaty *et al.* 2001), from OXPHOS to glycolysis (discussed in Van Blerkom *et al.* 2000). Interestingly, during pig oocyte maturation, mitochondria move from a peripheral location and disperse around the oocyte (Cran 1985, Sun *et al.* 2001) whilst also becoming more heterogeneous and granulated (Torner *et al.* 2004). After fertilisation, changes in mitochondrial distribution continue as they cluster around the nuclei during cell division in the early cleavage stages (Sun *et al.* 2001). It is, however, evident from parthenogenetically activated pig oocytes that the lack of microtubule networks restricts these mitochondrial dynamics (Brevini *et al.* 2005).

The importance of ATP levels during *in vitro* maturation has been demonstrated in bovine oocytes. Higher quality oocytes, assessed by morphology, contained significantly higher ATP levels and produced significantly higher blastocyst rates after fertilisation (Stojkovic *et al.* 2001). In this respect, human oocytes require at least 2 pmol ATP at fertilisation to enhance development and implantation (Van Blerkom *et al.* 1995). Furthermore, a correlation between mitochondrial distribution in individual blastomeres and blastomere ATP content suggests that the ATP contained within early embryos is likely to be mitochondrially derived (Van Blerkom *et al.* 2000). To this extent, we have previously hypothesised that oocytes without sufficient mtDNA, and therefore the capacity to generate ATP, would not normally be ovulated. In the case of *in vitro* fertilisation (IVF), such oocytes result from superovulation protocols (St John 2002*a*). This could also apply to those oocytes harvested for *in vitro* embryo production.

Increases in production and storage of energy substrates are another key element of oocyte maturation and many of these production processes require the reducing power of NADPH (Marchal *et al.* 2001). NADPH is produced via the pentose phosphate pathway, of which the rate-limiting enzyme is glucose-6-phosphate dehydrogenase (G6PD). G6PD is synthesised and accumulates during the oocyte growth phase. Its activity is decreased once this phase has been completed and oocytes are then likely to have achieved developmental competence (Tian *et al.* 1998).

Brilliant cresyl blue (BCB) is a dye that can be broken down by G6PD (Tian *et al.* 1998). Oocytes undergoing growth and those that have completed their growth phase will therefore have differing levels of G6PD. Oocytes not containing sufficient G6PD to reduce the dye, stain blue and are more likely to have completed their growth phase and be developmentally competent. Those occytes not staining blue contain G6PD and reduce the dye to a colourless solution. These oocytes are likely to be still growing and not developmentally competent. Furthermore, blue, BCB positive (BCB^+) pig oocytes show higher sperm penetrability than BCB negative oocytes (BCB^-; Roca *et al.* 1998) and for goat oocytes, higher fertilisation rates have been recorded (Rodriguez-Gonzalez *et al.* 2002).

Cytoplasmic volume is also an important indicator of oocyte competence. In mice, it has been demonstrated that up to 50% of the oocyte cytoplasm can be lost with minimal effects on fertilisation and development. However, any further cytoplasmic loss, although resulting in fertilisation, prevents development to the 2-cell stage (Wakayama & Yanagimachi 1998). Similarly, removal of between 12 and 52% mouse oocyte cytoplasm resulted in some development to term, although fusion of two resulting 2-cell embryos increased the proportion of live offspring 4- to 10-fold (Zernicka-Goetz 1998). As the cleavage of early embryos does not include a growth phase, it would be likely that human oocytes with very low cytoplasmic volumes would simply not have enough cytoplasmic content to produce the number of divisions and dilutions required to sustain development (Van Blerkom *et al.* 1998, St John 2002*b*).

Cytoplasmic transfer (CT), the supplementation of an oocyte with donor cytoplasm, has been proposed as a mechanism for rescuing those oocytes from women with repeated developmental failure (Cohen *et al.* 1997, 1998). This is particularly the case for older women with poor quality oocytes (Barritt *et al.* 2001*a*), who following CT then progress to achieve on-going pregnancies and offspring (Barritt *et al.* 2001*a*). However, the contents of the transferred cytoplasm are likely to be mixed possibly containing mRNAs, mitochondria and mtDNA. Consequently, it is not clear whether this developmental arrest arises from insufficient cytoplasmic factors or insufficient numbers of mitochondria to support metabolic function.

Studies in the mouse and the human have indicated an important role for mtDNA copy number in fertilisation, whilst cytoplasmic supplementation has demonstrated an influence on embryonic development. However, there is little data related to mtDNA content in the pig oocyte and embryo. Furthermore, the importance of mitochondrial number to porcine fertilisation outcome and embryonic development needs to be clearly determined. Here, we have (1) used BCB to determine the relationship between mtDNA copy number and oocyte volume, growth and development, (2) analysed the distribution of

mitochondria in individual blastomeres post-fertilisation to determine whether cellular division regulates mitochondrial division, and (3) supplemented incompetent oocytes with pure populations of mitochondria from competent oocytes to determine whether supplementary mitochondria can influence fertilisation outcome. Our results demonstrate that mtDNA copy number in mature pig oocytes has a strong influence on fertilisation outcome.

Materials and Methods

All chemicals and reagents were purchased from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated. All statistical tests were performed using Minitab Statistical Software 13.1 (Minitab Inc., State College, PA, USA). All statistical comparisons performed were considered statistically significant at $P < 0.05$.

Oocyte retrieval

Ovaries were collected from a slaughterhouse in 0.9% saline (w/v) supplemented with 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate at 25–30 °C. They were rinsed three times with PBS at 39 °C and transferred to a laminar flow hood for cumulus oocyte complex (COC) aspiration from follicles with a diameter of 3–6 mm.

In vitro maturation of oocytes

Oocytes were matured in 100 μl *in vitro* maturation $(IVM)_1$. This consisted of NCSU-23 (Petters & Wells 1993; see Table 1), supplemented with 10% (v/v) porcine follicular fluid, and 20 U/ml human chorionic gonadotrophin (Profasi 5000, Sigma), 0.1 μg/ml epidermal growth factor (EGF) and 0.1 mg/ml cysteine overlaid with equilibrated mineral oil and incubated at 39 °C, 5% CO_2 in air for 20–22 h. After 22 h, the oocytes were washed three times in IVM_2 (NCSU-23 supplemented with 0.1 μg/ml EGF) before being placed in 100 μl droplets of IVM_2 overlaid with equilibrated mineral oil, and incubated at 39 °C, 5% CO_2 in air for a further 20–22 h.

Table 1 Composition of base media used during the IVF protocol.

	Concentration (mM, unless otherwise specified)		
Ingredient	NCSU-23	IVF media	TL-Hepes
NaCl	108.73	113.1	113.7
KCl	4.78	3	3.22
KH_2PO_4	1.19	0	0
$MgSO_4.7H_2O$	1.19	0	0
$NaHCO_3$	25.07	0	2
Glutamine	1	0	0
Glucose	5.55	11	0
Taurine	7	0	0
Hypotaurine	5	0	0
$CaCl_2.2H_2O$	1.7	7.5	2.04
BSA (mg/ml)	0	4	0
Tris	0	20	0
Pyruvate	0	5	0.2
Na lactate	0	0	12.95
NaH_2PO_4	0	0	0.34
Phenol red	0	0	0.027
$MgCl_2$ $6H_2O$	0	0	0.50
FCS (μl/ml)	0	0	10
Penicillin-G	0	0	1.08
Gentamycin (μg/ml)	0	0	25
Hepes	0	0	10

Brilliant cresyl blue (BCB) test

Oocytes were washed 3 times in modified Dulbecco PBS (DPBSm, Dulbecco PBS supplemented with 0.4% (w/v) BSA (fraction V), 0.34 mM pyruvate, 5.5 mM glucose and 50 μg/ml gentamycin). Oocytes were exposed to 13 μM BCB diluted in DPBSm, and then incubated at 39 °C, 5% CO_2 in air for 90 min. The control oocyte group was incubated in DPBSm without BCB for 90 min. After incubation, the oocytes were transferred to DPBSm and washed three times. During the washing procedure, the oocytes were examined under a stereomicroscope and classified according to: (i) blue cytoplasmic staining (BCB^+ group), (ii) a colourless cytoplasm (BCB^- group), and a non-BCB-treated group (control).

Sperm preparation

Sperm were obtained from pure bred boars supplied by JSR Genetics (Driffield, East Yorkshire, UK). The heavy particles were removed by centrifugation of the whole semen sample at 50 r.p.m. for 3 min. The supernatant was then centrifuged at 1200 r.p.m. for 5 min and the pellet resuspended in 3 ml sperm preparation media (DPBS supplemented with 1 mg/ml BSA, 100 μg/ml potassium penicillin G and 75 μg/ml streptomycin) followed by centrifugation at 1200 r.p.m. for 5 min. The pellet was resuspended in 3 ml sperm preparation media and layered onto a 95%:45% Percoll gradient before centrifugation at 1500 r.p.m. for 25 min. The 90% fraction was resuspended in 2 ml IVF media (Koo *et al.* 2000; see Table 1). The supernatant was finally centrifuged at 1500 r.p.m. for 5 min and the pellet resuspended in 1 ml IVF media. Sperm counts were performed and samples were diluted to give a final concentration of 1×10^6/ml.

In vitro fertilisation

Following IVM, oocytes were washed three times in IVF media and were repeatedly drawn through a fine pipette to strip the cumulus cells; they were then placed in 100 μl drops of IVF media overlaid with equilibrated mineral oil. Diluted sperm (50 μl) were added to 100 μl of the IVF media containing the mature oocytes and these were incubated at 39 °C, 5% CO_2 in air for 15–18 h.

Embryo maturation

After sperm–oocyte co-incubation, putative zygotes were washed three times in IVP medium (NCSU-23 supplemented with 0.4% BSA; Abeydeera & Day 1997) and cultured in 100 μl of the same media overlaid with equilibrated mineral oil and incubated at 39 °C, 5% CO_2 in air. Cleavage and blastocyst formation were evaluated under a stereomicroscope, at 48 and 144 h after insemination respectively.

Measurement of oocyte diameter

Individual oocyte diameters (D) were measured immediately after BCB incubation. Ten oocytes from each culture dish were individually pipetted into 5 μl droplets of IVM_1 media, and examined at ×200 magnification on an inverted microscope at 39 °C. Oocyte volume (V) was then calculated using the standard formula: $V = 4/3\,\pi\,(D/2)^3$. The volumes of BCB^+, BCB^- and control oocytes were analysed by one-way ANOVA and Tukey's pairwise comparisons.

Preparation of oocytes and blastomeres for mtDNA analysis

Both zona pellucida (ZP) and attached sperm were removed by exposing each fertilised or unfertilised oocyte to 10 U/ml pronase in IVP media for 1–1.5 min (Fong *et al.* 2001). ZP-free unfertilised oocytes and intact embryos were washed several times in IVP media and transferred to 0.2 ml PCR tubes (Appleton Wood Laboratory Equipment, Birmingham, UK). Individual blastomeres were disassociated by exposing the intact denuded embryos to 10 U/ml pronase in IVP media for 1 min followed by repeated pipetting in IVP media. Each blastomere was transferred to a separate 0.2 ml PCR tube.

DNA extraction

Autoclaved sterile double distilled H_2O (ddH_2O; 20 μl) was added to each individual frozen oocyte, zygote or embryo and 10 μl were added to each blastomere. The samples were then subjected to freeze–thaw disruption as previously described (Findlay *et al.* 1996). Total DNA was isolated from blood samples from 37 sows representative of the whole dam line. DNA was extracted according to the whole blood DNA isolation protocol using the Puregene DNA Isolation Kit (Flowgen, Nottingham, UK).

Generation of DNA standards for real time PCR

The external standard of 369 bp was generated as previously described (Reynier *et al.* 2001) using primers designed to amplify the cytochrome B gene (see Table 2). Reactions were performed in 50 μl using 1 × PCR buffer (Bioline, London, UK), 1.5 mM $MgCl_2$ (Bioline), 0.5 μM each primer, 200 μM dNTP mix (Bioline) and 2 U *BioTaq* polymerase (Bioline). It was assumed that 1 ng of the 369 bp PCR product contains 2.47×10^9 molecules of double stranded DNA. These samples were then serially diluted 10-fold in order to construct a standard curve for PCR quantification.

Real time PCR quantification

Real time PCR was performed on an iCycler iQ Real Time Detection System (BIO-RAD, USA) in 20 μl reactions using a 96-well PCR plate (Abgenix, Epson, UK) containing 1 × buffer (Bioline), 0.2 mM dNTP Master Mix (Bioline), 4 mM $MgCl_2$ (Bioline), 0.5 μM each primer, 0.25 U Sybr Green, 2 U *BioTaq* DNA polymerase (Bioline) and 2 μl of each oocyte or blastomere sample. The reaction conditions were 1 cycle at 95 °C for 3 min followed by 50 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. iCycler software generated a standard curve. This then allowed the determination of the starting copy number of mtDNA in each sample. A melting curve was analysed in order to check for the absence of mispriming and the quality of amplifications. All samples were tested twice and the mean starting quantity of mtDNA was calculated by the iCycler software.

The difference in mtDNA copy number between fertilised and unfertilised oocytes in each group was analysed by unpaired Student's *t*-test. The data from the three BCB groups were analysed by one-way ANOVA and Tukey's pairwise comparisons and the distribution of mtDNA copy number in different blastomeres of each embryo was analysed by one-way ANOVA and Multi-Vari chart. The Student's *t*-test and the one-way ANOVA for mtDNA copy number were performed after logarithmic transformation of the data.

Fractionation of mitochondria

The ZP of BCB^+ oocytes were removed as described above, and then washed in IVM_2 medium. The ZP-free oocytes were cultured in IVM_2 supplemented with 0.5 μg/ml MitoTracker Green, a mitochondrial-specific

Table 2 Primer sequences for PCR and real time PCR.

Product	Forward primer	Reverse primer	Annealing temp	Product size (bp)
Cytochrome B	GGA ATC TCA TCA GAC ATA GAC	GAG GTC TGC TAC TAG TAT TC	55	368
β-Actin	CCA TGT ACG TGG CCA TCC AGG CTG	GGA GAT GGC CAC CGC GGC CTC	67	259
Cyclin B DNA	GTC GTG AAG TCA CTG GAA AC	CCA TCT GCC TGA TTT GGT AC	55	470
Cyclin B cDNA	GTC GTG AAG TCA CTG GAA AC	CCA TCT GCC TGA TTT GGT AC	55	236
D-loop	GCA TTC CAT TCG TAT GCA AAC C	TCA TGA TCC GGC CGA CAA T	67	606

fluorescent probe, for 30 min. After preloading of the fluorescent probe, ZP-free oocytes were cultured in 100 μl IVM_2 supplemented with 1 μg/ml cytochalasin D (CCD) for 1 h at 39 °C in 5% CO_2 in air. The ZP-free BCB^+ oocytes were centrifuged in 100 μl IVM_2 at 1500 ***g*** for 10 min to pellet the nuclei and unbroken cells. The supernatant was centrifuged at 9500 ***g*** for another 10 min to concentrate the cytoplasts. These were then transferred in 0.5 ml IVM_2 medium containing 1 μg/ml CCD to the surface of 48% Percoll solution and centrifuged at 19 600 ***g*** for 2 h. The cytoplasts were recovered from the lower portions of the centrifuge tube and transferred to a 20 μl droplet of TL-Hepes medium (see Table 1).

Mitochondrial supplementation

Recipient BCB^- oocytes that had matured *in vitro* for 24 h were incubated at 39 °C in IVM_2 medium supplemented with CCD (1 μg/ml) for 15 min prior to injection. For microinjection, oocytes were transferred to 5 μl microdroplets of TL-Hepes medium plus 0.3% BSA under mineral oil. The temperature of the microdroplet was maintained at 39 °C by means of a heated stage under the injection dish. The mitochondrial suspension was aspirated into the injection pipette until it reached a predetermined volume between 3 and 5 pl. Injection was at 3 o'clock in relation to the polar body at 6 o'clock directly into BCB^- oocytes (Nikon T200 Microinjection system, Kingston upon Thames, Surrey, UK). Another group of BCB^- oocytes was used for sham injection. The oocyte was penetrated with an injection pipette containing only some media. Immediately after injection, BCB^- oocytes were transferred to IVM_2 medium and cultured for another 24 h at 39 °C in 5% CO_2 in air. After 24 h of culture, BCB^+ oocytes, BCB^- oocytes, mitochondrial supplemented BCB^- oocytes and sham-injected BCB^- oocytes from each ovary were inseminated either by IVF, as described previously, or by intracytoplasmic sperm injection (ICSI). Putative zygotes, subjected to either IVF or ICSI, were cultured in IVP medium and checked for cleavage after 48 h. The fertilisation rates of BCB^+, BCB^-, supplemented BCB^- and sham-injected oocytes were analysed by one-way ANOVA and Tukey's pairwise comparisons.

In a separate experiment, we again isolated pure populations of mitochondria and assessed the number of mtDNA genomes that would have been injected into the cytoplasms of BCB^- oocytes.

ICSI

Following 48 h of *in vitro* maturation, oocytes were washed three times in TL-Hepes medium and were denuded of cumulus cells. The sperm suspension was added to a 10% polyvinyl pyrrolidone (PVP) solution and individual sperm were immobilised by squeezing the tail between the bottom of the dish and the injection pipette. Microinjection was performed in IVF media under mineral oil, as described above.

Quality control of the mitochondrial preparation

The enrichment of the mitochondrial preparation was assessed by visualising the mitochondria after staining the oocytes with 0.5 μg/ml Mito Tracker Green (Molecular Probes, Paisley, Strathclyde, UK) for 30 min. Absence of contamination was confirmed by PCR. β-Actin gene specific primers were used to identify the presence of any nuclear debris that may be contaminating the mitochondrial preparation. Cyclin B mRNA specific primers were used to identify any contaminating gene transcripts. mtDNA D-loop primers were used to confirm the presence of mtDNA in the mitochondrial preparation. Primer details are displayed in Table 2. RNA extraction was performed using the RNAqueous-4-PCR kit (Ambion Europe Ltd, Huntingdon, Cambs, UK) according to the manufacturer's protocol. Reverse transcription was performed using the Reverse Transcription System (Promega, Southampton, Hants, UK) according to the manufacturer's protocol. PCR using 2 U *BioTaq* polymerase was performed in 50 μl volumes in 1 × PCR buffer and 1.5 mM $MgCl_2$. The primer and nucleotide concentrations were 0.5 μM and 200 μM dNTP mix respectively. PCR reaction conditions were initial denaturation at 94 °C for 5 min then 35 cycles of denaturation at 94 °C for 1 min, annealing at the appropriate temperature for 1 min (see Table 2) and extension at 72 °C for 3 min. PCR products were resolved on 2% agarose gels. DNA preparations extracted from the oocytes, follicular fluid and sperm were used as positive controls. RNA extracted from follicular fluid was used as a positive control for cyclin B analysis.

mtDNA fingerprinting analysis

PCR amplifications of 660 bp of the porcine D-loop region (Accession No. AF034253; nt12 – 640) were performed in 50-μl reactions. Each reaction contained 200 ng total DNA, 1 × PCR buffer (BioLine), 1.5 mM $MgCl_2$ (BioLine), 200 μM dNTPs (BioLine), 0.5 μM of each primer and 2.5 U *BioTaq* DNA polymerase (BioLine). D-loop forward and reverse primers are listed in Table 2. Reaction conditions were 95 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 45 s, and then 72 °C for 3 min using an MJ Research PTC-200 DNA engine (GRI, Braintree, Essex, UK). PCR products were resolved on 2% agarose gels (Gibco BRL, UK) at 100 V for 1 h and product size confirmed against a 100 bp DNA ladder (Gibco BRL). The PCR products were excised from the agarose gels and purified for DNA sequencing using the QIAquick Gel Extraction Kit (Qiagen, London, UK), as described in the manufacturer's protocol. The purified mtDNA was then sequenced using the automated direct sequencing protocol (Hopgood *et al.* 1991).

Results

In order to determine the relationship between mitochondrial content, oocyte volume, oocyte growth, oocyte development and fertilisation outcome, we stained a cohort of oocytes with BCB. In each instance, we calculated their relative volumes and assessed their mitochondrial DNA copy number. There was a significant difference in oocyte volume between those oocytes staining positively for BCB (BCB^+) and those that reduced the dye (BCB^-; $P < 0.001$; see Table 3). BCB selection was also reflected in fertilisation rates between the two groups, where 46.6% of BCB^+ inseminated oocytes cleaved compared with 22.7% for the BCB^- set ($P < 0.001$; see Table 3).

In order to determine whether mtDNA content could influence fertilisation outcome, we analysed the number of mtDNA copies per oocyte between fertilised and unfertilised oocytes and between BCB^+ and BCB^- oocytes (see Table 3). In the BCB^+ oocyte group, the mean mtDNA copy number for fertilised oocytes was 404 550 ± 173 888 (mean ± S.D.) copies ($n = 20$) compared with 49 014 ± 30 416 for oocytes that failed to fertilise ($n = 21$), representing a significant difference of $P < 0.001$. For those BCB^- fertilised oocytes, mean mtDNA copy number was 209 737 ± 101 042 ($n = 19$) whilst for unfertilised oocytes mean mtDNA copy number was 25 686 ± 18 930 ($n = 20$; $P < 0.001$). Furthermore, there was a significant difference for mtDNA copy number between those oocytes predicted to fertilise and those predicted to fail, as assessed by BCB staining (Table 3). In addition, there was a significant difference in mtDNA copy number and volume between fertilised BCB^+ and unfertilised BCB^+ oocytes ($P < 0.05$) and between fertilised BCB^- and unfertilised BCB^- oocytes ($P < 0.02$) from the same ovary (data not shown).

The distribution of mitochondria among blastomeres was examined by determining mtDNA copy number in each disassociated blastomere. We analysed 10 fertilised control embryos ranging from the 2-cell to the 5-cell stage. In three of the 4-cell embryos, cleavage was not complete and therefore two blastomeres could not be separated. Combined measurements were taken and divided by 2, resulting in the most even distribution possible being presented (see Table 4). Despite this, there is considerable asymmetrical mitochondrial distribution amongst blastomeres from the same embryo, and amongst different embryos (Table 4).

To determine whether mitochondrial content has a direct effect on oocyte development, we supplemented BCB^- oocytes with purified populations of mitochondria containing 139 200 ± 34 800 copies of mtDNA. We compared their fertilisation rates after both IVF and ICSI treatment with BCB^+ oocytes, BCB^- oocytes, and sham-injected BCB^- oocytes. After both IVF and ICSI, BCB^+ and BCB^- oocytes with mitochondrial supplementation had significantly higher fertilisation rates ($P < 0.002$) than unsupplemented BCB^- oocytes and BCB^- sham-injected

Table 3 Properties of BCB positive and BCB negative oocytes. BCB positive oocytes have large diameters, higher copy numbers of mtDNA and are more likely to fertilise than BCB negative oocytes. Mean values are presented with standard deviations.

	Number of Oocytes inseminated	Mean oocyte complex volume (μm^3)	Fertilisation rate (%)	Oocyte mtDNA copy no.		mtDNA copy no. of unfertilised oocytes		mtDNA copy no. of fertilised embryos	
				Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
BCB^+	360	1.55×10^{6a}	46.6^a	$222\,446 \pm 217\,250^a$ ($n = 41$)	17 300–697 000	$49\,014 \pm 30\,416^d$ ($n = 21$)	17 300–108 000	$404\,550 \pm 173\,888^a$ ($n = 20$)	110 000–697 000
BCB^-	291	1.35×10^{6b}	22.7^b	$115\,352 \pm 117\,052^b$ ($n = 39$)	10 000–443 000	$25\,686 \pm 18\,930^e$ ($n = 20$)	10 000–81 600	$209\,737 \pm 101\,042^b$ ($n = 19$)	100 000–443 000
Control	257	1.45×10^{6c}	32.3^c	$138\,022 \pm 153\,841^c$ ($n = 46$)	10 100–700 000	$32\,800 \pm 20\,963^f$ ($n = 24$)	10 100–72 900	$248\,000 \pm 157\,486^c$ ($n = 22$)	100 000–700 000

[a,b,c]Values in the same column with different superscripts differ ($P < 0.001$); [d,e,f]Values in the same column with different superscripts differ ($P < 0.005$).

Table 4 Variation in mtDNA copy number within individual blastomeres from the same embryo, which can be more than an order of magnitude.

	Number of blastomeres within embryo									
	2	2	2	2	3	3	4	4	4	5
mtDNA copy number in blastomere	34 500	314 00	12 000	97 000	71 000	390 000	21 900	153 000	28 000	244 000
	117 000	297 000	88 300	192 000	37 200	100 000	21 900	171 000	48 300	106 000
					52 000	210 000	42 500*	145 500*	162 000*	97 400
							42 500*	145 500*	162 000*	16 700
										15 000
Total mtDNA copy number for embryo	151 500	611 000	100 300	289 000	160 200	700 000	128 800	615 000	400 300	479 100

* Some blastomeres had not fully divided and therefore could not be separated by pronase treatment. Values presented assume equal division of mtDNA and demonstrate minimum variation.

oocytes (see Table 5). Furthermore, the post-injection ova survival rate after both IVF and ICSI was significantly higher ($P < 0.001$) for mitochondrial-injected BCB^- oocytes (IVF = 77.8%, ICSI = 78.7%) than unsupplemented BCB^- oocytes (IVF = 70.5%, ICSI = 69.4%) and sham-injected oocytes (IVF = 67.9%, ICSI = 68%). There was no significant difference ($P > 0.05$) in the percentages of oocyte fertilisation after both fertilisation techniques (BCB^+: IVF = 37.5%, ICSI = 40.4%; BCB^- supplemented with mitochondria: IVF = 31.0%, ICSI = 34%; BCB^-: IVF = 17.6%, ICSI = 19.8%).

The donor mitochondria, derived from maternally related BCB^+ oocyte cytoplasts, were purified on Percoll density gradients and verified for the presence of contaminants and purity by fluorescence microscopy (data not shown) and through PCR. Figure 1A shows the presence of some nuclear debris contamination, in the form of β-actin, in the mitochondrial fraction prepared without the Percoll density gradient (lane 8), although very faint compared with the positive controls (lanes 11–13). However, it is absent in the Percoll density gradient mitochondrial preparation (lane 9). Similar results were obtained from analysis of cyclin B mRNA transcripts (Fig. 1B), which would be expected to persist in the oocyte cytoplasm (Hue *et al.* 1997). Cyclin B transcripts were present in the RNA extracted from follicular fluid, a positive control (lane 5). No cyclin B transcripts were present in either mitochondrial preparation, i.e. the Percoll density gradient (lane 6) and the non-Percoll density gradient (lane 7). However, some contaminating cyclin B DNA was observed in the non-Percoll density gradient mitochondrial preparation (lane 7) and in the intermediate supernatant from the purification process (lane 8).

Interestingly, the oocytes used in these studies were obtained from a commercial dam line of pure white pigs. Our mtDNA sequence analysis of 37 pigs representative of several breeding groups demonstrates that all the pigs arose from one original mtDNA ancestor belonging to haplotype A, as defined by Alves *et al.* (2003). This indicates that all the reconstructed oocytes were homoplasmic. Furthermore, the mean litter size for these pigs is 11.33 ± 2.96, demonstrating the variable fertilisation efficiency of the oocytes.

Table 5 Fertilisation rates after IVF or ICSI on oocytes with (supplemented) and without (sham injection) mitochondrial supplementation.

Treatment	IVF fertilisation rate (%)	ICSI fertilisation rate (%)
BCB^+	37.5^a	40.4^c
BCB^-	17.6^b	19.8^d
BCB^- supplemented	31.0^a	34.0^c
BCB^- sham injected	17.0^b	10.0^d

[a,b]Values in the same column with different superscripts differ ($P < 0.002$); [c,d]values in the same column with different superscripts differ ($P < 0.001$).

Discussion

It is well established that mammalian immature oocytes are heterogeneous in quality and developmental competence (Shea *et al.* 1975). There is considerable morphological variability among oocytes capable of normal development, and assessment of morphology has led to some improvements in the identification of oocytes that would develop *in vitro* (De Loose *et al.* 1992). Although morphology can indicate maturity and fertilisation potential (Madison *et al.* 1992), even after careful selection on the basis of visual appearance, 10–20% of harvested immature oocytes from slaughterhouse pig ovaries degenerate during sperm co-incubation (Matas *et al.* 1996). This suggests that other approaches warrant investigation.

Increases in the production and storage of energy substrates are vital to oocyte growth and therefore oocyte maturation and early embryonic development. These processes require NADPH production via the pentose phosphate pathway (Cetica *et al.* 2002). The pentose phosphate pathway has also been demonstrated to be involved in the meiotic induction of mouse oocytes (Downs *et al.* 1998). Completion of the pentose phosphate pathway activities during oocyte maturation is therefore likely to significantly increase fertilisation and developmental success, which we have demonstrated through

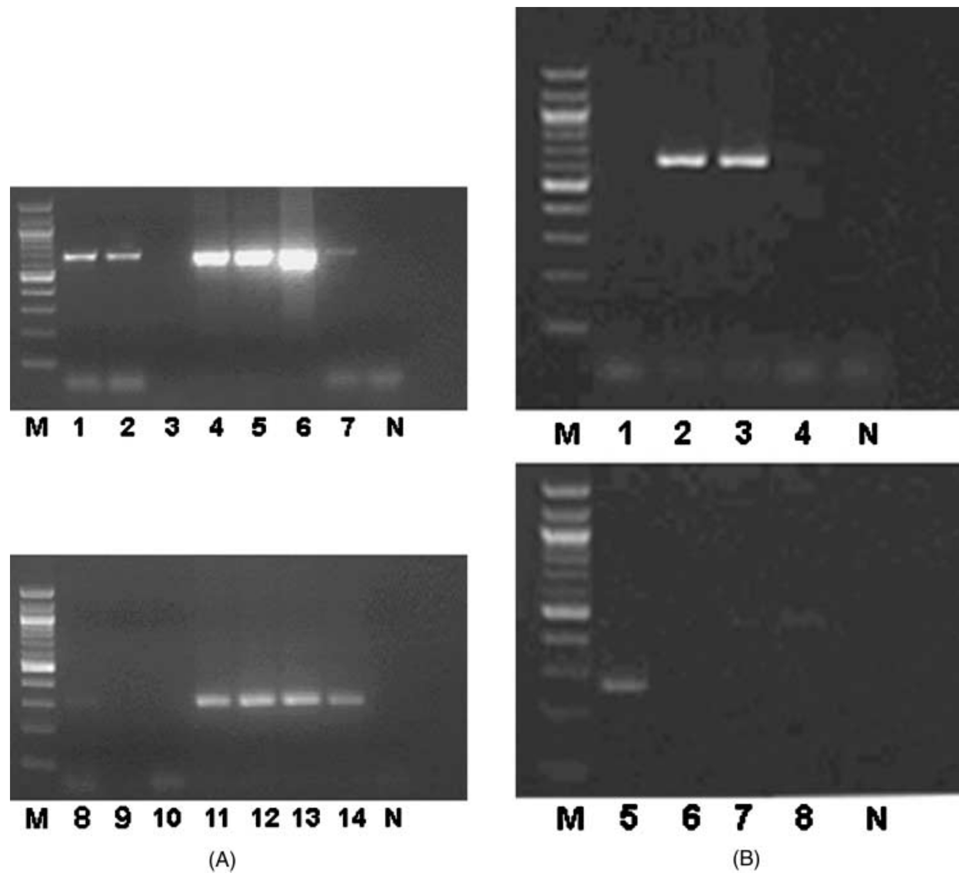

Figure 1 Confirmation of the purity of mitochondrial preparation. (A) β-Actin analysis to determine nuclear DNA contamination. Lanes 1 to 7 are mtDNA PCR product; lanes 8 to 14 are β-actin product. The mitochondria prepared without the Percoll density gradient (lanes 1 and 8) have some β-actin contamination; the Percoll density gradient mitochondrial preparation (lanes 2 and 9) contains no β-actin contamination; positive controls are supernatant (lanes 3 and 10), sperm (lanes 4 and 11), oocytes (lanes 5 and 12), and follicular fluid (lanes 6 and 13). Some mitochondria were still present in the nuclear pellet (lanes 7 and 14). M, 100 bp ladder (Gibco BRL); N, negative PCR control. (B) Cyclin B mRNA analysis to determine transcript contamination. Lanes 1 to 4 are mtDNA PCR product; lanes 5 to 8 are cyclin B transcripts. Lanes 2 to 4 demonstrate the presence of mtDNA in mitochondrial preparations purified using the Percoll density gradient (lane 2), without the Percoll density gradient (lane 3) and in an intermediate supernatant from the purification process (lane 4). No mtDNA D-loop (the only non-encoded region of the mtDNA genome) product was present in RNA extracted from follicular fluid (lane 1). Cyclin B transcripts are only present in the follicular fluid RNA extract (lane 5). However, there are faint traces of cyclin B DNA in the non-Percoll density gradient mitochondrial preparation (lane 7) and the supernatant (lane 8). No cyclin B transcripts or DNA are present in the Percoll density gradient mitochondrial preparation used for supplementation (lane 6). M, 100 bp ladder (Gibco BRL); N, negative PCR control.

BCB staining of porcine oocytes. In this instance, BCB^+ oocytes that had completed the growth phase of maturation and no longer contained G6PD, the rate-limiting enzyme of the pentose phosphate pathway, were more likely to fertilise and to survive to the later stages of preimplantation development compared with the BCB^- oocytes still containing G6PD.

We have further analysed the role of mtDNA in successful fertilisation and development of pig oocytes. In this respect, BCB^+ oocytes have a larger cytoplasmic volume ($1.55 \times 10^6\ \mu m^3$ vs $1.35 \times 10^6\ \mu m^3$), are more likely to fertilise and contain far higher numbers of mtDNA than BCB^- oocytes. This is in accordance with previous reports where individually both diameter (Raghu *et al.* 2002), an essential component of volume, and mtDNA copy number (Reynier *et al.* 2001), have been linked to fertilisation outcome in the buffalo and human respectively. Additionally, increased mitochondrial number correlates to cytoplasmic volume in cattle (Smith & Alcivar 1993). However, our porcine oocyte data would suggest that a correlation exists between increased cytoplasmic volume and increased mtDNA content, and that there appears to be a distinct relationship between mtDNA content and fertilisation outcome.

The likely outcome for those oocytes with low mtDNA copy numbers ($<$100 000), based on the lowest copies of mtDNA associated with the control group, and consequently those that fail to fertilise is that in the advanced blastocyst they would be compromised through severe mtDNA depletion. Throughout early embryogenesis, there is a continual dilution of the mtDNA genome due to the ever-increasing numbers of blastomeres and the lack of

mtDNA replenishment. In the mouse, mtDNA replication does not appear to ensue until after implantation (Larsson *et al.* 1998), or at least until the post-blastocyst stage (Piko & Taylor 1987). This is exemplified in both undifferentiated human embryonic stem cells (Sathananthan *et al.* 2002, St John *et al.* 2005) and primordial oocytes (Jansen & De Boer 1998) where low levels of mitochondria have been observed. Mitochondrial content would then exponentially increase during the differentiation processes leading to final cellular fate or, in the case of oocytes, up to MII (Smith & Alcivar 1993, Jansen & De Boer 1998). This highly regulated process ensures that appropriate numbers of mitochondria would be available to support cellular metabolism in fully functional cells.

The absence of mtDNA replication during the cleavage stages of embryonic development (Piko & Taylor 1987) is thought to function as a genetic 'bottleneck', thereby restricting segregation to the primordial germ cells, and consequently inheritance to the next generation, in order to maintain homoplasmy (Marchington *et al.* 1997). A homoplasmic population of mtDNA would be preferable to produce an efficient ETC since single point mutations and large-scale deletions can lead to the onset of mtDNA-type disease (Wallace 1999). The onset of mtDNA disease is determined by the ratio of mutant to wild-type present (heteroplasmy; Chinnery *et al.* 2001). In those oocytes carrying mtDNA-type mutations or deletions (Blok *et al.* 1997), their survival during early embryo development would be dependent on the degree of mutant molecule present. As long as these oocytes contained significant copies of the wild-type mtDNA genome to ensure successful fertilisation, it is likely that they would progress to the onset of mtDNA replication. It is then during gastrulation that the anticipated segregation of mtDNA mutations would take place, most likely affecting high-ATP requiring tissues such as neuronal and muscle tissue (Moyes *et al.* 1998).

The precise onset of mtDNA transcription and replication relies on nuclear-encoded transcription factors being translocated to the mitochondria (Clayton 1998). One particular transcription factor, mitochondrial transcription factor A (TFAM), has been implicated in mtDNA copy number regulation and is associated with mtDNA-depletion disease (Larsson *et al.* 1994, Poulton *et al.* 1994, Spelbrink *et al.* 1998, Tessa *et al.* 2000). TFAM is first expressed post-gastrulation in murine embryos (Larsson *et al.* 1998) as is the mtDNA-specific polymerase γ (PolG; Hance *et al.* 2005). Failure to initiate mtDNA replication shortly after implantation would probably result in embryos failing to survive, as is the case in homozygous knockout mice for TFAM (Larsson *et al.* 1998) and the PolG (Hance *et al.* 2005). Consequently, it would appear that the fertilised oocyte must have an appropriate amount of mtDNA present in order for TFAM and PolG to initiate mtDNA replication. Failure to do so would result in the onset of mtDNA-depletion type syndromes, as evidenced in heterozygous TFAM knockouts which exhibit severe cardiomyopathy (Li *et al.* 2000). Interestingly, a recent report indicates that an early window of mtDNA turnover takes place at the one- to two-cell stage in murine embryos (McConnell & Petrie 2004). Although no expansion of mtDNA copy number takes place at this stage, acquired levels could be maintained, thus potentially safeguarding those oocytes being borderline for fertilisation success and subsequent embryonic development.

Our analysis of developmentally competent and incompetent oocytes suggests a mitochondrial threshold relative to fertilisation outcome and subsequent embryonic development. This is implied by our supplementation of developmentally incompetent oocytes, as indicated by their BCB^- status, with donor mtDNA. In this instance, these oocytes were rescued from probable fertilisation and developmental failure by purified and concentrated mitochondrial preparations containing $139\,200 \pm 34\,800$ copies of mtDNA. This would enhance the mean mtDNA copy number for these BCB^- oocytes from 115 352 to 254 552 copies, which lies within the range for BCB^+ oocytes. Consequently, it would appear that mitochondria and/or mtDNA alone could influence fertilisation outcome. This clearly discriminates between the other factors, such as maternal RNAs that were most likely transferred during CT (Cohen *et al.* 1997), and have been suggested to be a putative trigger for subsequent development. In addition, the similarity in fertilisation outcomes between ICSI and IVF post-mitochondrial supplementation would indicate an oocyte- rather than a sperm-related factor affecting developmental outcome. This approach also eliminates the bias that might result from polyspermic fertilisation in the IVF group.

Interestingly, our data not only indicate considerable variation in oocyte mtDNA copy number (10 000 to 700 000) but we also observe this trend amongst intra-embryo blastomeres (see Table 4). This is in agreement with a previous report in the human (Lin *et al.* 2004) suggesting that this might not be a species-specific event. This unequal distribution may explain why some blastomeres survive and divide, i.e. those having sufficient mtDNA copy numbers, while others fragment due to insufficient mtDNA copy number, resulting in diminished ATP generation (Van Blerkom *et al.* 2000) or mtDNA-driven atresia (Krakauer & Mira 1999). Indeed, blastomere fragmentation is associated with blastocysts presenting with lower cell numbers, a factor well known to be associated with reduced developmental success (Park *et al.* 2005).

The ratio of mtDNA copies to the mitochondrion tends to be cell specific (see St John *et al.* 2004*a*,*b* for discussion). In the mouse (Piko & Taylor 1987) and frog (Marinos & Billett 1981), the oocyte mitochondrion contains only one copy of the mitochondrial genome, as is also hypothesised for human oocytes (Jansen & De Boer 1998). Other cell types, such as neurones and muscle cells possess

between 8 and 10 mtDNA copies per mitochondrion (reviewed in Moyes *et al.* 1998). Consequently, the mtDNA copy numbers we observe in porcine oocytes could also reflect their respective mitochondrial number. Should the pig possess more than one mtDNA copy per oocyte mitochondrion, then the number of mtDNA copies present would still be proportional to the number of mitochondria also present.

Cellular ATP is most effectively generated through OXPHOS and therefore relies on gene products for the ETC being derived from both the mitochondrial and nuclear genomes. The importance of ATP levels in developing oocytes has been demonstrated in bovine oocytes where higher quality oocytes, assessed by morphology, contained significantly higher ATP levels and produced significantly higher blastocyst rates after fertilisation (Stojkovic *et al.* 2001). In the pig during the maturation process, oxidative capacity is highest in those oocytes exhibiting granulated heterogeneous and clustered heterogeneous distributions of mitochondria which would indicate their readiness for fertilisation (Torner *et al.* 2004). It is, however, evident that inhibition of OXPHOS function at the compaction stage in porcine embryos can improve embryo viability (Machaty *et al.* 2001). Indeed, similar outcomes have been demonstrated in other mammalian species where the switch from OXPHOS to glycolysis takes place during cavitation (see Van Blerkom *et al.* 2000 for discussion). However, one report in the mouse indicates that total oxygen consumed by OXPHOS is higher in blastocysts than in other earlier staged embryos (Trimarchi *et al.* 2000). For the porcine system, this developmental switch to glycolysis would support the view that the non-replenishment of mitochondrial proteins of the ETC was reaching a critical phase and that a compensatory mechanism would be required to maintain embryo survival. A similar situation exists in somatic cells undergoing depletion of their mtDNA genomes. They cannot replicate mtDNA, nor can they survive if they are not supported by metabolic substrates that drive glycolysis (King & Attardi 1989). This is further evident in those blastomeres with very low mtDNA copy number where survival based on OXPHOS alone would be disadvantageous. This switch from OXPHOS to glycolysis would thus rescue them from metabolic crisis and support their persistence to participate at blastocyst.

The high numbers of mitochondria and mtDNA required to support fertilisation may also facilitate the distribution of ATP throughout the cell. Mitochondria form networks throughout the cell, with the distribution pattern being specific for different stages of the cell cycle (Margineantu *et al.* 2002). Mature and immature oocytes also have distinct patterns of mitochondrial distribution in cattle (Stojkovic *et al.* 2001), mice (Nishi *et al.* 2003), and the pig (Torner *et al.* 2004). Consequently, those oocytes with low mtDNA copy number may be unable to form the networks required for developmental competence, as observed in some porcine parthenogenetically activated oocytes (Brevini *et al.* 2005). Furthermore, mitochondria have been shown to act as calcium sinks in sea urchin oocytes (Eisen & Reynolds 1985). Through their uptake of Ca^{2+}, mitochondria are able to maintain the Ca^{2+} wave pacemaker that is necessary for completion of meiosis (Dumollard *et al.* 2003). Failure to maintain the Ca^{2+} wave pacemaker, for which mitochondria are also well-known mediators (Zamzami *et al.* 1996), results in apoptotic cell death of the oocyte (Liu *et al.* 2001).

Our supplementation protocol has enabled us to generate homoplasmic viable oocytes which would thus maintain the genetic integrity of the embryo and any subsequently derived offspring. This could overcome one of the major failings related to human clinical cytoplasmic transfer (St John & Barratt 1997, St John 2002*a*, St John *et al.* 2004*a*,*b*), where supplementation with donor cytoplasm has been proposed as a means of enhancing embryonic development for those women with repeated embryo development failure. Current cytoplasmic transfer practises can result in the transmission of donor and recipient oocyte mtDNA to the offspring (Brenner *et al.* 2000). Similar heteroplasmic patterns of mtDNA transmission have been observed in nuclear transfer generated embryos and offspring, discussed in St John *et al.* (2004*a*,*b*). Furthermore, the transfer of the same mtDNA source would reduce possible compromised OXPHOS function that could arise through mixing of mtDNA genotypes (McKenzie & Trounce 2000, and discussed extensively in St John & Barratt 1997 and St John 2002*a*). As a strong point of caution, our data do not, however, suggest that any of the resulting disorders associated with cytoplasmic transfer (Barritt *et al.* 2001*b*) might be overcome.

It is becoming increasingly apparent that mitochondria could be vital to oocyte maturation and subsequent embryonic development. They also provide a vehicle for the transmission of mtDNA to subsequent generations. Whilst OXPHOS is vital for early porcine embryonic development, the switch to glycolysis at compaction in the porcine system allows this critical mass of mitochondria to be maintained and its contents transmitted to subsequent generations. Mitochondrial assessment prior to any oocyte reconstruction protocol would allow fertilisable oocytes to be selected. Consequently, this might enhance success rates for those technologies which currently have low efficiencies, such as nuclear transfer (Wilmut *et al.* 1997), as these technologies may currently be hampered by the removal of some mitochondria during the enucleation procedure.

Acknowledgements

This work was partly funded by a research grant from Birmingham Womens Health Care Trust. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 29 October 2004
First decision 5 January 2005
Revised manuscript received 1 July 2005
Accepted 11 October 2005