Oocyte mitochondrial deletions and heteroplasmy in a bovine model of ageing and ovarian stimulation

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Submitted on September 25, 2015; resubmitted on November 17, 2015; accepted on January 11, 2016

STUDY HYPOTHESIS: Maternal ageing and ovarian stimulation result in the accumulation of mitochondrial DNA (mtDNA) deletions and heteroplasmy in individual oocytes from a novel bovine model for human assisted reproductive technology (ART).

STUDY FINDING: The levels of mtDNA deletions detected in oocytes increased with ovarian ageing. Low levels of mtDNA heteroplasmy were apparent across oocytes and no relationship was identified with respect to ovarian ageing or ovarian stimulation.

WHAT IS KNOWN ALREADY: Oocyte quality decreases with ovarian ageing and it is postulated that the mtDNA may have a role in this decline. The impact of ovarian stimulation on oocyte quality is poorly understood. Human studies investigating these effects are often limited by the use of low quality oocytes and embryos, variation in age and ovarian stimulation regimens within the patients studied, as well as genetic and environmental variability. Further, no study has investigated mtDNA heteroplasmy in individual oocytes using next-generation sequencing (NGS), and little is known about whether the oocyte accumulates heteroplasmic mtDNA mutations following ageing or ovarian stimulation.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: A novel bovine model for the effect of stimulation and age in human ART was undertaken using cows generated by somatic cell nuclear transfer (SCNT) from one founder, to produce a homogeneous population with reduced genetic and environmental variability. Oocytes and somatic tissues were collected from young (3 years of age; n = 4 females) and old (10 years of age; n = 5 females) cow clones following multiple natural ovarian cycles, as well as oocytes following multiple mild (FSH only) and standard (based on human a long GnRH agonist protocol) ovarian stimulation cycles. In addition, oocytes were recovered in a natural cycle from naturally conceived cows aged 4–13.5 years (n = 10) to provide a heterogeneous cohort for mtDNA deletion studies. The presence or absence of mtDNA deletions were investigated using long-range PCR in individual oocytes (n = 62). To determine the detection threshold for mtDNA heteroplasmic levels in individual oocytes, a novel NGS methodology was validated; artificial mixtures of the Bos taurus and Bos indicus mitochondrial genome were generated at 1, 2, 5, 15 and 50% ratios to experimentally mimic different levels of heteroplasmy. This NGS methodology was then employed to determine mtDNA heteroplasmic levels in single oocytes (n = 24). Oocyte mtDNA deletion and heteroplasmy data were analysed by binary logistic regression with respect to the effects of ovarian ageing and ovarian stimulation regimens.

MAIN RESULTS AND THE ROLE OF CHANCE: Ovarian ageing, but not ovarian stimulation, increased the number of oocytes exhibiting mtDNA deletions (P = 0.04). A minimum mtDNA heteroplasmy level of 2% was validated as a sensitive (97–100%) threshold for variant detection in individual oocytes using NGS. Few mtDNA heteroplasmies were detected across the individual oocytes, with only 15 oocyte-specific variants confined to two of the 24 oocytes studied. There was no relationship (P > 0.05) evident between ovarian ageing or ovarian stimulation and the presence of mtDNA heteroplasmies.

LIMITATIONS, REASON FOR CAUTION: The low number of oocytes collected from the natural ovarian cycles limited the analysis. Fertilization and developmental potential of the oocytes was not assessed as the oocytes were destroyed for mtDNA deletion and heteroplasmy analysis.

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Introduction

The oocyte is the major determinant of embryo developmental potential, and the decline in oocyte quality following ageing is well documented (Fragouli et al., 2011; Keefe et al., 2015). Ovarian ageing is associated with increased chromosomal aneuploidy, mitochondrial dysfunction and altered metabolic output of the oocyte, as well as changes in the function of the surrounding cumulus cells, although the underlying processes are incompletely understood (Fragouli et al., 2011; Pacella et al., 2012; Bentov and Casper, 2013). Maternally inherited, the mitochondrial genome (mtDNA) is thought to play a key role in ovarian ageing (Lagouge and Larsson, 2013). The mtDNA is a 16.5 kb closed circular, double stranded multi-copy genome that encodes 13 components of the respiratory chain, as well as two rRNAs and 22 tRNAs needed for mitochondrial translation (Larsson, 2010). Normal respiratory chain activity requires adequate numbers of intact and functional mitochondrial genomes (Larsson, 2010).

Mitochondria account for a large fraction of the cytoplasmic volume of the mature oocyte, and human oocytes have been shown to harbour between 240 000 and 1 550 000 mtDNA copies (Barritt et al., 2002; Dell’Aquila et al., 2009; Cotterill et al., 2013). In contrast, the mtDNA copy number of somatic cells is considerably lower, at around 100–10 000 copies per cell, with the number reflecting the energy requirements of a particular cell type (Lagoure and Larsson, 2013). The mtDNA is particularly susceptible to damage due to its close proximity to reactive oxygen species (ROS) produced as a by-product of oxidative phosphorylation. In addition, mtDNA lacks protective histones, has limited DNA repair mechanisms and is prone to replication errors (Larsson, 2010). Through accumulated damage, mitochondrial dysfunction is implicated in the ageing process, and increased mtDNA mutations, mtDNA deletions and respiratory chain deficiency are apparent in many tissue types following ageing (Bua et al., 2006; Kazachkova et al., 2013; Lagoure and Larsson, 2013). These may be present as mtDNA heteroplasm, the term used to describe the presence of a mixture of wild type and mutated mtDNA molecules in a cell.

Oocytes remain arrested in prophase I for up to 50 years in the human and it is unclear to what extent the mtDNA of the oocyte remains in a protected quiescent state during ovarian ageing. It is possible that during this arrest, the mtDNA may accumulate damage or replication errors that may manifest as mtDNA heteroplasm and mtDNA deletions. These in turn may lead to mitochondrial dysfunction, but there is limited work investigating this theory. What is known is that during the transition from a primordial follicle to a mature oocyte, there is a large increase in the number of mtDNA copies (Cree et al., 2008; St. John et al., 2010). During the process of maturation, the oocyte is supported by the transfer of high energy substrates through gap junction mediated transfer from cumulus cells, where the metabolic activity of the oocyte itself is initially low (Coticchio et al., 2015). Following maturation oocyte mitochondria are involved in ATP synthesis, ROS production and calcium signalling to support chromosomal segregation and the process of fertilization, as well as being integral to embryo development (Schon et al., 2000; Reynier et al., 2001; Van Blerkom, 2004; Shoubridge and Wai, 2007; Ramalho-Santos et al., 2009). Evidence suggests that mtDNA content may modulate oocyte quality, the ploidy status and the implantation potential of an embryo (Reynier et al., 2001; Cummins, 2002; Diez-Juan et al., 2015; Fragouli et al., 2015). In contrast, little is known about the effect of mtDNA deletions on oocyte and embryo quality, and even fewer studies have assessed mtDNA heteroplasm in this context (Barritt et al., 1999; Hsieh et al., 2002; Chan et al., 2005; Shamshi et al., 2013; Fragouli et al., 2015). In the early preimplantation embryo, mtDNA is apportioned between individual blastomeres with each cell division (Cree et al., 2008). mtDNA only starts to replicate in the post-implantation stage of development (Cree et al., 2008; St. John et al., 2010). Therefore, it is feasible that an oocyte requires a minimum number of functionally active mtDNA genomes for normal development and implantation.

Like ovarian ageing, the effects that ovarian stimulation regimens have on oocyte quality are poorly understood. Ovarian stimulation often appears to produce a cohort of oocytes that are heterogeneous in their developmental potential and work to date shows that individual oocytes have varied gene expression profiles, possibly due to the differing ovarian microenvironments present within the ovary (McNatty et al., 2010; Cree et al., 2015). It has been proposed that milder ovarian stimulation produces a smaller cohort of higher quality oocytes (van der Gaast et al., 2006; Santos et al., 2010; Ji et al., 2013). However, ovarian stimulation may also impact endometrial receptivity as evidenced by higher pregnancy rates in thawed embryo transfer cycles (Verberg et al., 2009; Allatoonian et al., 2010; Shapiro et al., 2011a, b). One study in the rhesus macaque has suggested that mtDNA deletions may be more common in oocytes following ovarian stimulation, and this should be investigated further (Gibson et al., 2005). To date, no study has assessed whether mtDNA heteroplasm is more common in oocytes following ovarian stimulation. Specifically, stimulation may rescue oocytes with mtDNA deletions or high levels of mtDNA heteroplasm that would otherwise undergo atresia in a natural ovarian cycle.

Studies are needed to assess how ovarian ageing and ovarian stimulation may influence the accumulation of mtDNA heteroplasm and mtDNA deletions, since many women having fertility treatment are...
near the end of their reproductive period. Studying these effects in humans is difficult due to wide variation in both the genetic and environmental background. Generally oocytes are only recovered from a stimulated cycle (not a natural cycle) from couples receiving fertility treatment, and only unfertilized oocytes are available for study. Unlike rodents and sheep, cattle are an attractive species to model human reproduction, as they are mono-ovular, have a similar follicle size at ovulation and have a nine-month gestation period (Adams et al., 2008). The current study employs a novel bovine model, in which a cohort of young and old cloned females generated from the same founder by somatic cell nuclear transfer (SCNT), underwent natural ovarian cycles, mild ovarian stimulation and standard ovarian stimulation. This study, included stimulation based on the long GnRH agonist down-regulation regimen used in human fertility clinics. This model allowed for the independent effects of ovarian ageing and ovarian stimulation on mtDNA heteroplasmy and mtDNA deletions to be assessed in individual oocytes. The clones were maintained as one herd, reducing both genetic and environmental variability to a level that could not be obtained in human studies. It is hypothesized that ovarian ageing and increasing doses of ovarian stimulation will be associated with an increased frequency of mtDNA deletions and heteroplasmy in oocytes from this novel bovine model. Knowledge gained from the current study will enable further understanding of how the mitochondrial genome may be involved in determining oocyte developmental potential following ovarian ageing and ovarian stimulation.

### Materials and Methods

**Animals and tissues**

Oocytes were collected from a young cohort and an old cohort of genetically identical bovine clones as previously described in Cree et al. (2015). The young and old bovine clones were created by somatic cell nuclear transfer (SCNT) using mural granulosa cells from a single 3-year-old Friesian cow, thus the donor cells used were of the same genetic origin across the two cohorts (Wells et al., 1999). Following their creation by SCNT, the young females were aged 3 years and the old females were aged 10 years. Donor oocytes were collected from abattoir ovaries, with each clone being formed as previously described by Cree et al. (2015). The Friesian cows were non-lactating, maintained as one herd and kept under the herd, reducing both genetic and environmental variability to a level that could not be obtained in human studies. It is hypothesized that ovarian ageing and increasing doses of ovarian stimulation will be associated with an increased frequency of mtDNA deletions and heteroplasmy in oocytes from this novel bovine model. Knowledge gained from the current study will enable further understanding of how the mitochondrial genome may be involved in determining oocyte developmental potential following ovarian ageing and ovarian stimulation.

### Ethical approval

This study was approved by the Ruakura Animal Ethics Committee (AEC1817).

### Ovarian stimulation regimen and oocyte recovery

Ovarian stimulation and oocyte recovery for the bovine clones was performed as previously described by Cree et al. (2015). Briefly, over the course of 3 years, young (n = 4) and old (n = 5) females were each subjected to four natural, three mild and two standard ovarian stimulation cycles with at least one 21-day natural cycle between every sampled cycle to minimize any carry over effects from previous stimulation on subsequent follicular or luteal characteristics. For natural cycles, females were synchronized by administering two i.m. injections of 500 μg Cloprostenol (EstroPLAN; New Zealand) 12 days apart and oestrus (Day 0) was identified by monitoring twice daily, and the use of tail paint. The size of the dominant ovulatory follicle was mapped by ultrasonography on Days 9, 12, 15, 17 and 19 post-oestrus. Ovulation was induced on Day 19 by administering i.m. 1500 IU hCG (Chorulon; MSD Animal Health, New Zealand), and oocyte recovery was undertaken 22 h later (Day 20). For mild cycles, females were synchronized, and administered 160 mg of FSH i.m. (Folltropin-V; Vetpharm Canada) in a 5 day step-down regimen (Day 13 50 mg, Day 14 40 mg, Day 15 30 mg, Day 16 20 mg and Day 17 20 mg), divided in to twice daily injections. On Day 18, once lead follicles were >14 mm, 1500 IU hCG was administered i.m. and oocyte recovery was undertaken 22 h later (Day 19). In the bovine mature oocytes are obtained once the follicle diameter reaches 12–14 mm, equivalent to 17–18 mm in human IVF. For standard stimulation, local anaesthesia (2 ml 2% v/v lidocaine; Bomacaine, Bomac Laboratories Ltd New Zealand) was administered in the ear of each female and a GnRH agonist implant (4.7 mg Supreflan implant; PepTech Animal Health, Australia) was inserted sub-dermally. Every 7 days, follicles ≥14 mm were ablated until Day 35, when each female received i.m. 320 mg of FSH (Folltropin-V; Vetpharm, Canada) in a 7 day step-down regimen (Day 36 100 mg, Day 37 70 mg, Day 38 50 mg, Day 39 40 mg, Day 40 20 mg, Day 41 20 mg and Day 42 20 mg) divided in to twice daily injections, with ovarian dynamics mapped and measured at each of these days. On Day 42, once the lead follicle diameters were >14 mm, 1500 IU of hCG (Chorulon; MSD Animal Health) was administered i.m. and oocyte recovery was undertaken 22 h later (Day 43). For the 10 naturally conceived females, oestrous cycles were synchronized as described for the natural cycles of the cloned cows, and oocytes recovered from follicles >10 mm on Day 14 of a FSH stimulated cycle following euthanasia. For female clones, prior to ovum pick-up (OPU), i.v. administration of 2 ml 2% v/v xylocaine (Rompun; Bayer Animal Health, New Zealand) was undertaken for mild sedation, and epidural anaesthesia was administered (4 ml 2% v/v lidocaine; Bomacaine, Bomac Laboratories Ltd, New Zealand). OPU was undertaken using a 7.5 mHz transvaginal sector probe (PieMed 2005; Pie Medical Imaging BV, Netherlands) with a sampling wand employing a 19G × 1.5” BD Precision-Glide needle (Becton Dickinson, New Zealand) attached to a 25 mm Hg vacuum aspiration pump (Karl Storz, Tuttlingen, Germany). To recover each cumulus-oocyte complex (COC), follicles were aspirated with repeated curetting and flushing with warm (39°C) modified morpholinosperminepropanesulfonic acid (MOPS) saline pH 7.4 (0.9% v/v NaCl, 2.5 mM MOPS, 5.56 mM d-glucose, 1 mM sodium pyruvate, 1.8 mM CaCl₂, 0.98 mM MgCl₂, 5.36 mM KCl, 133.1 IU penicillin G, 250.5 IU streptomycin, 150 IU Heparin, 0.5% (w/v) Phenol red and 1%
(v/v) BSA (ICP Bio, New Zealand)]. For both naturally conceived and cloned females, cumulus cells were stripped from the recovered COC by manual pipetting following brief exposure to 1 mg/ml hyaluronidase (Sigma-Aldrich, Australia). For naturally conceived females, oocytes were pooled before being snap frozen, and for cloned females individual oocytes were snap frozen. All oocyte samples were stored at −80°C until required for analysis.

DNA extraction

Total DNA was isolated from individual oocytes, blood, liver and muscle tissue using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, USA), according to the manufacturer’s instructions. The amount of DNA isolated from individual oocytes was too low to permit an accurate quantity assessment. The concentration of DNA isolated from blood, liver and muscle tissue was determined using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies; Thermo Scientific; New Zealand).

mtDNA deletion and mtDNA heteroplasmy analyses

A total of 62 oocytes were allocated to long-range PCR for mtDNA deletion analysis to assess the potential effects of ovarian ageing and stimulation on mtDNA deletions. These oocytes came from four young cloned cows (n = 29 oocytes) and five old cows (n = 33 oocytes) and where possible, oocyte numbers were balanced between old and young for the different levels of ovarian stimulation. Oocytes were collected following natural cycles (n = 9 oocytes), mild ovarian stimulation (n = 26) and standard ovarian stimulation (n = 27 oocytes). Blood, liver and muscle tissue from each clone also underwent mtDNA deletion analysis, in order to assess multiple tissue references for each animal. Additionally, a total of 107 antral oocytes (> 10 mm) were subjected to mtDNA deletion analysis and were collected from the ten naturally conceived females aged between 4 and 13.5 years. This validated the mtDNA deletion methodology and confirmed the presence of mtDNA deletions in oocytes collected from a heterogeneous cohort of naturally conceived females, across a wide age range. These oocytes underwent mtDNA deletion analysis for each animal, in which an average of 11 oocytes were pooled prior to analysis and assessed for each naturally conceived female. A total of 24 oocytes from cloned cows were allocated to both long-range PCR and NGS for a paralleled mtDNA deletion and heteroplasmy analysis. These oocytes were collected from two young clones and two old clones, with each animal contributing one oocyte from a natural cycle, two oocytes from mild stimulation and three oocytes from standard ovarian stimulation, representing a balanced group with which to study mtDNA heteroplasmy. While the clones had identical nuclear genetics, the SCNT procedure used to create the clones resulted in each clone inheriting the mitochondrial genome from the oocyte used from each donor cow, and therefore subtle mtDNA variants were expected to exist between the different clones. To account for differences between clones, and also unique differences that exist within each animal compared with the published Bos taurus reference sequence, a genomic DNA reference from blood was used for each clone as an internal reference for mtDNA heteroplasmy and mtDNA deletion analysis.

Long-range PCR

A 15 kb PCR product, encompassing the entire mitochondrial genome excluding the D-loop, was used for NGS and mtDNA deletion analysis in oocytes, blood, liver and muscle samples. The long-range PCR was performed as a two-step nested protocol using two primer sets that are located at positions bMT551 to bMT15334 and bMT660 to bMT15171 of the bovine mitochondrial genome (Supplementary Table S1). The long-range PCR reactions were performed using the PrimeSTAR GXL DNA Polymerase system (Clontech, USA). A 25 µl PCR reaction was performed according to the manufacturer’s instructions, with 2 µl of purified DNA being used as a template for the first round of PCR. PCR cycling conditions were; initial denaturation for 1 min at 94°C, followed by 39 cycles of denaturation for 10 s at 98°C and extension for 10 min at 68°C, with a final extension of 72°C for 12 min (Verity, Applied Biosystems, New Zealand). The primary PCR product was diluted 1:40, and 1 µl was used as a template for a second round of PCR using a 50 µl reaction and the same conditions as above. Negative controls with H2O added to the reaction in the place of DNA were included, and showed no evidence of amplification. Following both the first and second round of PCR, the PCR product was separated on a 0.7% agarose containing ethidium bromide and viewed under UV light. Deletion analysis was performed following the second round of PCR in which the PCR product was abundant. The intact 15 kb PCR product was extracted from the agarose gel using the QIAquick Gel Extraction Kit (Analytikjena, Germany), and quantified using the Qubit dsDNA HS assay kit (Life Technologies, New Zealand) according to the manufacturer’s instructions, prior to library preparation.

mtDNA heteroplasmy threshold detection

To determine the detection threshold for mtDNA heteroplasmy analysis, mixtures of the Bos taurus (AF492351.1) and Bos indicus (AF492350.1) mitochondrial genome, which have 217 sequence differences between them, were generated at 1, 2, 5, 15 and 50% ratios. These mixtures were representative of the Bos taurus mitochondrial genome relative to the Bos indicus mitochondrial genome. These mixtures were generated as follows; the entire mitochondrial genome was amplified in four overlapping fragments of 4 kb in length, using TaKaRa LA Taq DNA Polymerase (Clontech, USA) according to the manufacturer’s instructions. The primer pairs for each overlapping fragment, along with nested primers for each fragment are shown in Supplementary Table S1. PCR cycling conditions were; initial denaturation for 1 min at 94°C, followed by 38 cycles of denaturation for 10 s at 98°C and extension for 5 min at 60°C, with a final extension of 72°C for 10 min (Verity, Applied Biosystems). The PCR products were separated on a 0.7% agarose gel containing ethidium bromide, viewed under UV light and extracted using the QIAquick Gel Extraction Kit (Qiagen, Netherlands). In order to create a pure population of mtDNA, each fragment was cloned into the pGEM-T vector (Promega, USA) and transformed into TOP10 Chemically Competent E. coli (Invitrogen, New Zealand) using the manufacturers protocol. Plasmids were extracted using the Plasmid Midi Kit (Qiagen, Netherlands) and quantified using the Qubit dsDNA HS assay kit (Life Technologies). Each of the plasmid samples harbouring the pure Bos taurus and Bos indicus mtDNA fragments were mixed at 1, 2, 5, 15 and 50% ratios. Each of the four fragments from the same mixed ratio was pooled at equal molarity prior to library preparation.

Library preparation and next-generation sequencing

Libraries were prepared from 2.0 µg of PCR product using the TruSeq DNA library preparation kit (illumina, USA). Barcoded adapters (Illumina) were ligated onto each end of the fragments according to the manufacturer’s instructions. Adaptor-ligated products were then size selected (400 bp) by gel purification and amplified to obtain DNA libraries. The libraries for the 1, 2, 5, 15 and 50% Bos taurus/Bos indicus mtDNA mixtures were pooled by equal molarity and sequenced using the MiSeq 2X 250 base PE platform (Illumina). The libraries from the oocytes and genomic DNA reference samples from blood were pooled by equal molarity and sequenced using the same methodology as that used in the validation experiment.

Next-generation sequencing analysis

Next-generation sequencing (NGS) was performed by New Zealand Genomics Limited (NZGL; Dunedin, New Zealand) in conjunction with
massey genome services (palmerston north, new zealand), on an illumina msseq platform. reads that failed to pass the dynamic trim filter set at the p = 0.01 quality level, and those under 65 bp determined using length-sort from solexaqa ++ were removed from the analysis (cox et al., 2010).

for the validation experiments, reads for the 1, 2, 5, 15 and 50% bos taurus/bos indicus mtDNA mixtures were aligned to the bos taurus reference sequence (AF492351.1). for the oocyte samples, reads were aligned to a genomic DNA reference sequence from blood for each animal. briefly, de novo assembly of genomic DNA reference sequences were performed, and those assemblies with a K value of 55, at quality p = 0.01 and sub-sampling level of 10%, were used for alignment. these genomic DNA reference sequences were realigned to the bos taurus reference using the geneious multiple align algorithm (http://www.geneious.com, kearse et al., 2012). the aligned BAM files underwent variant detection using the genome analysis toolkit (Gatk) (mckenna et al., 2010) and varscan (koboldt et al., 2009). the variant detection for the oocyte samples was performed using a minimum 2% frequency, as determined by the mixed ratio validation experiment. bos indicus mtDNA haplotypes are present in the new zealand population of European cattle (bos taurus) and this phenomenon was previously confirmed for these bovine clones (steinborn et al., 2002). therefore, known variants between bos taurus and bos indicus mtDNA were excluded prior to oocyte variant analysis. a schematic diagram of the overall experimental design of the current study is shown in Fig. 1.

Statistical analyses

COC recovery rates and follicle diameters were compared between old and young clone females using the Mann–Whitney test. A one way analysis of variance Kruskal–Wallis test and Dunn’s multiple comparison tests was undertaken to investigate differences in COC recovery rates and follicle diameters between natural, mild and standard stimulation regimens. The number of follicles per female was analysed for the effects of maternal age and ovarian stimulation regimen, with regimen replicate as a random factor included in the model. all analyses were run using the Proc Mixed procedure of SAS software version 9.1 (SAS institute, USA). For the validation experiment, sensitivity was calculated as a measure of the proportion of variants that were correctly identified for the bos taurus/bos indicus mtDNA mixtures. The percentage of oocytes exhibiting mtDNA deletions or heteroplasms was calculated. Binary logistic regression was used to analyse the relationship between ageing and ovarian stimulation for the presence of mtDNA heteroplasmy in individual oocytes, as well as for the presence of mtDNA deletions in individual oocytes, blood, liver and muscle samples. Binary logistic regression was performed on the IBM SPSS statistics version 22 statistics package (IBM, USA). Each follicle was treated as an independent observation due to the experimental design reducing genetic and environmental variability. Statistical significance was taken as $p < 0.05$.

Results

Ovarian stimulation regimen and oocyte recovery outcomes

For the four young and five old female clones 100% of the natural cycles, 83% of the mild ovarian stimulation and 72% of the standard ovarian stimulation cycles were sampled. Reasons for cycle cancellation were poor response to stimulation or early ovulation. The overall successful COC recovery rate was 61%, with no difference with respect to female age or the natural/stimulated cycle type sampled ($p > 0.05$). As expected, the number of follicles (mean ± SEM) increased with increasing stimulation dose (1.00 ± 0.00 for natural cycle, 5.36 ± 0.65 for mild and 9.31 ± 0.85 for standard stimulations, $p = 0.004$). The mean follicle diameter (mean ± SEM) was largest in the natural cycles (16.8 ± 0.4 mm), compared with both mild (14.0 ± 0.2 mm) and standard stimulation (13.9 ± 0.1 mm) regimens ($p < 0.0001$), but was not affected by maternal age ($p > 0.1$). The number of oocytes (mean ± SEM) recovered for analysis per female per sampling was 0.6 ± 0.1 for a natural cycle, 1.9 ± 0.4 for the mild and 3.4 ± 0.2 for a standard ovarian stimulation cycle.

mtDNA deletion analysis

The deletion analysis for oocytes was performed by long-range PCR which amplified a 15 kb fragment of mtDNA. Of the oocytes collected from 10 naturally conceived females, representing a heterogeneous
cohort across a wide age range \((n = 107\) oocytes\), 80% of the animals showed the presence of mtDNA deletions in their pooled oocyte samples. The deletion analysis revealed a 15 kb band in addition to bands with lengths ranging from 2.2 to 5.0 kb representing deleted mtDNA. This analysis validated the long-range PCR methodology used in the current study and confirmed the presence of mtDNA deletions in oocytes from a heterogeneous cohort of naturally conceived females.

For each female clone mtDNA deletion analysis was performed for oocytes, blood, liver and muscle. The presence or absence of mtDNA deletions was determined, with the intact 15 kb band being detected in addition to bands with lengths ranging from 0.65 to 9.0 kb representing deleted mtDNA. The frequency of deletions was different across the tissue types, with this trend being apparent following analysis within the same animal (shown in Fig. 2). Unlike in humans, no ‘common’ deletion was apparent (Chan et al., 2005). As such, the exact percentage of deleted compared to intact mtDNA for a given sample was not quantified, due to numerous different deletions being detected, within the same sample. In total, 48% of individual oocytes showed at least one deletion \((n = 62)\). For the reference tissue, 22% of the clones showed at least one deletion in their blood sample, 89% in their liver and 100% in their muscle \((n = 9\) clones\). There was a statistically significant difference between the frequency of mtDNA deletions and tissue type (oocytes, blood, liver and muscle) \((P = 0.001)\). Of the tissue samples that contained deletions, 67% had one deletion, 27% had two deletions and 6% had multiple deletions. Across all tissue types, older clones had a significantly higher number of deletions compared with the young clones \((P = 0.02)\). This was particularly apparent for mtDNA deletions in oocytes, as 35% of oocytes from young clones had deletions \((n = 29\) oocytes\) compared with 61% of oocytes from old clones \((n = 33\) oocytes\, \(P = 0.04)\) (Table I). Although only 22% of oocytes from a natural ovarian cycle \((n = 9\) oocytes\) showed deletions, compared with 62 and 44% from mild \((n = 26\) oocytes\) and standard ovarian stimulation \((n = 27\) oocytes\) respectively, there was no significant difference with respect to ovarian stimulation regimen \((P > 0.05)\) (Table I).

**Heteroplasmy threshold detection**

The average sequence coverage in the validation experiment using the *Bos taurus/Bos indicus* mixed ratio samples was 14 000, however the coverage was not uniform between the four mtDNA fragments, as they showed two highly represented regions at 500–4600 bp and 8800–13 000 bp of the bovine mitochondrial genome (AF492351.1). This bias confirmed that the optimal methodology for subsequently sequencing the oocyte mtDNA was to use a single 15 kb PCR product spanning the complete mitochondrial genome (excluding the D-loop). While this option was deemed technically unfeasible for the validation experiment due to the difficulties associated with cloning a 15 kb large insert, it did confirm that high quality sequencing data could be obtained from the use of a single PCR product in the current study. Of the 217 substitutions present between *Bos taurus* and *Bos indicus*, 100% were detected at the 50, 15 and 5% mixed ratios, 97% were detected at the 2% mixed ratio, and 81% were detected at the 1% mixed ratio (Fig. 3). Together, these observations suggest that the NGS methodology used in the current study has a high sensitivity \((97–100\%)\) down to the 2% heteroplasmy level, and this was deemed an accurate threshold for analysing oocyte mtDNA heteroplasmy. While the majority of the substitutions were detected at the 1% level, the sensitivity at this level was too low

![Figure 2](https://academic.oup.com/molehr/article-abstract/22/4/261/2459838/Oocyte-mitochondrial-deletions-and-heteroplasmy-in/1595468?median embargo=2018-05-16&max embargo=2018-05-16)

**Figure 2** Representative gel electrophoresis image of mitochondrial deletions present in bovine clones. Mitochondrial deletion analysis was performed following long-range PCR of a 15 kb fragment of the mitochondrial DNA (mtDNA). This representative image demonstrates the mitochondrial deletion analysis performed for one clone from five oocytes across a natural cycle, mild ovarian stimulation and standard ovarian stimulation, as well as blood, liver and muscle tissue. Multiple bands with lengths ranging from 0.85 to 4.0 kb are observed, with deletions being apparent in four out of the six oocytes across the different levels of ovarian stimulation. Mitochondrial deletions are absent in blood, faint deletions are present in liver, and a single deletion is present in muscle.

**Table 1** Univariate binary logistic regression of the presence of mitochondrial DNA deletions in oocytes \((n = 62)\) on age of bovine clones \((young n = 4\) and old \(n = 5)\) and ovarian stimulation level \((natural, mild or standard)\).

<table>
<thead>
<tr>
<th>Condition tested</th>
<th>(\beta)</th>
<th>95% CI</th>
<th>95% CI</th>
<th>(P)-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.342</td>
<td>0.121</td>
<td>0.964</td>
<td>0.042*</td>
</tr>
<tr>
<td>Standard ovarian stimulation (reference category)</td>
<td></td>
<td></td>
<td></td>
<td>0.128</td>
</tr>
<tr>
<td>Mild ovarian stimulation</td>
<td>2.000</td>
<td>0.669</td>
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<td>0.215</td>
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<td>Natural ovarian cycle</td>
<td>0.357</td>
<td>0.062</td>
<td>2.045</td>
<td>0.248</td>
</tr>
</tbody>
</table>

\(\text{CI, confidence interval.}\)

\(*P < 0.05.\)
(81%) to permit an accurate mtDNA heteroplasmy analysis, therefore the 2% level was selected for mtDNA heteroplasmy analysis.

mtDNA heteroplasmy analysis

The mitochondrial genomes from a total of 24 oocytes (6 oocytes per female, \( n = 4 \) females), as well as 4 genomic DNA reference samples from blood underwent successful NGS, supporting the use of this methodology for obtaining high quality sequencing data for single oocytes limited by low DNA template. Following de novo assembly of the mitochondrial genome of the internal genomic DNA reference sequence from blood for each animal, mtDNA variant analysis was performed for individual oocytes. As expected, known variants between \( \text{Bos taurus} \) and \( \text{Bos indicus} \) mtDNA were detected across individual oocytes, and these were excluded from further analysis. Compared with the genomic DNA references from blood, a total of 32 variants were detected across the individual oocytes, ranging from a heteroplasmy level of 2 to 37%. Of these variants, 17 variants were present at a low heteroplasmy level (average of 4%) in two or more oocytes from the same animal, suggesting that they represented oocyte tissue specific heteroplasmy compared with the blood, or were also present at very low levels in the blood and were therefore a germ line heteroplasmy of the individual animal. The remaining 15 variants were detected at variable heteroplasmy levels and detected in a single oocyte from each animal, and thus were deemed to represent mtDNA heteroplasmies exclusive to individual oocytes (Table II). These variants were detected at a heteroplasmy level ranging from 2 to 37%, with an average heteroplasmy level of 14% and specific nucleotide changes shown (Table II). The bMT-RNR1 and bMT-ND5 genes showed the highest number of mtDNA heteroplasmic variants, with three variants detected for each gene and an average heteroplasmy level of 5% for bMT-RNR1 (an rRNA that plays a role in mitochondrial protein translation), and 15% for bMT-ND5 (a mitochondrial gene which encodes for a subunit of Complex I in the respiratory chain). These 15 variants were however confined across only two oocytes, with one oocyte being from a young clone following a standard ovarian stimulation, contributing five variants, and the other being from an old clone following a natural ovarian cycle, contributing 11 variants. Given that no other mtDNA variants were detected in any other oocytes, the current study found no evidence in the cohort analysed that there was a relationship between mtDNA heteroplasmy levels and either ovarian ageing or ovarian stimulation in this bovine model (\( p > 0.05 \)).

Discussion

The current study used a novel bovine model to study the frequency of mtDNA deletions and heteroplasmy from young and old clones, at three

![Figure 3](https://academic.oup.com/molehr/article-abstract/22/4/261/2459838/Oocyte-mitochondrial-deletions-and-heteroplasmy-in/267)

Figure 3 Detected and theoretical mtDNA heteroplasmy levels (%) for the 217 substitutions between \( \text{Bos taurus} \) and \( \text{Bos indicus} \). To determine the detection threshold for mtDNA heteroplasmy, mixtures of the \( \text{Bos taurus} \) and \( \text{Bos indicus} \) mtDNA were generated at 1, 2, 5, 15 and 50% ratios. \( \text{Bos taurus} \) and \( \text{Bos indicus} \) mtDNA have 217 substitutions between them, shown on the X axis. The detected heteroplasmy level for each substitution at each different ratio is represented by the spots, and the theoretical mtDNA heteroplasmy levels (1, 2, 5, 15 and 50%) are represented by the lines.
levels of ovarian stimulation (natural, mild and standard) based on that used for human ART. The methodology for detecting the mtDNA deletions by long-range PCR was validated in a heterogeneous population of naturally conceived females across a wide age range. Data from the current study show that mtDNA deletions could be detected in 48% of the oocytes from clones, with oocytes from old clones showing higher numbers of deletions (61%) compared with young clones (35%). This finding suggests that oocytes collected from bovine clones have increased rates of mtDNA deletions following ovarian ageing. By necessity, the majority of oocytes were collected following stimulated cycles. Higher deletion levels were detected in oocytes from mild and standard ovarian stimulation (62 and 44% respectively) compared with those from a natural cycle (22%). However the difference was not statistically significant, most likely due to the small sample size of the natural cycle oocytes. It has previously been suggested that the presence of the ‘common’ deletion was more frequent in oocytes following ovarian stimulation, compared with immature or unstimulated oocytes in the rhesus macaque, which may support this finding (Gibson et al., 2005).

A limited number of studies have assessed the presence of mtDNA deletions in oocytes. Previous human studies have demonstrated that 51–66% of oocytes that are either immature or fail to fertilize exhibit deletions in oocytes. Previous human studies have demonstrated that (Barritt et al., 1999; Hsieh et al., 2002). Multiple rearrangements have been detected, however the 4977 bp ‘common’ deletion appears to be the most frequently detected (Barritt et al., 1999; Hsieh et al., 2002). In support of these previous findings, the current study reports a similar frequency of oocytes exhibiting mtDNA deletions (48%). Previous studies have also identified that 32–35% of Day 3 embryos that arrest or are abnormal show mtDNA deletions, but there is no comparison available for normally developing embryos (Barritt et al., 1999; Hsieh et al., 2002). The apparent decrease in the level of deletions from the oocyte to the Day 3 embryo could suggest that mtDNA deletions may become selected against, possibly by a purifying selection mechanism (Stewart et al., 2008). In terms of live born children, increasing age of the maternal grandmother does not seem to influence the presence of mtDNA deletions (Elson et al., 2010). A strength of the current study was the assessment of the mtDNA deletion frequency of endogenous reference tissues within each clone, enabling oocyte deletion levels to be compared to post-mitotic tissue (muscle), and regenerative tissues (blood and liver) within females. Interestingly, all clones showed at least one deletion in their muscle, regardless of age. Given that mtDNA deletions were detected at a lower frequency in oocytes, this may provide evidence that the oocyte mtDNA may be partially protected from accumulating mtDNA deletions by remaining in a metabolically quiescent state during maturation in the ovary (Coticchio et al., 2015), compared to muscle which has a high metabolic activity, or alternatively oocytes may undergo purifying selection to reduce the presence of deletions.

Double the number of mtDNA deletions in oocytes from old clones, compared with young clones, suggests that ageing may affect the frequency of these deletions. Human and mice studies have identified that the percentage of deleted mtDNA increases following ageing in a variety of tissues, especially those with high energy demands and in post-mitotic tissues, such as muscle (Bua et al., 2006; Kazachkova et al., 2013). However, for oocytes, studies in humans are conflicting as to whether the presence of the mtDNA deletions increases with age, and may be biased due to the use of low quality oocytes and embryos (Barritt et al., 1999; Hsieh et al., 2002; Chan et al., 2005). In the current study, multiple oocytes from each clone were assessed following ovarian stimulation, allowing for an assessment of oocytes with differing developmental potential, rather than the sampling of exclusively poor quality oocytes. This along with the study of a genetically homogenous cohort may explain why the current study found an age-related increase in the number of oocytes showing mtDNA deletions, in contrast to previous human studies (Barritt et al., 1999; Hsieh et al., 2002).

The current study validated a novel methodology for accurately quantifying mtDNA heteroplasmy in individual oocytes by generating artificial heteroplasmy at various frequencies prior to NGS analysis to determine

### Table II Mitochondrial DNA mutations identified in bovine oocytes.

<table>
<thead>
<tr>
<th>Gene name(s)</th>
<th>Gene symbol</th>
<th>Nucleotide change</th>
<th>Heteroplasy level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrially encoded 12S RNA</td>
<td>bMT-RNR1</td>
<td>m.791C&gt;A</td>
<td>2.82</td>
</tr>
<tr>
<td>Mitochondrially encoded 12S RNA</td>
<td>bMT-RNR1</td>
<td>m.865G&gt;A</td>
<td>9.06</td>
</tr>
<tr>
<td>Mitochondrially encoded 12S RNA</td>
<td>bMT-RNR1</td>
<td>m.942T&gt;C</td>
<td>2.65</td>
</tr>
<tr>
<td>Mitochondrially encoded 16S RNA</td>
<td>bMT-RNR1</td>
<td>m.2254G&gt;A</td>
<td>15.33</td>
</tr>
<tr>
<td>Mitochondrially encoded NADH dehydrogenase 1</td>
<td>bMT-ND1</td>
<td>m.2943G&gt;A</td>
<td>11.14</td>
</tr>
<tr>
<td>Mitochondrially encoded NADH dehydrogenase 2</td>
<td>bMT-ND2</td>
<td>m.4266G&gt;A</td>
<td>23.48</td>
</tr>
<tr>
<td>Mitochondrially encoded cytochrome c oxidase I</td>
<td>bMT-COII</td>
<td>m.5346C&gt;T</td>
<td>11.92</td>
</tr>
<tr>
<td>Mitochondrially encoded ATP synthase 8</td>
<td>bMT-ATP8</td>
<td>m.7903T&gt;C</td>
<td>2.34</td>
</tr>
<tr>
<td>Mitochondrially encoded ATP synthase 6</td>
<td>bMT-ATP6</td>
<td>m.8625C&gt;T</td>
<td>2.51</td>
</tr>
<tr>
<td>Mitochondrially encoded cytochrome c oxidase III</td>
<td>bMT-COIII</td>
<td>m.9009C&gt;T</td>
<td>23.55</td>
</tr>
<tr>
<td>Mitochondrially encoded NADH dehydrogenase 4</td>
<td>bMT-ND4</td>
<td>m.10382G&gt;A</td>
<td>22.52</td>
</tr>
<tr>
<td>Mitochondrially encoded NADH dehydrogenase 5</td>
<td>bMT-ND5</td>
<td>m.11875T&gt;C</td>
<td>2.76, 9.47</td>
</tr>
<tr>
<td>Mitochondrially encoded NADH dehydrogenase 5</td>
<td>bMT-ND5</td>
<td>m.12001T&gt;C</td>
<td>11.85</td>
</tr>
<tr>
<td>Mitochondrially encoded NADH dehydrogenase 5</td>
<td>bMT-ND5</td>
<td>m.13012T&gt;C</td>
<td>36.87</td>
</tr>
<tr>
<td>Mitochondrially encoded cytochrome b</td>
<td>bMT-CYB</td>
<td>m.14723T&gt;C</td>
<td>15.28</td>
</tr>
</tbody>
</table>
a detection threshold. It was established that mtDNA heteroplasmy could accurately be detected at the 2% level. A previous study employed an alternative NGS platform using artificial heteroplasmy created at different ratios from two human subjects, however unlike the current study, the mtDNA fragments were not cloned prior to assessment, meaning that a pure population may not have been obtained, and a lower number of variant positions were assessed (Tang and Huang, 2010). The current study also used an internal genomic DNA reference sequence from blood for each clone, strengthening this methodology. To the best of our knowledge, the current study is one of the first studies to perform a validated mtDNA heteroplasmy analysis on individual oocytes using NGS.

The current study identified that half of the heteroplasmic mtDNA oocyte variants were present in only one oocyte per animal, suggesting that these were not low-level germline heteroplasmas, and were present at an average heteroplasmy level of 14%. These particular variants were confined across only two of the 24 oocytes studied. These two oocytes may have exhibited a lower developmental potential but this could not be determined from the current study. Given these results, the current study found no evidence that the oocytes from these bovine clones accumulated mtDNA point mutations, nor evidence that the levels of mtDNA heteroplasmy increase following either ageing or ovarian stimulation. It has been suggested however that some tissues preferentially accumulate mtDNA point mutations and others deletions with age, but the underlying mechanism is poorly understood, although it may relate to the mitotic activity of the tissue (Larsson, 2010).

It is plausible that the oocyte, despite being arrested in the ovary for up to 50 years, may remain in a protected quiescent state, accumulating limited mtDNA heteroplasmic mutations, but further studies are needed to assess this hypothesis. Mitochondria in oocytes contain few cristae, and show reduced oxygen consumption, supporting this theory (Trimarchi et al., 2000; Van Blerkom, 2011). Secondly, replication of mtDNA and clonal expansion of mutated mtDNA is not linked to the cell cycle and can occur in the absence of cell division (Larsson, 2010) introducing the possibility that this could occur in the oocyte during its arrest, however on the basis of this study there is little evidence for this in terms of point mutations. The mtDNA does play an important role in preimplantation development however, and recent reports suggest that elevated levels of mtDNA at the blastocyst stage are associated with aneuploidy and decreased implantation potential for euploid human embryos (Diez-Juan et al., 2015; Fragogli et al., 2015). These findings suggest that there is strict control of mitochondrial biogenesis, and having the correct number of functionally correct genomes may be an important factor that determines implantation success. However, more studies are required to fully characterize the mitochondrial genome in terms of abundance, heteroplasmic levels and deletions throughout oocyte and embryo development in order to understand the segregation patterns of mtDNA within the embryo and its role in normal embryo development and quality. While one study found that mtDNA heteroplasmy did not appear to be related to mtDNA abundance in trophectoderm samples from 23 human blastocysts (Fragagli et al., 2015), the sequencing coverage was low (150 X), which would not have permitted an in-depth mtDNA heteroplasmy analysis comparable to that used in the current study. Additionally, a study using a Sanger sequencing approach found that disease associated mtDNA mutations were correlated with a low fertilization rate in oocytes and poor quality embryos; however the methodology used only permitted assessment of the presence or absence of the mtDNA mutation and did not allow for an accurate quantification of heteroplasmy levels (Shamsi et al., 2013). While the current study determined that mtDNA heteroplasmas were generally low in this cohort of bovine oocytes, it will be important for future studies to assess mtDNA heteroplasmy, mtDNA deletions and mtDNA abundance in the developing oocyte and preimplantation embryo, and relate this to implantation success in the context of ovarian ageing and ovarian stimulation.

In conclusion, this novel model was developed to overcome the fact that it would be unethical for human studies to assess the effects of ageing and ovarian stimulation on the mitochondrial genome because by necessity the oocytes would need to be destroyed. The current findings show that ovarian ageing increased the number of oocytes exhibiting mtDNA deletions. Unlike previous human studies analysing mtDNA deletions in non-viable oocytes and Day 3 embryos, cloned cows representing a homogenous population with reduced nuclear genetic and environmental variability each had a complete cohort of oocytes assessed with a range of developmental competencies. Additionally, unlike other studies, mtDNA deletions in oocytes were verified by comparison with blood, liver and muscle tissue for the same female, strengthening the analysis. Previous results from the same cohort of clones show age-related changes in the expression levels of key mitochondrial genes in the ovarian follicle (Cree et al., 2015). Together, these results suggest that there are some age-related mitochondrial changes in the ovarian follicle, additional to or concomitant with the known increase in aneuploidy. While there were a justifiably low number of animals, this novel model minimized the genetic and environmental variability that is endemic of human studies. A further reduction in variability was obtained through all animals undergoing natural, mild and standard ovarian stimulation. This large animal model has the advantage of oocytes being obtained from a mono-ovular species with endocrinology and follicle dynamics that are similar to the human. The ovarian stimulation used in the study is autologous to that used in fertility clinics for IUI and IVF. The animal model allowed the entire heterogeneous cohort of oocytes to be examined, avoiding the analysis of an abnormal subset of oocytes, which is characteristic of human studies. A limitation of this animal model was the inherently low number of oocytes collected from natural ovarian cycles, meaning that a comparison between a natural ovarian cycle and ovarian stimulation was underpowered. Further research is needed to determine what constitutes effective stimulation, especially in the context of consecutive cycles of natural or mild stimulation and freezing all embryos for later use. In order to further study the mitochondrial genome in human oocytes, future studies could make use of oocytes that have been cryopreserved and discarded due to lysing, or surplus cryopreserved oocytes obtained from donors. The methodologies performed in the current study importantly provide an accurate way to identify and quantify mtDNA heteroplasmas in individual oocytes, and this methodology could be confidently applied to future studies investigating mtDNA heteroplasmy in oocytes and embryos.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

**Acknowledgements**

The authors wish to thank Drs Debbie Berg, Rita Lee and Blake Paget (AgResearch Ltd, New Zealand), as well as Dr Penny Back (Massey
University, New Zealand) for help with cattle trials and sample collection. Also, the authors would like to thank Dr Dave Wells (AgResearch Ltd, New Zealand) for the creation and access to the cloned females. The authors wish to thank Patrick Biggs (Massey University, New Zealand) and Mauro Truglio (Massey University, New Zealand) for their help with bioinformatics.

Authors’ roles

L.M.C. and A.N.S. conceived the study design, and supervised the laboratory work which was carried out by E.R.H. M.P.G. and J.C.P. developed the animal model and the animal work was carried out by M.P.G. and M.C.B. NGS and preliminary bioinformatics analysis was performed by New Zealand Genomics Limited (NZGL). Data analysis was carried out by E.R.H. under the supervision of L.M.C. E.R.H. wrote the first draft, which was modified after critical appraisal from the other authors.

Funding

These studies were supported by funding from The Nurture Foundation for Reproductive Research (grant number 3702463); The Fertility Society of Australia (grant number 201200010); Fertility Associates Ltd (grant number 3621889); The Pearce Trust, University of Auckland (grant number 3700186) awarded to M.P.G. and L.M.C.; E.R.H. is supported by The Auckland Medical Research Foundation Doctoral Scholarship (reference number 1 212 001).

Conflict of interest

J.C.P. is a shareholder of Fertility Associates and M.P.G. received a fellowship from Fertility Associates. The other authors of this manuscript have nothing to declare and no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References


Keefe D, Kumar M, Kalmbach K. Oocyte competency is the key to embryo potential. Fertil Steril 2015;103:317–322.


