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human reproduction **ORIGINAL ARTICLE** ***Reproductive biology***

Maternal age and ovarian stimulation independently affect oocyte mtDNA copy number and cumulus cell gene expression in bovine clones

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STUDY QUESTION: Does maternal ageing and ovarian stimulation alter mitochondrial DNA (mtDNA) copy number and gene expression of oocytes and cumulus cells from a novel bovine model for human IVF?

SUMMARY ANSWER: Oocytes collected from females with identical nuclear genetics show decreased mtDNA copy number and increased expression of an endoplasmic reticulum (ER) stress gene with repect to ovarian stimulation, whilst differences in the expression of genes involved in mitochondrial function, antioxidant protection and apoptosis were evident in relation to maternal ageing and the degree of ovarian stimulation in cumulus cells.

WHAT IS KNOWN ALREADY: Oocyte quality declines with advancing maternal age; however, the underlying mechanism, as well as the effects of ovarian stimulation are poorly understood. Human studies investigating these effects are often limited by differences in age and ovarian stimulation regimens within a patient cohort, as well as genetic and environmental variability.

STUDY DESIGN, SIZE, DURATION: A novel bovine cross-sectional maternal age model for human IVF was undertaken. Follicles were aspirated from young (3 years of age; $n = 7$ females) and old (10 years of age; $n = 5$ females) Holstein Freisian clones following multiple unstimulated, mild and standard ovarian stimulation cycles. These bovine cloned females were generated by the process of somatic cell nuclear transfer (SCNT) from the same founder and represent a homogeneous population with reduced genetic and environmental variability. Maternal age and ovarian stimulation effects were investigated in relation to mtDNA copy number, and the expression of 19 genes involved in mitochondrial function, antioxidant protection, oocyte–cumulus cell signalling and follicle development in both oocytes and cumulus cells.

MATERIALS, SETTING, METHODS: Young (3 years of age; $n = 7$ females) and old (10 years of age; $n = 5$ females) Holstein Freisian bovine clones were maintained as one herd. Stimulation cycles were based on the long GnRH agonist down-regulation regimen used in human fertility clinics. Follicle growth rates, numbers and diameters were monitored by ultrasonography and aspirated when the lead follicles were >14 mm in diameter. Follicle characteristics were analysed using a mixed model procedure. Quantitative PCR (qPCR) was used to determine mtDNA copy number and reverse transcriptase–qPCR (RT–qPCR) was used to measure gene expression in oocytes and cumulus cells.

MAIN RESULTS AND THE ROLE OF CHANCE: Method of ovarian stimulation ($P = 0.04$), but not maternal age ($P > 0.1$), was associated with a lower mtDNA copy number in oocytes. Neither factor affected mtDNA copy number in cumulus cells. In oocytes, maternal age had no effect on gene expression; however, ovarian stimulation in older females increased the expression of *GRP78* ($P = 0.02$), a gene involved in ER stress. In cumulus cells, increasing maternal age was associated with the higher expression of genes involved in mitochondrial maintenance (*TXN2* $P = 0.008$ and *TFAM* $P = 0.03$), whereas ovarian stimulation decreased the expression of genes involved in mitochondrial oxidative stress and apoptosis (*TXN2* $P = 0.002$, *PRDX3* $P = 0.03$ and *BAX* $P = 0.03$).

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LIMITATIONS, REASON FOR CAUTION: The low number of oocyte and cumulus cell samples collected from the unstimulated cycles limited the analysis. Fertilization and developmental potential of the oocytes was not assessed because these were used for mtDNA and gene expression quantification.

WIDER IMPLICATIONS OF THE FINDINGS: Delineation of the independent effects of maternal age and ovarian stimulation regimen on mtDNA copy number gene expression in oocytes and cumulus cells was enabled by the removal of genetic and environmental variability in this bovine model for human IVF. Therefore, these extend upon previous knowledge and findings provide relevant insights that are applicable for improving human ovarian stimulation regimens.

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Key words: oocyte ageing / ovarian stimulation / mtDNA copy number / gene expression / cumulus cells

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Introduction

The lowering of human oocyte quality with maternal ageing is associated with chromosomal aneuploidy, mitochondrial dysfunction and altered metabolic output, as well as extrinsic follicular factors, such as changes in the functions and viability of the surrounding cumulus cells (Fragouli et al., 2011; Pacella et al., 2012; Bentov and Casper, 2013; Pacella-Ince et al., 2014). In many species, including humans and cattle, the complex bi-directional communication between the oocyte and its nurturing cumulus cells regulates the health of the ovarian follicle (Gilchrist et al., 2008). The oocyte secretes paracrine factors such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) regulating proliferation, expansion and metabolism within the cumulus cells (Gilchrist et al., 2008; Sutton-McDowall et al., 2010). It has been proposed that analysis of gene expression of key genes within cumulus cells, for example, those involved in cumulus cell expansion, metabolism, steroidogenesis and signalling may be a useful non-invasive tool for assessing oocyte quality and may reflect embryo quality, pregnancy and live birth outcomes (Fragouli et al., 2013; Uyar et al., 2013). In particular, genes regulating cumulus cell expansion, including gremlin 1 (*GREM1*), versican (*VCAN*) and hyaluronic acid synthase 2 (*HAS2*), have been related to IVF outcomes. However, there is limited consensus between the findings of different studies (McKenzie et al., 2004; Cillo et al., 2007; Gebhardt et al., 2011; Wathlet et al., 2012; Ekart et al., 2013), possibly explained by the heterogeneity of human tissue, the effects of underlying maternal ageing and different ovarian stimulation regimens. For example, maternal age is known to affect genes relating to mitochondrial function, oocyte–cumulus cell signalling and antioxidants in oocytes and cumulus cells from human and bovine studies (Steuerwald et al., 2007; Grøndahl et al., 2010; McReynolds et al., 2012; Takeo et al., 2013).

The effects of ovarian stimulation regimens on oocyte quality are also poorly understood. While providing a suitable method to increase the number of oocytes available for fertilization in a cycle, ovarian stimulation regimens are likely to yield oocytes that are heterogeneous in their developmental potential, in which some are of lower quality than others within the same cohort (McNatty et al., 2010). Notably, the cAMP responsiveness of granulosa cells differs and is not overridden by exogenous hormone administration (McNatty et al., 2010). In recent years, research has been directed towards using milder ovarian stimulation in human fertility treatment, with a focus on collecting a cohort of high-quality oocytes, rather than focusing on obtaining a high number of oocytes of variable quality (van der Gaast et al., 2006; Verberg et al., 2009; Santos et al., 2010; Ji et al., 2013; Steward et al., 2014). The standard GnRH agonist ovarian stimulation regimens, commonly undertaken in fertility clinics, may also negatively impact oocyte quality, endometrial receptivity and thus lower pregnancy rates. This is supported by better pregnancy rates in thawed embryo transfer cycles (Aflatoonian et al., 2010; Shapiro et al., 2011a,b; Evans et al., 2014). Data investigating the effect of ovarian stimulation on oocyte quality are less clear. Some studies suggest that ovarian stimulation may rescue those follicles that without stimulation would be destined for atresia or alternatively, it may create a suboptimal follicular microenvironment (Ziebe et al., 2004; Baart et al., 2007; Verberg et al., 2009). Several studies have identified ovarian stimulation to modulate the expression of genes related to angiogenesis, follicle growth and signalling in human and bovine oocytes and cumulus cells (Chu et al., 2012; de los Santos et al., 2012; Dias et al., 2013). Moreover, ovarian stimulation is known to perturb the methylation of imprinted genes in human and mouse oocytes and embryos (Borghol et al., 2006; Sato et al., 2007; Market-Velker et al., 2010). Notably, the frequency of abberant methylation increases with hormone dosage, resulting in gene translation changes in the oocyte that affect imprint maintenance required during preimplantation embryo development (Market-Velker et al., 2010; Denomme et al., 2011).

Oocyte quality has also been investigated in terms of mitochondrial DNA (mtDNA) content of the mature human oocyte, as it is considered an important factor that determines the competence of the developing embryo (Reynier et al., 2001; Cummins, 2002; Santos et al., 2006). Mitochondria are the most abundant organelles in oocytes, where they account for a large fraction of the cytoplasmic volume (Dell'Aquila et al., 2009). These organelles play a role in determining oocyte quality where they are involved in providing ATP synthesis, reactive oxygen species production and calcium signalling to support chromosomal segregation, fertilization and subsequent embryonic development (Schon et al., 2000; Reynier et al., 2001; Van Blerkom, 2004; May-Panloup et al., 2007; Shoubridge and Wai, 2007; Ramalho-Santos et al., 2009). Mitochondrial DNA is strictly maternally inherited, and following fertilization, the mtDNA pool is subdivided into individual blastomeres upon successive cell divisions, due to the lack of mtDNA replication in the preimplantation embryo (Cree et al., 2008). This suggests mitochondrial number might influence preimplantation development. Indeed, cytoplasmic transfer studies, where the cytoplasm of younger oocytes was transferred to older recipient oocytes, led to improved implantation

and live birth rates, suggesting that enriching the mitochondrial pool could be one factor responsible for the rescue of poor quality oocytes (Lanzendorf et al., 1999; Barritt et al., 2001; Dale et al., 2001). Therefore, changes in mtDNA copy number and mitochondrial gene expression could be one mechanism that explains ageing in human oocytes (Grøndahl et al., 2010; Murakoshi et al., 2013). Conversely, little is known about how many mtDNA copies human cumulus cells have, and whether this is altered during the ageing process (Tsai et al., 2010).

It is hypothesized that mtDNA copy number and expression of genes relating to mitochondrial function, antioxidant protection, signalling and follicle development in both oocytes and cumulus cells will be affected by increasing age and intensity of ovarian stimulation regimen. The effect that maternal age has on the biological function of both the oocyte and cumulus cells is not fully understood, and more studies are needed to identify alterations that could reflect the lowering of oocyte quality with age. However, studying the effects of maternal age and ovarian stimulation on oocyte quality is problematic in humans, as studies are often confounded by the diversity of genetic and environmental factors. Cattle, unlike rodents, are an attractive model species when studying human reproduction, as they have a similar follicle size, are mono-ovular with the selection of a single dominant follicle and have a 9-month gestation (Adams et al., 2008). In this study, the unique use of young and old cows generated by somatic cell nuclear transfer (SCNT), maintained as one herd, reduced the genetic and environmental variability to a level that would be ethically impossible to achieve with humans. Knowledge generated from the current study will inform and aid human fertility treatment, with the potential to design stimulation regimens based on oocyte quality rather than number.

Materials and Methods

Animals and treatments

Oocytes and cumulus cells were collected from two cohorts of genetically identical bovine clones that were created by SCNT using mural granulosa donor cells from the same genetic origin (Wells et al., 1999). The young cohort consisted of seven clones that were aged 3 years old at the start of the study; which is equivalent to woman aged in their mid-20s (Malhi et al., 2005; Dias et al., 2014). The old cohort consisted of five clones that were aged 10 years old at the start of the study, equivalent to woman aged around 37 years (Malhi et al., 2005; Dias et al., 2014). All cows were non-lactating and under the same management and nutritional regimen, kept as one herd for more than 3 years at AgResearch Ltd (Hamilton, New Zealand). At the completion of the study, all females were euthanized on Day 14 of an unstimulated cycle by i.v. administration of 500 mg/ml sodium pentobarbitone (Pentobarb 500; National Vet Supplies, New Zealand). Blood (10 ml) was centrifuged at 1500*g* and the resulting buffy coat collected, snap frozen in liquid nitrogen and stored at −80°C. Liver samples (100 mg) were collected, snap frozen and stored at −80°C.

Ethical approval

This study was approved by the Ruakura Animal Ethics Committee (AEC11817).

Ovarian stimulation regimen

Each of the young ($n = 7$) and old ($n = 5$) females were subjected, as a single group, to four unstimulated, three mild and two standard ovarian stimulation cycles in that order, respectively. At least, one 21-day natural cycle occurred between every sampled cycle, to ensure no carryover effects on subsequent follicular or luteal characteristics. In total, the duration of the study was 18 months. Briefly, for the unstimulated cycles, females were synchronized by the administration of two i.m. injections of 500 μg Cloprostenol (Estro-PLAN; Provet, New Zealand) 12 days apart. Oestrus (Day 0) was identified by twice-daily monitoring, as well as the use of tail paint to identify oestrus behaviour. Ovarian dynamics were mapped and measured by ultrasonography on Days 9, 12, 15, 17 and 19 post-oestrus, to follow the growth of the dominant ovulatory follicle. On Day 19, to induce ovulation, 1500 IU hCG (Chorulon; MSD Animal Health, New Zealand) was administered i.m. and 22 h later (Day 20), ovum pick up (OPU) was undertaken. Following OPU for each unstimulated cycle, females were allowed to go through one full oestrous cycle, before being resynchronized by one i.m. injection of 500 μg of Cloprostenol on Day 7 post-oestrus of the next cycle. The timing of ovarian monitoring by ultrasonography and hCG administration were identical for all four unstimulated cycles.

After completion of the unstimulated cycle sampling, females were synchronized, as described, by administration of two i.m. injections of 500 μg Cloprostenol 12 days apart. On Day 10 post-oestrus, all follicles larger than 4 mm were ablated using 19G × 1.5″ BD Precision-Glide needles (Becton Dickinson, New Zealand) with 25 mmHg vacuum provided by an aspiration pump (Karl Storz, Germany). Between Days 13 and 18 post-oestrus, ovarian dynamics were mapped and measured by daily ultrasonography. Mild ovarian stimulation was accomplished in each female by the i.m. administration of 160 mg of FSH (Folltropin-V, Vetrapharm, 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 into twice-daily injections. The increased number of recruited follicles compared with the unstimulated cycle resulted in an increased chance of early ovulation, so on Day 18, once lead follicle diameters were >14 mm, 1500 IU hCG (Chorulon) was administered i.m. and 22 h later (Day 19), OPU undertaken. In the bovine, a follicle diameter of 12 mm and above is associated with a high chance of obtaining a mature oocyte that will establish a pregnancy. This regimen was repeated twice more with a full oestrous cycle between each sampling.

For the two standard stimulation cycles, a novel bovine regimen was developed based on the long GnRH agonist down-regulation regimen used in human fertility clinics. Local anaesthesia (2 ml 2% v/v lidocaine; Bomacaine, Bomac Laboratories Ltd, New Zealand) was administered in the ear of each female and a single GnRH agonist implant (4.7 mg Suprelorelin implant; PepTech Animal Health, Australia) inserted subdermally using a trocar applicator. Every 7 days, until Day 35 post-insertion, follicles larger than 4 mm were ablated as described. On Day 35 after implant insertion, a standard ovarian stimulation regimen was undertaken in which each female received i.m. 320 mg of FSH 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 into twice-daily injections. On each of these days, ovarian dynamics were mapped and measured by daily ultrasonography. Due to the increased number of stimulated follicles, on Day 42, once lead follicle diameters were >14 mm, 1500 IU of hCG (Chorulon; MSD Animal Health) was administered i.m. and 22 h later (Day 43), OPU undertaken. Every 7 days for the next 21 days, ovarian dynamics were monitored and any residual follicles larger than 4 mm were ablated. The standard stimulation regimen was then repeated and following OPU implants removed under local anaesthesia.

Recovery of cumulus–oocyte complexes

Individual cumulus–oocyte complexes (COC) were collected from dominant follicles using ultrasound-guided transvaginal OPU. Prior to OPU, a mild sedation was induced by i.v. administration of 2 ml 2% v/v xylazine (Rompun; Bayer Animal Health) and females received epidural anaesthesia

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(4 ml 2% v/v lidocaine; Bomacaine, Bomac Laboratories Ltd). Sampling was undertaken using a 7.5 mHz transvaginal sector probe (PieMed 200S; Pie Medical Imaging BV, The Netherlands) with a sampling wand employing a 19G × 1.5″ BD Precision-Glide needle (Becton Dickinson) attached to a 25 mmHg vacuum aspiration pump (Karl Storz, Tuttlingen, Germany). To recover the COC, each follicle was aspirated with repeated curetting and flushing with warm (39°C) modified morpholinopropanesulphonic 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 $CaCl_2$, 0.98 mM $MgCl_2$, 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)]. Recovered COC were briefly exposed to 1 mg/ml hyaluronidase (Sigma-Aldrich, Australia) for 30 s prior to manual stripping of cumulus cells. A cohort of oocytes from both young and old females were visualized on an Olympus microscope (Olympus, Tokyo, Japan) with a UV light for the presence of a polar body to verify that oocytes were at the expected MII stage of development. Individual cumulus cell masses and oocytes were snap frozen and stored at −80°C until required. Where possible, comparable numbers of samples from individual females, within each stimulation group, were randomly assigned to either the mtDNA copy number or gene expression assays.

mtDNA copy number assays

Total DNA was isolated from individual oocyte, cumulus cells, liver and blood samples using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies) according to the manufacturer's instructions and the concentration determined using a NanoDrop ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies; Thermo Scientific). mtDNA copy number was quantified by quantitative PCR (qPCR) using a SYBR green (Invitrogen, Auckland, New Zealand) assay on an ABI 7900 HT Fast Real-Time PCR system (Applied Biosystems) to target a template spanning nt2837 and nt2939 of the *MTND1* gene (accession number AF492351.1). Prior to studying individual oocyte and cumulus cell samples, three different qPCR assays directed at different regions of the mitochondrial genome were compared: ND1 (nt2837–nt2939), ND4 (nt10370–nt10510) and ND5 (nt12646–nt12782). The results obtained from each assay were tightly correlated ($r^2 > 0.99$) over a wide linear range of mtDNA concentrations. The ND1 assay was arbitrarily selected for all subsequent analyses. The DNA content of the *MTND1* gene was normalized with that of the *GAPDH* gene (target template spanning nt277 and nt358; accession number NM_001034034) in order to calculate the mtDNA copy number of each cumulus cell sample (Supplementary data, Table SI). This normalization was not necessary for oocyte samples, as mtDNA copy number was being measured in a single cell. The standard curve method was used for absolute quantification of mtDNA copy number. A target template spanning nt2363 and nt4583 of the mitochondrial genome and nt166 and nt1035 of the *GAPDH* gene was purified by gel extraction (Qiagen, Auckland, New Zealand), quantified using the NanoDrop ND-1000 UV–Vis spectrophotometer and serially diluted in order to generate standard curves for absolute quantification of mtDNA copy number in the samples.

The qPCR cycling conditions were: 50°C for 2 min, 95°C for 2 min, then 40 amplification cycles with 15 s of denaturation at 95°C, 1 min of annealing and extension at 60°C. Each qPCR reaction included 0.5 μM of each forward and reverse primer, 5 μl of SYBR Green Mix and 5 ng of DNA to a final volume of 10 μl. All reactions were performed in triplicate. For oocyte samples, the mtDNA copy number (*MTND1*) of 1 μl of sample per qPCR reaction was multiplied by the elution volume of the oocyte DNA (35 μl) to calculate the mtDNA copy number of each oocyte. Conversely, the relative mtDNA copy number (MTND1/GAPDH) of each cumulus cell mass was calculated by normalizing for the number of copies of the nuclear genome per qPCR reaction.

Measurement of gene expression by RT–qPCR in oocytes and cumulus cells

The expression profiles of 19 genes associated with mitochondrial function, antioxidant protection, signalling and follicle development (as summarized in Fig. 1) were determined in individual oocytes and cumulus cell samples collected from old and young females following unstimulated, mild and standard ovarian stimulation cycles. RNA from oocyte and cumulus samples was isolated using the RNAqueous-Micro kit (Ambion) according to the

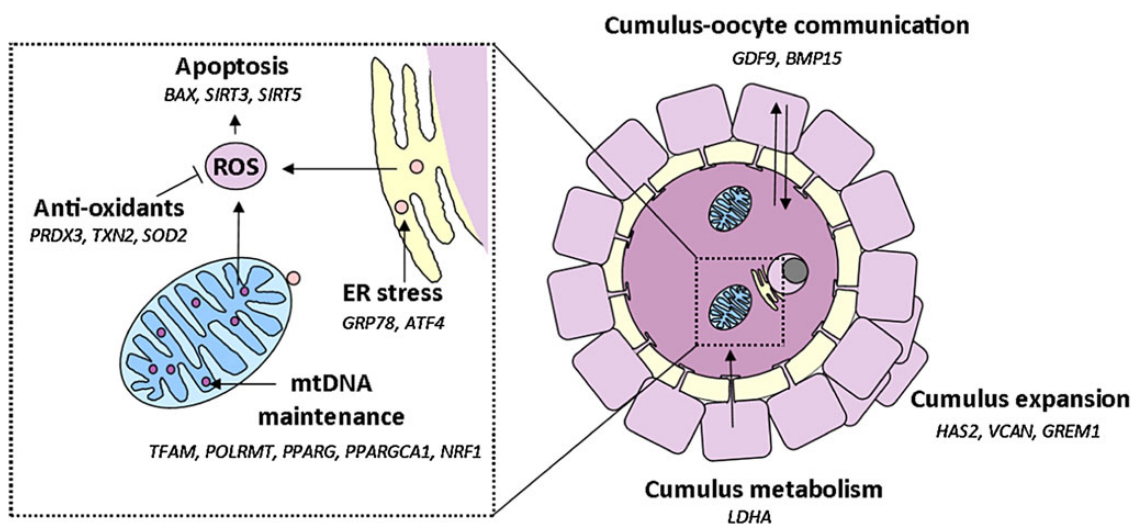

Figure 1 Summary of selected genes of interest, presented in functional categories, measured in oocytes and cumulus cells. Genes involved specifically in follicular development were selected based on their roles in cumulus–oocyte communication, cumulus expansion and cumulus metabolism. Mitochondrial and ER candidate genes were selected based on their roles in apoptosis, oxidative stress, mtDNA maintenance and ER stress. ROS, reactive oxygen species. Full gene names and accession numbers are given in Supplementary data, Table SI.

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Table I Aspirated follicle numbers and diameters (mm) from multiple unstimulated, mild or standard ovarian cycles in a cohort of young ($n = 7$) and old ($n = 5$) clones.

Parameter	Maternal age (number of follicles)			Ovarian stimulation regimen (number of follicles)			
	Young ($n = 151$)	Old ($n = 76$)	*P*-value	Unstimulated ($n = 46$)	Mild ($n = 85$)	Standard ($n = 96$)	*P*-value
Follicle number per cycle per female							
Mean (±SEM)	4.8 ± 0.63	3.3 ± 0.56	0.04	1.0 ± 0.03[a]	5.2 ± 0.55[b]	9.4 ± 0.81[c]	<0.0001
Median	3.0	2.0		1.0	4.0	10.0	
Range	1.0–16.0	1.0–12.0		1.0–2.0	2.0–12.0	4.0–16.0	
Follicle diameter per female (mm)							
Mean (±SEM)	14.5 ± 0.17	14.6 ± 0.29	>0.1	16.9 ± 0.35[a]	13.4 ± 0.16[b]	14.0 ± 0.13[b]	<0.0001
Median	14.0	14.0		16.0	13.0	14.0	
Range	12.0–22.0	12.0–22.0		12.0–22.0	12.0–16.0	12.0–16.0	

Different superscript letters denote significant ($P < 0.05$) differences between treatments.

manufacturer's instructions. Total RNA was eluted in 20 μl of elution buffer and subsequently treated with 1 U of DNase I (Ambion, Auckland, New Zealand). The quantity of RNA present in samples was too low to permit an accurate quantity assessment. For each sample, cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen) in conjunction with 3 μg of Random Primers (Invitrogen). Three rounds of cDNA synthesis were performed for each sample. These were pooled for each individual oocyte and cumulus cell mass prior to analysis. RT–qPCR was performed on an ABI 7900 HT Fast Real-Time PCR system (Applied Biosystems) and each reaction included 0.5 μM of each forward and reverse primer, 5 μl of SYBR Green Mix (Invitrogen), 3 μl of RNAse free water and 1 μl of cDNA to a final volume of 10 μl. All reactions were performed in triplicate. Primers were designed using Primer3 (Untergasser *et al.*, 2012) and a BLAST analysis was performed using the NCBI database to confirm primer specificity. The nucleotide sequences of the selected qPCR primers are provided in Supplementary data, Table SI. The RT–qPCR cycling conditions were: 50°C for 2 min, 95°C for 2 min, then 40 amplification cycles with 15 s of denaturation at 95°C, 1 min of annealing and extension at 60°C. Exclusion of the cDNA template and SuperScript III enzyme were both used as negative controls. The cycle threshold was kept the same across all samples for each assay, and the expression levels of the genes of interest were normalized to a geometric mean of the two reference genes, TATA box binding protein (*TBP*) and hypoxanthine guanine phosphoribosyl transferase (*HPRT*), using the delta CT method. All gene expression assays were performed in accordance with the MIQE guidelines (Bustin *et al.*, 2009).

Statistical analysis

Follicle characteristics (number and daily growth rate) were analysed for effects of stimulation regimen and maternal ageing in each female, with regimen replicate as a random factor included in the model. Each follicle was treated as an independent observation due to the experimental design that reduced genetic and environmental variability. All analyses were run using the Proc Mixed procedure of SAS software version 9.1 (SAS Institute, Cary, NC, USA) in which interactions between the main effects of maternal age and stimulation regimen were also examined. Follicle diameter, mtDNA copy number and normalized gene expression were compared between old and young female groups using the Mann–Whitney test. A one-way analysis of variance Kruskal–Wallis test and Dunn's multiple comparison tests were performed to detect differences in follicle diameter, mtDNA copy number and normalized gene expression between unstimulated, mild and standard stimulation regimens. mtDNA and normalized gene expression differences with a probability value of ≤ 0.05 were considered statistically significant. All data are presented as mean ± SEM, unless otherwise stated.

Results

Stimulation outcomes

In total, 96, 69 and 79% of the unstimulated, mild and standard cycles were sampled, respectively. Reasons for cycle cancellations included poor or no response to stimulation, early ovulation prior to sampling or the presence of a cystic follicle, which was independent of age. The mean COC recovery rates for the unstimulated, mild and standard cycles were 57, 56 and 66%, respectively, which was also independent of age. Thus, the overall successful COC recovery rate was 59%, with no difference between young and old female cohorts. Differences in aspirated follicle numbers and diameters with respect to maternal age and ovarian stimulation are summarized in Table I. A greater number of follicles were aspirated per female from young compared with old females ($P = 0.04$), although follicle diameter or daily growth rate did not differ with maternal age (young 1.43 ± 0.07 mm/day, old 1.44 ± 0.08 mm/day; $P > 0.1$). The mean number of aspirated follicles in the unstimulated, mild and standard cycles increased with increasing stimulation severity ($P < 0.0001$, Table I). Ovarian stimulation also resulted in the mean follicle diameter of both mild and standard stimulation regimens being smaller when compared with those of unstimulated cycles ($P < 0.0001$).

mtDNA copy number of oocytes and cumulus cells

The mtDNA copy number was measured in both oocytes ($n = 85$) and cumulus cell ($n = 88$) samples from a subset of follicles, collected from young ($n = 7$ females) and old ($n = 5$ females) cloned cows following unstimulated, mild and standard ovarian stimulation. Oocytes collected from young females following a unstimulated cycle had on average 380 000 ± 104 000 copies of mtDNA ($n = 6$ females) (Fig. 2A). Similarly, oocytes collected from the old females following a unstimulated cycle had on average 296 000 ± 55 000 copies of mtDNA ($n = 3$ females). There was no significant ($P > 0.1$) difference in mtDNA copy number

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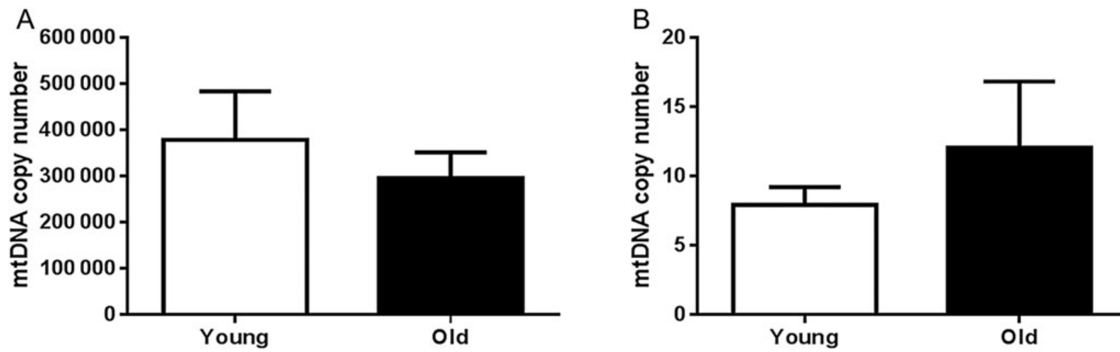

Figure 2 mtDNA copy number of oocytes (**A**) and cumulus cells (**B**) collected from unstimulated ovarian cycles in young ($n = 6$ females) and old ($n = 3$ females) clones. No differences ($P > 0.1$) were evident with respect to maternal age for mtDNA copy number of oocytes (young $n = 7$ oocytes, old $n = 5$ oocytes) or cumulus cell samples.

of oocytes between age groups within different stimulation cycles (Fig. 2 and Supplementary data, Fig. S1). Similarly, no difference ($P > 0.1$) in mtDNA copy number was evident in cumulus cells collected following a unstimulated cycle from young (8 ± 1 copies of mtDNA; $n = 6$ females) and old females (12 ± 5 copies of mtDNA; $n = 3$ females) (Fig. 2B). Data were therefore combined for analysis of stimulation effects. Blood and liver mtDNA copy number was also assessed in the cohort of old and young clones, where there was no evidence that copy number changed following ageing (Supplementary data, Fig. S2).

When data were analysed with respect to effects of ovarian stimulation, no differences ($P > 0.1$) were evident between oocyte mtDNA copy numbers of old and young female within any of the three regimens (Supplementary data, Fig. S3). Thus, oocyte data from old and young females were combined. The average mtDNA copy number was 345 000 ± 63 800 ($n = 12$) following a unstimulated ovarian cycle, decreasing to 294 000 ± 37 900 ($n = 23$) copies in a mild ovarian stimulation and 210 000 ± 12 800 ($n = 50$) in a standard ovarian stimulation ($P = 0.04$) (Fig. 3). Variation in mtDNA copy number was similar both within and between animals (coefficient of variation <20% for both), supporting a homogeneous sample set, due to the reduced genetic and environmental variability. In cumulus cells, mtDNA copy number did not change ($P > 0.1$) following ovarian stimulation. Cumulus cells from old and young females combined had 10 ± 6 ($n = 23$) copies of mtDNA following a unstimulated ovarian cycle, 11 ± 9 ($n = 20$) copies following mild ovarian stimulation and 9 ± 5 ($n = 45$) copies following standard ovarian stimulation.

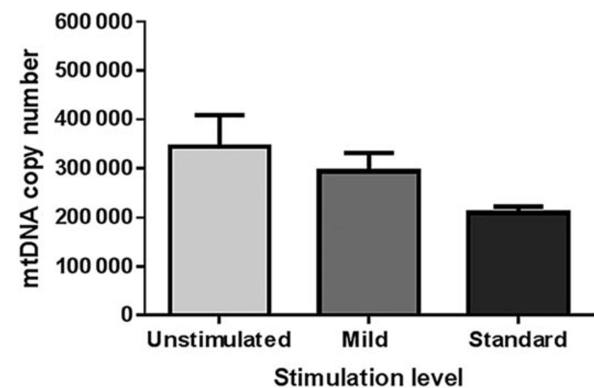

Figure 3 mtDNA copy number in oocytes collected from young ($n = 7$ females) and old ($n = 5$ females) clones subjected to multiple unstimulated, mild and standard ovarian stimulation cycles. Data were combined, due to the lack of difference with respect to maternal age ($P > 0.1$). Lower average mtDNA copy numbers were evident following increasing ovarian stimulation ($P = 0.04$). *Post hoc* analysis, while approaching significance between unstimulated and standard ovarian stimulation ($P = 0.08$), determined no differences between mild ovarian stimulation and either unstimulated or standard ovarian stimulation ($P > 0.05$).

Gene expression in individual oocytes and cumulus cell samples

The expression profiles of genes relating to mitochondrial function, antioxidant protection, signalling and follicle development were determined in both oocytes ($n = 30$) and cumulus cells ($n = 36$) collected from young ($n = 4$ females) and old ($n = 5$ females) clones following unstimulated, mild and standard ovarian stimulation. In oocytes collected from a unstimulated cycle, maternal age did not affect expression levels of any of the genes studied. However, maternal age effects were evident in cumulus cells, as greater expression of the mitochondrial antioxidant thioredoxin 2 (*TXN2*) ($P = 0.008$) and the mtDNA maintenance gene, mitochondrial transcription factor A (*TFAM*) ($P = 0.03$), was observed in old ($n = 5$ samples) compared with young clones ($n = 5$ samples) following a unstimulated cycle (Fig. 4).

When analysed with respect to ovarian stimulation, the expression of the endoplasmic reticulum (ER) stress marker 78 kDa glucose-regulated protein (*GRP78*) was higher ($P = 0.02$) in oocytes after mild ovarian stimulation compared with unstimulated cycles in old clones ($n = 5$ females) (Fig. 5).

In cumulus cells, the expression of *TXN2*, apoptotic activator BCL2-associated X protein (*BAX*) and mitochondrial antioxidant peroxiredoxin 3 (*PRDX3*) were found to differ ($P < 0.05$) between unstimulated, mild and standard ovarian stimulation in old clones ($n = 5$ females) (Fig. 6). Specifically, lower expression of *TXN2* ($P = 0.002$)

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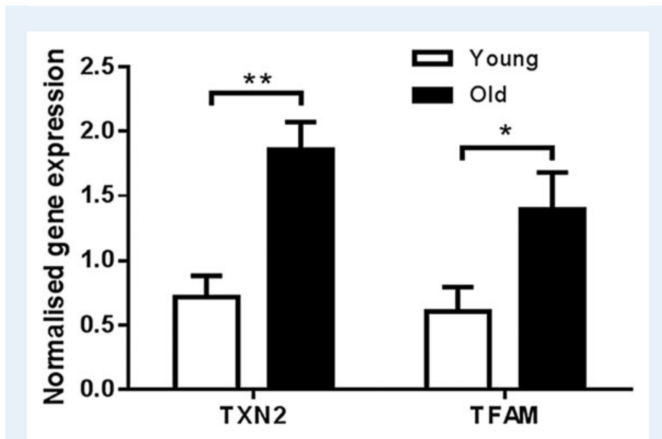

Figure 4 Normalized gene expression in cumulus cells ($n = 10$) of thioredoxin 2 (*TXN2*) and transcription factor A, mitochondrial (*TFAM*) from unstimulated ovarian cycles in young ($n = 5$ females) and old ($n = 5$ females) clones. Expression of *TXN2* ($P = 0.008$) and *TFAM* ($P = 0.03$) was higher in cumulus cells from old females compared with young females.

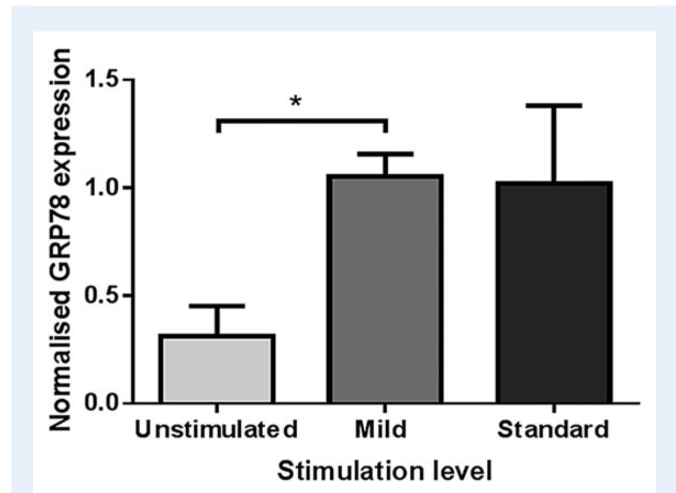

Figure 5 Normalized gene expression of glucose-regulated protein, 78 kDa (*GRP78*) in oocytes ($n = 18$) collected from old ($n = 5$ females) clones recovered in unstimulated cycles, mild and standard ovarian stimulation regimens. Overall normalized gene expression was significantly different between stimulation groups ($P = 0.004$), with a difference observed between unstimulated and mild ovarian stimulation groups ($P = 0.02$).

and *BAX* ($P = 0.03$) were identified after standard ovarian stimulation regimens compared with that in unstimulated cycles. Lower expression of *PRDX3* ($P = 0.03$) was also evident after mild ovarian stimulation regimens compared with that in unstimulated cycles.

Additional genes analysed in both oocytes and cumulus cells are shown in Fig. 1 and analysis outcomes summarized in Supplementary data, Table SII. No other genes showed a significant difference ($P < 0.05$) in normalized gene expression levels between the young and old cohort, or between unstimulated, mild and standard ovarian stimulation.

Discussion

The current study reports that oocytes collected from females subjected to ovarian stimulation with identical nuclear genetic backgrounds had decreased mtDNA copy number irrespective of age, and older females had increased expression of the ER stress marker *GRP78*. Furthermore, changes in the expression of genes involved in mitochondrial function and antioxidant protection, in response to either maternal ageing or severity of ovarian stimulation, were evident in cumulus cells. Specifically, maternal ageing was found to increase the expression of mitochondrial maintenance genes *TXN2* and *TFAM* in cumulus cells. Whereas in old clones, ovarian stimulation decreased the expression of *TXN2* and *PRDX3*, genes involved in mitochondrial antioxidant protection, as well as *BAX*, involved in apoptosis.

The present study confirmed that bovine oocytes contain around 300 000 copies of mtDNA, in line with levels in humans and mice (Barritt et al., 2002; Cree et al., 2008). This study also reports that bovine cumulus cells have on average 10 copies of mtDNA. Notably, in the present study, there was no maternal age-related effect on mtDNA copy number in oocytes, which contrasts with some previous bovine (Iwata et al., 2011), mouse (Kushnir et al., 2012) and human (Chan et al., 2005; Murakoshi et al., 2013) oocyte studies. The current study employed a unique large mammalian model that allowed the direct comparison between mtDNA copy number in oocytes and cumulus cells following a unstimulated ovarian cycle without the presence of ovarian stimulation and, therefore, represents a more physiologically relevant, controlled cohort.

Previous human and animal studies have suggested huge variability in the mtDNA copy number varying from 240 000–1 550 000 in human oocytes, even within oocytes from the same individual (Barritt et al., 2002; Cotterill et al., 2013). This variability may be due to differences in study design where mtDNA copy number was examined in immature oocytes, or mature oocytes that failed to fertilize. Interestingly, variability was also apparent in the current study, despite controlling both genetics and the environment to create a uniquely homogeneous cohort. In human studies, it is unethical to analyse the entire cohort of oocytes leading to a bias towards poorer quality oocytes. However, an advantage of the current study is that the full cohort of oocytes from a single cycle were examined, therefore representing oocytes with a range of developmental competencies, as previously demonstrated in sheep and postulated to exist in many mammalian species (McNatty et al., 2010). In addition, in the current study, data from the unstimulated cycle were used as a reference within each female. This suggests real biological variability both within and between individuals, which may be reflective of the unique microenvironment from which the follicle develops.

The present study demonstrated that mtDNA copy number is likely to be affected by ovarian stimulation in oocytes, but not cumulus cells. Irrespective of age, oocyte mtDNA copy number was lowest following standard ovarian stimulation. This finding provides evidence that oocytes collected following ovarian stimulation have fewer mtDNA copies compared with those from a unstimulated ovarian cycle, supporting the idea that oocytes from stimulated cycles are potentially metabolically compromised compared with those from unstimulated ovarian cycles. These findings have clinical relevance, as oocytes retrieved from our bovine model were generated based on human stimulation protocols, including criteria for follicle size, where women undertaking ART do not produce a single unstimulated large dominant follicle. Considering

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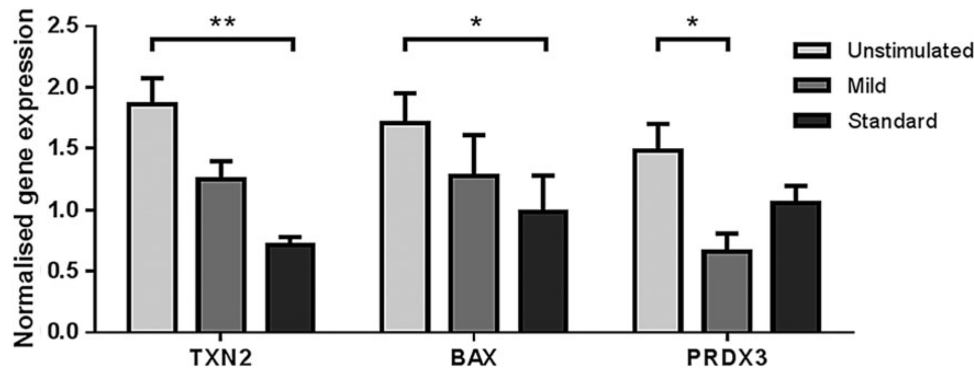

Figure 6 Normalized gene expression of thioredoxin 2 (*TXN2*), BCL2-associated X protein (*BAX*) and Peroxiredoxin 3 (*PRDX3*) in cumulus cells ($n = 17$) collected from old ($n = 5$ females) clones recovered in unstimulated, mild and standard ovarian stimulation cycles (*TXN2*, $P = 0.002$; *BAX* and *PRDX3*, $P = 0.03$).

this, it is possible that the development of oocytes from stimulated cycles may not be synchronized, with oocytes from some smaller follicles not reaching their optimal developmental potential compared with those from unstimulated cycles. Therefore, insufficient mtDNA in these oocytes may result in their inability to successfully develop post-fertilization. Alternatively, ovarian stimulation may override the selection of a single high-quality dominant oocyte and allow oocytes with a poorer capacity for mitochondrial biogenesis to mature. Examining the data within a single animal, and therefore using each animal as their own control, the current study indicated that there is a general trend towards all follicles from a stimulated cycle having fewer mtDNA copies than the single oocyte collected from the unstimulated cycle. This suggests that the stimulation itself may lower mtDNA copy number in all follicles, suppressing the development of a single dominant oocyte with high copy number. Ultimately, these results highlight mitochondrial differences between oocytes from unstimulated ovarian cycles and those from stimulated ovarian cycles, which may support the use of milder ovarian stimulation in human stimulation regimens. The functional effect that fewer mtDNA copies would have on the developing embryo is however unknown. Some studies report comprised development, as evidenced by a decrease in fertilization rates in oocytes with lower mtDNA copy number (El Shourbagy *et al.*, 2006), whereas other studies suggest that development is not compromised unless a critical threshold (50 000 mtDNA copies) is reached (Chiaratti *et al.*, 2010; Wai *et al.*, 2010).

In addition to differences in mtDNA copy number, several genes associated with mitochondrial function, antioxidant protection, signalling and follicle development were differentially expressed with maternal ageing and increasing ovarian stimulation regimen in oocytes and cumulus cells. In older females, cumulus cell expression of the mitochondrial antioxidant *TXN2* and mtDNA maintenance gene *TFAM* increased. These two novel changes could be indicative of mitochondrial dysfunction in the cumulus cells from older females or could reflect the cumulus cells working at higher capacity in response to signals from the oxidatively stressed older oocyte. The underlying mechanisms warrant further investigation. In humans, expression of mitochondrial and antioxidant genes become altered following ageing in cumulus cells (Lee *et al.*, 2010; McReynolds *et al.*, 2012; Pacella-Ince *et al.*, 2014), for example, ATP synthase and H^+ transporting, mitochondrial Fo complex, subunit E (*ATP5I*); however, data for *TXN2* and *TFAM* have not been previously reported. Following ovarian stimulation, *TXN2* and *PRDX3* expression in cumulus cells of older females was lower which suggests that the oxidative environment in the mitochondria may differ in response to the stimulatory conditions in the ovary. This is further supported by higher cholesterol and free fatty acid concentrations, key oxidative agents, in the follicular fluid of these older bovine females following stimulation (Green *et al.*, 2011). Interestingly, cumulus cell levels of the apoptotic activator *BAX* decreased following ovarian stimulation, suggesting that promotion of apoptosis may be altered. However, interpretation of these data is problematic without investigating other apoptotic factors. In relation to cumulus cell biomarkers of oocyte and embryo competence, some of the novel genes identified in this study, such as *TXN2* and *PRDX3*, warrant further investigation. In particular, evaluating their relationship to embryo development in human and animal ART studies in which a heterogeneous population is studied, in order to further understand the gene expression signature of viable embryos and how this is modulated by ovarian stimulation. It should be noted that no gene expression changes were observed in cumulus cells from younger females following ovarian stimulation. This suggests that gene expression changes caused by stimulation effects could have been exacerbated in older compared with younger females, making them more easily detected. One of the most striking findings in the older cohort is that oocyte levels of the ER stress marker, *GRP78*, dramatically increased from a unstimulated ovarian cycle to mild ovarian stimulation, suggesting that even a mild level of ovarian stimulation can promote ER stress within the older oocyte, a change which suggests that oocyte quality may be lowered following stimulation. This also warrants further investigation.

In conclusion, maternal age altered the expression of some genes involved in mitochondrial maintenance in cumulus cells. Ovarian stimulation also altered the expression of genes involved in mitochondrial maintenance in cumulus cells, as well as modulating genes involved in apoptosis. In oocytes, ovarian stimulation results in lower mtDNA copy number, as well as altering the expression of genes that are involved in ER stress. In this unique animal model, unlike previous studies, females in the form of cloned cows represented a homogeneous population with reduced genetic and environmental variability. These females were also

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used as their own controls, having multiple replicates of different protocols, suggesting that the changes observed were most likely due to differences in ovarian stimulation regimens. This study was inherently limited by a justifiably small cohort and a limited number of replicates from unstimulated ovarian cycles. In addition, the stimulations were performed sequentially from unstimulated, though mild to standard, rather than randomized in order. This was undertaken to avoid stimulation having carry-over into unstimulated cycles. This may have contributed to the contrast with other studies, suggesting that mitochondrial and signalling genes have altered expression following ageing in bovine and human oocytes (Grøndahl *et al.*, 2010; Takeo *et al.*, 2013). Additionally, more knowledge would be gained by investigating oocyte quality in terms of maturation, fertilization, embryo development and live birth outcome; however, mtDNA copy number and gene expression assays required samples to be destroyed in order to generate these data. The current study, using a novel bovine model of human ovarian stimulation with reduced variability, found that both maternal age and ovarian stimulation alter the follicular microenvironment, potentially negatively impacting oocyte quality. These results are applicable to human ovarian stimulation regimens and are consistent with the notion that follicles from standard ovarian stimulation may have delayed maturation compared with those from a unstimulated cycle, indicated by their altered mitochondrial profiles.

Supplementary data

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

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Authors' roles

L.M.C., J.C.P., A.N.S. and M.P.G. designed the study. M.P.G. and M.C.B. carried out the cycle monitoring, stimulation regimens and COC recovery in cloned cows. E.R.H. carried out mtDNA copy number assays, gene expression assays and data analysis. E.R.H. and L.M.C. drafted the manuscript. J.C.P., A.N.S. and M.P.G. critically reviewed and approved the final version of the manuscript.

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Conflict of interest

J.C.P. is a shareholder of Fertility Associates and M.P.G. received a fellowship from Fertility Associates. M.P.G. currently holds the position of Merck Serono Lecturer in Reproductive Biology. 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.

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