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Advancing maternal age predisposes to mitochondrial damage and loss during maturation of equine oocytes *in vitro*

B.P.B. Rambags [1], D.C.J. van Boxtel, T. Tharasanit [2], J.A. Lenstra, B. Colenbrander, T.A.E. Stout*

Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 112, 3584 CM Utrecht, The Netherlands

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ABSTRACT

In many mammalian species, reproductive success decreases with maternal age. One proposed contributor to this age-related decrease in fertility is a reduction in the quantity or functionality of mitochondria in oocytes. This study examined whether maternal age or (*in vitro* maturation). IVM affect the quantity of mitochondria in equine oocytes. Oocytes were collected from the ovaries of slaughtered mares categorized as young (<12 years) or aged (≥12 years) and either denuded and prepared for analysis immediately (not-IVM) or matured *in vitro* for 30 hours before preparation (IVM). The mean oocyte mitochondrial DNA copy number was estimated by quantitative polymerase chain reaction and found to be significantly lower in oocytes from aged mares and that had been subjected to IVM than in any other group. Transmission electron microscopy demonstrated that mitochondria in aged mare oocytes subjected to IVM experienced significantly more swelling and loss of cristae than in other groups. We conclude that maternal aging is associated with a heightened susceptibility to mitochondrial damage and loss in equine oocytes, which manifests during IVM. This predisposition to mitochondrial degeneration probably contributes to reduced fertility in aged mares.

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1. Introduction

In many mammalian species, there is a threshold maternal age beyond which reproductive success decreases markedly [1]. In women, fertility declines after 35 years [2], largely as a result of an increase in the incidence of spontaneous abortion [3]. The decline culminates in the cessation of fertility in most women at around 41 years, even though the menopause does not onset until approximately 51 years [4]. Similarly, the likelihood of a live birth per embryo transferred in human IVF programs decreases from 24% at maternal ages below 30 years to 8% at 42 years and 4% at 45 years or more [5]. The underlying causes of reproductive senescence have been proposed to include a decrease in the size of the resting follicle pool, a decrease in oocyte quality, and impaired endometrial receptivity. The size of the resting follicle pool certainly affects fertility in women, especially just before the menopause [6,7]. However, a reduction in oocyte number does not explain why many women are cyclic but unable to conceive in the last 10 years before the menopause [4]. Similarly, because the negative effects of advanced maternal age on IVF success can largely be overcome using an oocyte donated by a younger woman (≤35 years), impaired endometrial receptivity seems to be a relatively minor contributor to the premenopausal decline in fertility [5,8]. Instead, the primary contributor to reduced

* Corresponding author. Tel.: +31 30 2531350; fax: +31 30 2537970.
E-mail address: t.a.e.stout@uu.nl (T.A.E. Stout).
[1] Present address: uQinisa, Houten, The Netherlands.
[2] Present address: Department of Obstetrics, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

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fertility is thought to be reduced oocyte quality; previous studies have reported a relatively low expression of spindle assembly checkpoint mRNA [9] and high incidences of spindle aberrations [10] and chromosomal abnormalities [11,12] in oocytes from aged women. Similarly, oviductal transfer of oocytes from aged mares (20–26 years) produced significantly fewer embryonic vesicles (31%) in inseminated young mare recipients than transfer of oocytes from young (6–10 years) donors (92%) [13].

One postulated underlying cause of the age-dependent decrease in oocyte quality is a decline in mitochondrial function [14,15]. Mitochondria play several important metabolic roles in eukaryotic cells, including energy generation by oxidative phosphorylation, steroid production, β-oxidation, and calcium homeostasis. However, mitochondria are also implicated in processes associated with cell deterioration, such as the production of potentially harmful reactive oxygen species (ROS) [16,17]. Because mtDNA is located close to the site of ROS generation, it may be particularly at risk for accumulating oxidative damage. Moreover, mtDNA is more sensitive to mutagens than nuclear DNA because it lacks introns, protective histones [18], and DNA repair mechanisms. Indeed, the mutation rate of mtDNA is more than 10 times higher than that of nuclear DNA [18], and point mutations, deletions, and duplications have been reported to accumulate in mtDNA over time, particularly in slowly or nondividing, postmitotic tissues with high energy demands, such as brain and muscle [14,19–22]. Mammalian oocytes are also nondividing, postmitotic cells; however, their energy demands are modest because they arrest after entering meiosis and remain in a resting phase until reactivation during final follicle development before ovulation. Nevertheless, because the resumption of meiosis may occur many years later, oocytes may accumulate mitochondrial DNA damage with increasing maternal age (for review, see Eichenlaub-Ritter et al. [23]). However, studies that have examined a possible age-related decline in oocyte mtDNA quality have produced conflicting results [24–30]. Even so, it was recently reported that increasing maternal age is accompanied by a decrease in oocyte mtDNA quantity in mice [31]. In this respect, earlier studies may have been biased by using relatively small populations of oocytes obtained from aged women attending IVF clinics for fertility problems.

The aim of the present study was to determine whether maternal age significantly affects either mitochondrial number, estimated via mtDNA copy number, or electron microscopic morphological quality of equine oocytes before and after maturation *in vitro*. The mare was an attractive animal model because the horse is a monovulatory species in which fertility decreases markedly with advancing maternal age [32], oocytes can be obtained from slaughtered animals, and the time interval to reproductive senescence is more comparable with women than that of laboratory species such as mice.

2. Materials and methods

2.1. Collection and culture of cumulus oocyte complexes

Ovaries were recovered from 268 horse (*Equus caballus*) mares immediately after slaughter. Age was estimated using standard parameters for equine dental eruption and wear [33,34]. Animals for which the age could not be estimated reliably were excluded from the study. The ovaries from young (<12 years) and aged mares (≥12 years) were transported separately to the laboratory in thermos flasks at 30 °C. On arrival at the laboratory within 4 hours of slaughter, the ovaries were washed with tap water (30 °C), freed of extraneous tissue, and maintained at 30 °C in 0.9% (wt/vol) saline supplemented with 0.1% (vol/vol) penicillin and streptomycin (Gibco BRL, Life Technologies, Paisley, UK). Cumulus oocyte complexes (COCs) were then recovered by aspirating, flushing, and scraping the contents of 5- to 30-mm follicles [35]. For each mare age group, recovered oocytes were divided randomly into 2 groups. In the first group (not-IVM), 127 oocytes from young mares and 106 from aged mares were immediately denuded of their cumulus cells by vortexing for 4 minutes in calcium- and magnesium-free Earles' balanced salt solution containing 0.25% (vol/vol) trypsin–EDTA (Gibco BRL, Life Technologies). After vortexing, oocytes were examined under an inverted stereomicroscope to confirm the absence of cumulus cells; denuded oocytes were stored for further analysis, and incompletely denuded oocytes were discarded. In the second group (*in vitro* matured: IVM), 114 COCs from young mares and 154 from aged mares were matured *in vitro* in maturation medium consisting of M199 tissue culture medium supplemented with 10% FCS, 0.01 units/mL porcine FSH, and 0.01 units/mL equine LH (Sigma-Aldrich Chemicals BV) for 30 hours at 38.7 °C in a humidified atmosphere of 5% CO_2 in air [35], before denuding and storage. Next, IVM oocytes were examined with an inverted stereomicroscope to confirm that cumulus removal was complete and determine whether a first polar body was visible. IVM oocytes were then divided into those that had reached the MII stage, i.e., had a visible first polar body (MII) and those that had not (not MII). The oocytes were then washed three times in phosphate buffered saline (PBS) containing 0.1% (wt/vol) polyvinyl alcohol (Sigma-Aldrich Chemicals BV) and three times in (Tris-EDTA) TE, consisting of 10 mM Tris (MP Biomedicals Inc., Eschwege, Germany) and 0.1 mM EDTA (BDH Ltd., Poole, UK) in double-distilled water, before being placed individually in eppendorf tubes in 5 μL of TE and stored at −20 °C until analysis.

2.2. DNA extraction

Oocytes were lysed by adding 5 μL of an alkaline lysis buffer (200 mM KOH and 50 mM dithiothreitol), incubating them at 65 °C for 10 minutes and vortexing. After addition of 5 μL of neutralization buffer (0.9 M Tris–HCl, 0.3 M KCl, and 0.2 M HCl), the lysates were further diluted to a total volume of 150 μL and stored at −20 °C.

2.3. Preparation of reference samples and internal controls

Two series of reference samples were produced to ensure that values obtained in different quantitative polymerase chain reaction (QPCR) plates were comparable. First, a DNA sequence of 428 bp, spanning the fragment used for QPCR, was amplified (Table 1, #1) and purified using the Qiaquick Purification Kit (Qiagen, Venlo, The

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