Mitochondrial DNA replication is initiated at blastocyst formation in equine embryos

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Abstract

Intracytoplasmic sperm injection (ICSI) is the technique of choice for equine *in vitro* fertilization (IVF) and, in a research setting, 18-36% of injected oocytes develop to blastocysts. However, blastocyst development in clinical programs is lower, presumably due to a combination of variable oocyte quality (e.g. from old mares), suboptimal culture conditions and marginal fertility of some stallions. Furthermore, mitochondrial constitution appears to be critical to developmental competence, and both maternal ageing and *in vitro* embryo production (IVEP) negatively influence mitochondrial number and function in murine and bovine embryos. This study examined the onset of mitochondrial DNA (mtDNA) replication in equine embryos and investigated whether IVEP affects the timing of this important event, or the expression of genes required for mtDNA replication (*TFAM*, *mtPOLB* and *SSB*). We also investigated whether developmental arrest was associated with low mtDNA copy number. MtDNA copy number increased ($P < 0.01$) between the early and expanded blastocyst stages both *in vivo* and *in vitro*, while the mtDNA/total DNA ratio was higher in IVEP embryos ($P = 0.041$). Mitochondrial replication was preceded by an increase in *TFAM* but, unexpectedly, not *mtPOLB* or *SSB* expression. There was no association between embryonic arrest and lower mtDNA copy numbers.

Additional keywords: embryonic development, horse, oocyte, *in vitro* fertilization, gene expression

Introduction

Success of conventional *in vitro* fertilization (IVF) with equine gametes is poor, and the primary reason for this failure appears to be inadequate activation of equine spermatozoa to penetrate the zona pellucida *ex vivo* (Hinrichs, 2012). As for human male factor infertility, this deficit can be overcome by injecting the spermatozoon directly into the oocyte's cytoplasm (i.e. intracytoplasmic sperm injection: ICSI). To date, however, only a handful of laboratories have been able to achieve acceptable blastocyst production (18-36% of injected oocytes) after *in vitro* oocyte maturation, ICSI and *in vitro* embryo culture (Galli *et al*., 2007; Hinrichs, 2012). A combination of factors including variable oocyte quality, suboptimal culture conditions and questionable fertility of donor stallions or semen, appears to compromise the developmental potential of zygotes and embryos in a commercial setting, and contribute to sub-optimal blastocyst production (Galli *et al.*, 2007; Hinrichs, 2013). In this respect, several processes are disturbed in the follicles, and the oocytes they contain, in aged mares (Carnevale, 2008), and during oocyte maturation and the cleavage stages of early embryo development *in vitro*. These deficits can in turn influence developmental kinetics, energy and glucose metabolism, and predispose to a higher incidence of embryonic cell apoptosis, and to an altered epigenetic constitution (Badr *et al.,* 2007). One critical contributor to normal early embryo development is mitochondrial number and activity (Bentov *et al.,* 2011). Impaired mitochondrial function is thought to be a key factor in the reduced developmental competence of oocytes from older women, and can be successfully overcome by the transfer of cytoplasm containing 'healthy' mitochondria from younger donors (Barritt *et al.,* 2001).

During oocyte development, the mitochondrial DNA (mtDNA) copy number increases from tens of copies to hundreds of thousands (McConnell and Petrie, 2004; Shoubridge and Wai, 2007), this passage through the so-called 'mitochondrial bottleneck',

i.e. via a step with very few mtDNA copies, is thought to present an opportunity for filtering out mtDNA copies carrying potentially detrimental mutations (Song *et al.*, 2014). By the time of germinal vesicle breakdown and the onset of oocyte maturation, the oocyte contains a large but stable quantity of mitochondria and copies of mitochondrial DNA. A species-specific minimum number of mitochondria is thought to be required to permit normal post-fertilization development of the early embryo (Shoubridge and Wai, 2007; Wai *et al.*, 2010; Fragouli *et al.,* 2015). This is primarily because mitochondrial DNA replication is transiently arrested between fertilization and the onset of cell lineage segregation (St. John *et al.,* 2010).. Indeed, the mtDNA copy number remains constant in mouse and rat embryos until gastrulation (Thundathil *et al.,* 2005; Facucho-Oliveira *et al.,* 2007; Kameyama *et al.,* 2007; Wai *et al.,* 2010), and even decreases over time in cattle, pig and human embryos up to the time of blastocyst formation (May-Panloup *et al.,* 2007; Spikings *et al.,* 2007; Hashimoto *et al.,* 2017). Coincident with blastocyst formation in the cow and pig (May-Panloup *et al.,* 2007; Spikings *et al.,* 2007), mitochondrial DNA replication is reinitiated in trophectoderm, but not in ICM, cells (Hashimoto *et al.,* 2017). In general, the ratio of mtDNA to mitochondria is cell-type specific and, since the ratio in oocytes and early embryos is 1-2 mtDNA copies per mitochondrion (Shoubridge and Wai, 2007), mtDNA copy number is a useful indicator of mitochondrial number. The developmentally programmed arrest in mtDNA replication at the early cell cleavage stages, despite a near exponential increase in the number of cells, underlines why the number of functional mitochondria present in a mature oocyte is of critical importance for subsequent developmental competence of the embryo (May-Panloup *et al.,* 2007). It is also noteworthy that mitochondrial inheritance in mammals is entirely maternal; although the paternal mitochondria in the sperm mid-piece enter the oocyte at the time of fertilization, they are tagged with ubiquitin and degraded via a combination of the ubiquitin-proteasome system

and lysome-mediated autophagy, in a process recently termed mitophagy (Chiaratti *et al*., 2018; Song *et al*., 2014).

In vitro embryo production (IVEP) predisposes oocytes and early embryos to alterations in mitochondrial function leading to mitochondrial damage (Wilding *et al.,* 2001; Wang *et al.,* 2009) and resulting in a decrease in mtDNA copy number, a reduction in mitochondrial gene expression, and an increased production of reactive oxygen species (ROS) within embryonic cells (Giritharen *et al.,* 2007; 2010). Reduced mtDNA quantity can also result in reduced expression of genes, such as mitochondrial transcription factor *(TFAM)* (Facucho-Oliveira *et al.,* 2007), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single stranded DNA binding protein (*SSB*: St. John *et al.,* 2010), involved in the various steps of the mitochondrial replication process. Expression of these genes is normally up-regulated shortly before mtDNA replication is reactivated (Piko and Taylor, 1987; Thundathil *et al.,* 2005; May-Panloup *et al.,* 2007; Spikings *et al.,* 2007).

We previously reported that equine IVEP embryos contain lower numbers of mitochondria than their *in vivo* counterparts (Hendriks *et al.,* 2015). However, IVEP embryos develop more slowly and have lower cell numbers at the same time point than *in vivo* embryos (Tremoleda *et al.,* 2003), such that mtDNA quantity per embryonic cell did not differ significantly between *in vivo* and IVEP embryos. At present therefore, it is not clear whether the reduced mtDNA copy number in IVEP embryos is primarily a factor of retarded development, whether it reflects a reduction in the starting number of mtDNA copies, or whether it results from alterations in the timing or efficiency of mtDNA replication or the selective removal of damaged or mutated mtDNA copies by mitophagy.

The present study was performed to determine the time of onset of mitochondrial DNA replication in equine embryos, and to examine whether this and the expression of genes required for mtDNA replication (*TFAM, mtPOLB* and *SSB)* were affected by IVEP.

Materials and methods

Collection of cumulus oocyte complexes

Ovaries from 11 mares (age < 12 years) were recovered immediately after slaughter, transported to the laboratory at 30°C in a thermos flask and processed within 4 hours. Cumulus oocyte complexes (COCs) were recovered as described previously (Tharasanit *et al.*, 2005). Only oocytes with a complete multi-layered cumulus investment (Hinrichs *et al.*, 1993) were selected and maintained in HEPES buffered M199 (Gibco BRL Life Technologies, Paisley, Strathclyde, UK) supplemented with 0.014% (w/v) BSA (Sigma-Aldrich Chemicals B.V., Zwijndrecht, The Netherlands). The oocytes were immediately denuded by vortexing for 4 min in calcium and magnesium free Earle's Balanced Salt Solution (EBSS; Gibco BRL Life Technologies) containing 0.25% (v/v) trypsin-EDTA (Gibco BRL Life Technologies). The denuded oocytes were washed twice in phosphate buffered saline (PBS; Sigma-Aldrich Chemicals B.V.) containing 0.1% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich Chemicals B.V.) and placed individually in 10 µl lysis buffer (RLT + β-mercaptoethanol; Qiagen, Venlo, The Netherlands) in a 0.5 ml eppendorf tube, snap-frozen and stored at -80°C until further processing (DNA/RNA extraction).

Collection of embryos

Embryos were recovered from ten Dutch Warmblood mares (aged 4 - 12 years) on day 7 after ovulation. All animal procedures were approved by Utrecht University's Institutional Animal Care and Use Committee (DEC 2007.III.02.036). During oestrus, the reproductive organs of the mares were examined daily by transrectal palpation and ultrasonography using a MyLab Five ultrasound machine (Esaote Pie Medical, Maastricht, The Netherlands) equipped with a 7.5 MHz linear array probe. Once the dominant follicle

exceeded 35 mm in diameter, ovulation was induced by the intravenous injection of 1500 IU hCG (Chorulon®, Intervet, Boxmeer, The Netherlands) and the mare was inseminated with >500 million motile sperm from a single fertile stallion. Thereafter, mares were examined daily until ovulation was detected by the evacuation of the pre-ovulatory follicle. Seven days after ovulation, embryos (n = 21) were collected by non-surgical uterine lavage using 3 x 1 L pre-warmed (37°C) Lactated Ringer's solution (LRS: Baxter, Lessines, Belgium) supplemented with 0.5% fetal calf serum (FCS: Greiner Bio-One, Alphen aan den Rijn, The Netherlands). After recovery, embryos were washed 10 times with LRS to remove maternal cells and residual FCS prior to assessment using a dissecting microscope (SZ60: Olympus, Zoeterwoude, The Netherlands). The embryos were classified by developmental stage (morula, early blastocyst or expanded blastocyst) and quality (1-4: good to degenerate) as described previously (Tremoleda *et al.*, 2003), and their diameter was measured using a calibrated eye-piece micrometer. Only grade 1 - 2 embryos were used further, and these were transferred with 10 µl LRS into 0.5ml eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C until DNA/RNA extraction.

In vitro embryos were produced as described by Galli *et al.* (2001), with minor modifications. Briefly, COCs recovered from the ovaries of slaughtered mares of mixed breed and unknown age were matured *in vitro* (IVM) by incubating for 22-24h in a 50:50 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 supplemented with 10% serum replacement (SR: Gibco BRL Life Technologies), ITS supplement (1.0 mg/ml insulin, 0.55 mg/ml transferrin, 0.5 µg/ml sodium selenite: Sigma-Aldrich Chemicals B.V.), 1 mM sodium pyruvate, 50 ng/ml EGF (Sigma-Aldrich Chemicals B.V.), and 0.1 IU LH and FSH (Pergovet, Serono, Italy), at 38.5ºC in a humidified atmosphere of 5% CO2-in-air. Next, oocytes were denuded by incubation for 5 min in HEPES-buffered SOF containing 2.5 µg/ml hyaluronidase followed by repeated pipetting, and then returned

to maturation medium for a further 2 - 4h (i.e. up to 26 - 28 h total duration of IVM). Oocytes with an extruded first polar body were then fertilised by intracytoplasmic sperm injection (ICSI) using frozen-thawed sperm from a single stallion known to yield a high blastocyst rate after ICSI; sperm for ICSI were selected by Redigrad (Amersham Biosciences AB, Uppsala, Sweden) density gradient centrifugation. Sperm injection was performed using a Piezo driven unit (PrimeTech, Japan) fixed to a micromanipulator equipped with a 37°C heated stage. Oocytes were held with the polar body orientated to the 6- or 12-o'clock position using a holding pipette with an inner diameter of 50 µm and an outer diameter of 150 µm, and injected with a blunt-ended needle with an inner diameter of 5 µm. Only motile sperm were selected for ICSI, and immobilized by two or three Piezo pulses before injection. After immobilization, the spermatozoa was aspirated tail-first into the injection pipette and the Piezo drill was used to first remove a 'plug of zona pellucida', and then to penetrate the oolemma to allow injection of the sperm into the ooplasm. The resulting presumptive zygotes were cultured at 38.5ºC in an humidified atmosphere containing 5%CO2 and 6%O2 in modified synthetic oviductal fluid (mSOF; Tervit *et al.,* 1972) supplemented with MEM essential and non-essential amino acids, glutamine and BSA, and examined for cleavage at day 2 post injection; those showing development to the 2-cell stage or further were cultured *in vitro* for an additional 6 days, replacing half of the medium at days 4 and 6 (Galli *et al*., 2007). At day 7 or 8, culture was stopped when embryos reached the morula, early blastocyst or expanded blastocyst stage.

IVP embryos (n = 32) that appeared to be developing normally were also harvested at various time-points during culture: 48 h (2 days), 96 h (4 days), 144 h (6 days) and 192 h (8 days) after ICSI (8 embryos per group). In addition, 15 embryos showing arrested development were collected at the same time points; not cleaved (n=5) on day 1, blocked at 2-cell stage (n=5) on day 2, blocked at 8-cell stage (n=2) on day 3 and blocked at >16-cell

stage (n=3) on day 4 after ICSI. All embryos were washed twice in phosphate buffered saline (PBS) containing 0.1% polyvinyl alcohol (PVA; Sigma-Aldrich Chemicals B.V.), and transferred individually to DNase-RNase free tubes in RLT buffer (Qiagen), snap-frozen in liquid nitrogen and stored at -80°C.

DNA and RNA extraction, and cDNA synthesis

Total RNA and DNA (tDNA) were extracted from individual oocytes and individual embryos in 350 or 600 µL RLT buffer respectively using an AllPrep DNA/RNA/Protein Mini Kit (Qiagen) and subjected to on-column DNase I digestion using an RNase-Free DNase Set (Qiagen) as described by Paris *et al.* (2011). tDNA was eluted in 50 µL elution buffer (Qiagen) and RNA was eluted in 35 µL RNase-free water (Qiagen). The RNA from individual oocytes or embryos was then transcribed into cDNA. For conventional polymerase chain reaction (PCR), the total reaction volume was 25 µL and contained 1 µL cDNA, 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs (Promega), 0.5 µM forward primer, 0.5 µM reverse primer and 0.625 U HotStarTaq DNA polymerase (all Qiagen). PCR cycle conditions consisted of 15 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at the primer-specific annealing temperature (Hendriks *et al.* 2015) and 1 min at 72°C, with a final extension for 10 min at 72°C. Products were visualised on 1% agarose gels. If suitable for PCR amplification, and free of genomic (g) DNA contamination, cDNA samples with and without reverse transcriptase were diluted 10-fold and frozen at −20°C before quantitative reverse transcription–polymerase chain reaction (RT-PCR).

DNA quantification

Cell number was estimated by quantifying tDNA using a Quant-iT PicoGreen dsDNA assay kit (Molecular Probes). A standard curve ranging from 25 ng mL^{-1} to 2.5 pg mL^{-1}

was created via a 1 : 10 dilution series. To quantify tDNA, 50 µL Pico Green reagent was mixed with 9.95 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and 40 µL was added to 20 µL sample (standard curve or embryo extract) per well in a FLUOTRAC 600 96-well microplate (Greiner Bio-One). During preparation and plate set-up, the materials were protected from the light. Fluorescence was measured using a multimode detector (Beckman Coulter DTX 880) and Fluorescein Top Read software for Anthos Multimode detectors (Anthos Microsystem).

Quantitative RT-PCR

Primers for the genes *TFAM, mtPOLB* and *SSB* were optimized and tested for specificity using an iQ5 RT-PCR Detection System with iQ5 Optical System Software v2.0 (BioRad, Hercules, CA, USA; Table 1); equine kidney was used as the positive control tissue.

Table 1. Primer details used for gene amplification in quantitative reverse transcription–polymerase chain reaction

T_A, annealing temperature

Symbol	Gene name	GenBank accession no.	Primer sequence	Product size (
PGK1	Phosphoglycerate kinase 1	XM_001502668	Forward: 5′-CAAGAAGTATGCTGAGGCTG-3′ Reverse: 5′-AGGACTTTACCTTCCAGGAG-3′	260
RPL4	60S ribosomal protein L4-like	XM_001497094	Forward: 5′-CATCCCTGGAATTACTCTGC-3′ Reverse: 5′-CGGCTAAGGTCTGTATTGAG-3′	203
TFAM	Transcription factor A, mitochondrial	NM_001034016	Forward: 5′-GGCAGGTATACAAGGAAGAG-3′ Reverse: 5′-GTTATAAGCTGAGCGAGGTC-3′	170
mtPOLB	DNA polymerase subunit gamma-2, mitochondrial	NM_015810	Forward: 5′-CCGAGTAAGGAACAGCTAGT-3′ Reverse: 5′-ACTCCAATCTGAGCAAGACC-3′	155
SSB	Mitochondrial single-stranded DNA binding protein	XM_003364867	Forward: 5′-CATGAGACAGGTGGAAGGAA-3′ Reverse: 5′-GATATGCCACATCTCTGAGG-3′	167
mtDNA	Mitochondrial DNA	NC_001640.1	Forward: 5′-CATGATGAAACTTCGGCTCC-3′ Reverse: 5′-TGAGTGACGGATGAGAAGGCAG-3′	118

Quantitative real-time PCR was performed separately for oocytes and embryos using the same equipment, settings and software as described by Paris *et al.* (2011). The total reaction volume was 25 µL per well, and included 1× iQ5 SYBR Green Supermix (BioRad), 0.5 µM forward primer and 0.5 µM reverse primer (Ocimum Biosolutions). Each well included 10 µL standard or sample, and the following were included in the final plates: (1) standard curve, consisting of a five-fold dilution series ranging from 100 fg to

6.4 ag ($n = 7$; fresh or frozen); (2) positive control tissue ($n = 1$; kidney); (3) 10-fold diluted sample cDNA (*in vivo*: $n = 5$ morulae, $n = 8$ early blastocysts, $n = 8$ expanded blastocysts; IVEP: $n = 8$ morulae, $n = 9$ early blastocysts, $n = 9$ expanded blastocysts) or 10-fold diluted sample cDNA (normal: $n = 8$ oocytes, $n = 8$ cleaved embryos 2 days after ICSI, $n = 8$ embryos 4 days after ICSI, $n = 8$ embryos 6 days after ICSI, $n = 8$ embryos 8 days after ICSI; arrested: $n = 5$ uncleaved embryos, $n = 5$ embryos blocked at the 2-cell stage, $n = 2$ embryos blocked at the 8-cell stage, $n = 3$ blocked at the >16-cell stage); (4) 10-fold diluted sample without reverse transcriptase (–RT); and (5) DNase- and RNase-free water (Invitrogen) as a no-template control.

All samples except for the –RT samples were run in duplicate. Both validated frozen and freshly -prepared standards were included on each plate. The –RT samples for the embryos were run on a separate plate, in a subsequent run on the same day using identical standards. PCR cycle conditions consisted of 4.5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at the optimal annealing temperature (T_A; Table 1) and 30 s at 72°C, during which the fluorescence was acquired, followed by a melting curve protocol consisting of 1 min at 95°C, 1 min at optimal T_A, then 10 s at optimal T_A increasing to 95°C by 0.5°C per cycle, during which a second round of fluorescence was acquired. Baseline and threshold (~100 relative fluorescence units (RFU)) were manually adjusted and samples with non-uniform or failed amplification, primer dimers or with amplified products in the corresponding –RT sample were excluded from further analysis for all genes (only three samples in total of all reactions for gene expression and mtDNA quantification). Raw gene expression data from single extracted oocytes were set at 1, and the results of the other groups were expressed relative to these values. Relative gene expression was calculated using iQ5 Optical Software v2.0 (BioRad).

For mtDNA quantification, DNA was used from individual oocytes and individual embryos. The same protocol was used as for cDNA quantification, and all samples were amplified on one plate using the same plate setup as above. Further calculations were based on the fact that the PCR product length for mtDNA is 118 base pairs; such that 1 mtDNA copy weighs 1.21×10^{-4} fg. Therefore, mtDNA copy number could be calculated by dividing the starting quantity (i.e. absolute quantity in fg measured during the amplification process) by 1.21×10^{-4}.

Statistical analysis

Data were analysed using SPSS 16.0 for Windows (SPSS Inc.). For gene expression, the effects of IVEP, stage of development and effect of developmental arrest were analysed using one-way analysis of variance (ANOVA), followed by a post hoc Bonferroni test. Data for mtDNA (mtDNA copy number, tDNA quantity and mtDNA : tDNA ratio) were $\log_{10}$ transformed before analysis to achieve equivalence of variance. After transformation, the data was analysed using univariate analysis. For gene expression comparisons, oocyte values were set as 1. The dataset was $\log_{10}$ transformed before analysis, followed by one-way ANOVA and post hoc Bonferroni testing. Differences were considered statistically significant if $P < 0.05$ (two-tailed). Data are given as the mean ± s.e.m.

In several instances, mRNA was below the detection limit in extracts from individual oocytes or IVP embryos. For statistical analysis, these data were subjected to 'left-censoring' (i.e. observations below the detection limit were replaced by a value just below the detection limit, rather than being recorded as 0, and were treated as left censored; Klein and Moeschberger 2003). The Akaike information criterion (AIC) was used to select the appropriate model for statistical analysis. According to the AIC, this

approach gave the best fit for the following distributions: Weibull, normal, log-normal, logistic, log-logistic, extreme value, Raleigh and the t-distribution.

Results

Mitochondrial quantity in normal in vivo and in vitro produced embryos

The number of mtDNA copies increased significantly between the early and expanded blastocyst stages in both *in vivo* and IVP embryos ($P < 0.01$; Fig. 1*a*). The quantity of tDNA (an indicator of cell number) did not differ significantly between the morula and early blastocyst stages; however, there was a marked increase in tDNA in *in vivo* but not *in vitro* expanded blastocysts ($P < 0.01$; Fig. 1*b*). When tDNA was used to correct for cell number, the mtDNA/tDNA ratio increased significantly between the morula and early blastocyst stages in both the *in vivo* and IVP embryos ($P = 0.03$; Fig. 1*c*). Despite the wide variation in the mtDNA/tDNA ratio, especially among IVP embryos (ranging from 7.7×10^{-6} to 1.4×10^{-4} in the expanded IVP blastocysts and from 1.6×10^{-5} to 1.4×10^{-4} in *in vivo* expanded blastocysts), there was a significant overall difference between embryo production methods; the mtDNA/tDNA ratio was higher in IVP than *in vivo* embryos ($P = 0.041$).

To further examine changes in mtDNA quantity during early embryo development *in vitro*, mtDNA copy numbers were compared for immature oocytes and developing zygotes or embryos 2, 4, 6 and 8 days after ICSI. There was an increase in mtDNA copy number from $0.3 \pm 0.1 \times 10^6$ in oocytes to $28.8 \pm 16.3 \times 10^6$ in normally developing embryos on Day 8 of culture (Fig. 2*a*). None of the embryos on Day 2 after ICSI contained more than 1×10^6 mtDNA copies. In contrast, on Day 4 after ICSI, six embryos contained more than 1×10^6 mtDNA copies and on Day 8 after ICSI four had more than 10×10^6 mtDNA copies. The mean tDNA quantity appeared to increase in embryos on Day 8 after

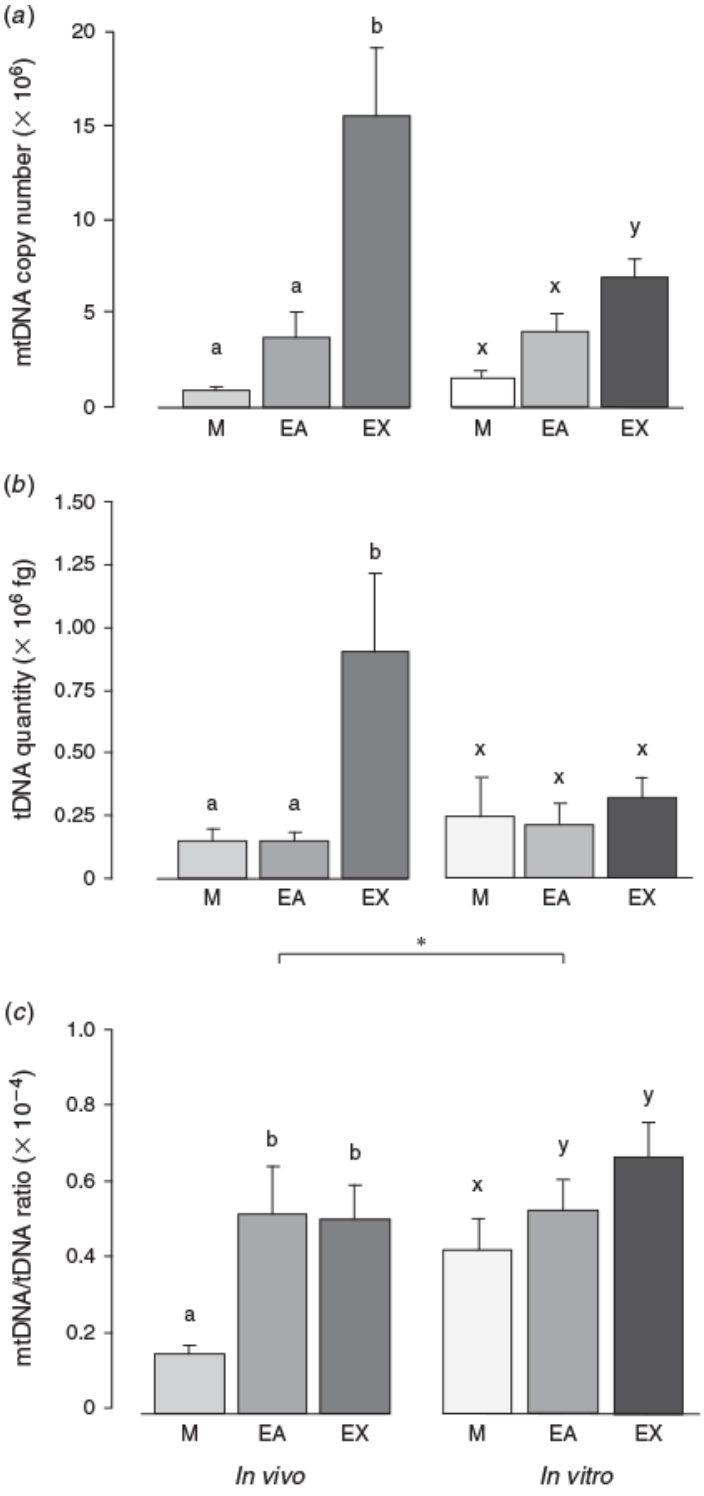

Fig. 1. (*a*) Mitochondrial (mt) DNA copy number, (*b*) quantity of total (t) DNA per embryo and (*c*) mtDNA/tDNA ratio in *in vivo* (i.e. flushed) horse embryos at the morula (M; $n = 5$), early blastocyst (EA; $n = 8$) and expanded blastocyst (EX; $n = 8$) stages, as well as *in vitro*-produced embryos (M, $n = 8$; EA, $n = 9$; EX, $n = 9$). Values are shown as the mean ± s.e.m. Within a production method (i.e. *in vivo* or *in vitro*), different letters above columns indicate significant differences ($P < 0.05$). The asterisk indicates significant differences ($P < 0.05$) between *in vivo* and *in vitro* overall.

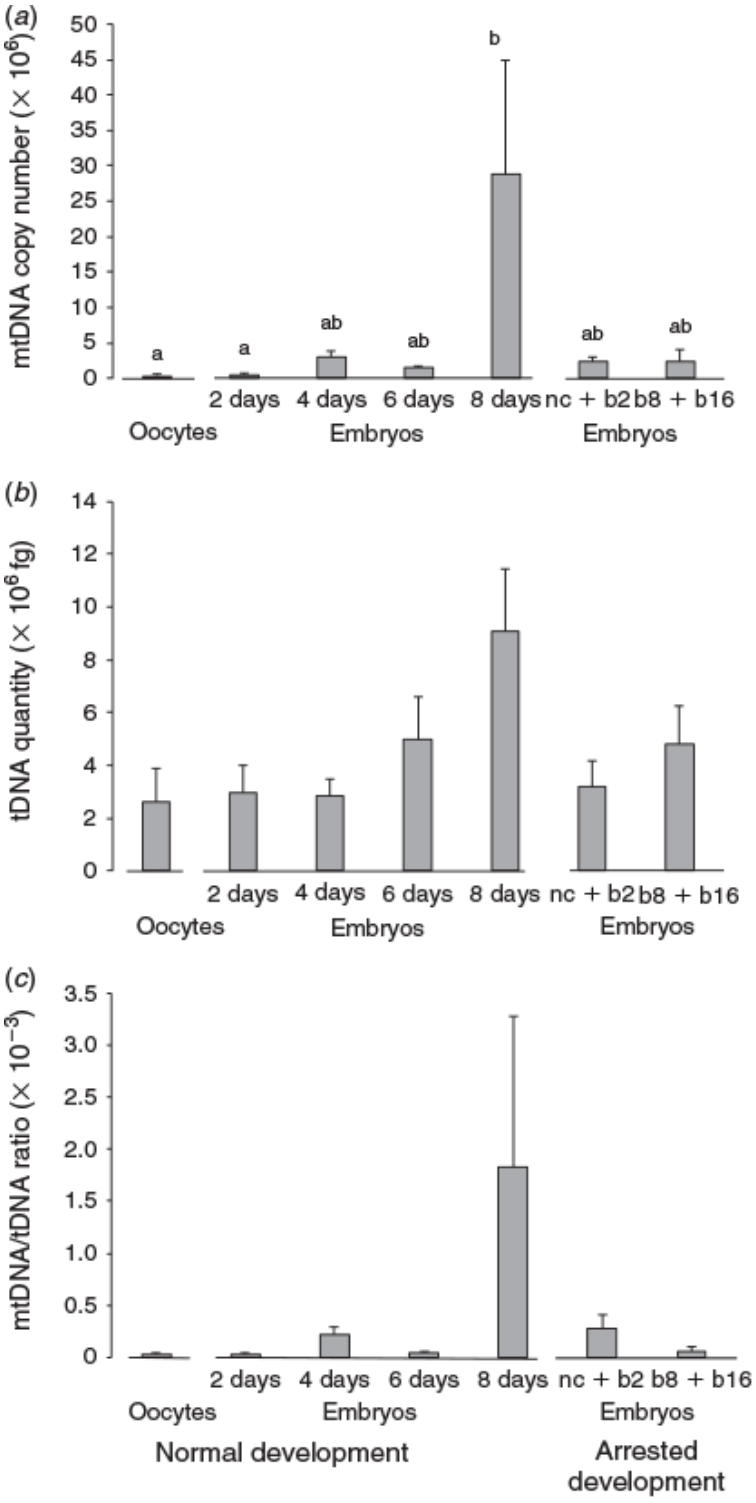

Fig. 2. (*a*) Mitochondrial (mt) DNA copy number, (*b*) total (t) DNA quantity per oocyte or embryo and (*c*) mtDNA/tDNA ratio per oocyte ($n = 8$) and in normally developing *in vitro*-produced (IVP) horse embryos (2, 4, 6 and 8 days after intracytoplasmic sperm injection; $n = 8$ per group; left panels) and arrested IVP embryos (uncleaved (nc), $n = 5$; blocked at the 2-cell stage (b2), $n = 5$; blocked at the 8-cell stage (b8), $n = 2$; blocked at the >16-cell stage (b16), $n = 3$; right panels). Values are shown as the mean ± s.e.m. Different letters above columns indicate significant differences ($P < 0.05$).

ICSI (Fig. 2*b*), as did the mtDNA/tDNA ratio (from $0.03 \pm 0.01 \times 10^{-3}$ in oocytes to $1.84 \pm 1.44 \times 10^{-3}$ in embryos on Day 8 after ICSI); however, there was a high degree of interembryo variation (ranging from 0.11×10^{-4} to 0.12×10^{-1} in embryos 8 days after ICSI; Fig. 2*c*), such that a significant effect of embryo developmental stage was not detected ($P = 0.266$).

Expression of genes involved in mitochondrial replication

Expression of two potential (reference) genes for normalizing gene expression between oocytes and embryos, *PGK1* (phosphoglycerate kinase 1) and *RPL4* (ribosomal protein L4), was evaluated in oocytes and in the *in vivo* and IVEP embryos described above. All –RT cDNA samples were free of gDNA contamination. Evaluation of the stability of gene expression for *PGK1* and *RPL4* (stably expressed in *in vivo* embryos: Paris *et al.*, 2011), across the single oocytes and IVEP embryos using geNorm v3.5 indicated that both *RPL4* and *PGK1* were suitable for use as reference genes (M values 1.44; 1.35). Since the combination of *RPL4* and *PGK1* did not lower the M value and some results for *PGK1* were close to the limit of detection, *RPL4* alone was used for normalization of gene expression values.

The normalised relative gene expression for *TFAM* increased significantly between fertilization and day 4 after ICSI, with a 16 fold increase compared to oocytes, but subsequently tended to decrease from day 4 to day 8 (Fig. 3A). *mtPOLB* expression was high in oocytes and IVEP embryos during the first 2 days of culture, but decreased to baseline levels thereafter (Fig. 3B). *SSB* expression had increased significantly by day 2 after ICSI, but thereafter decreased to levels similar to those seen in oocytes (Fig. 3C).

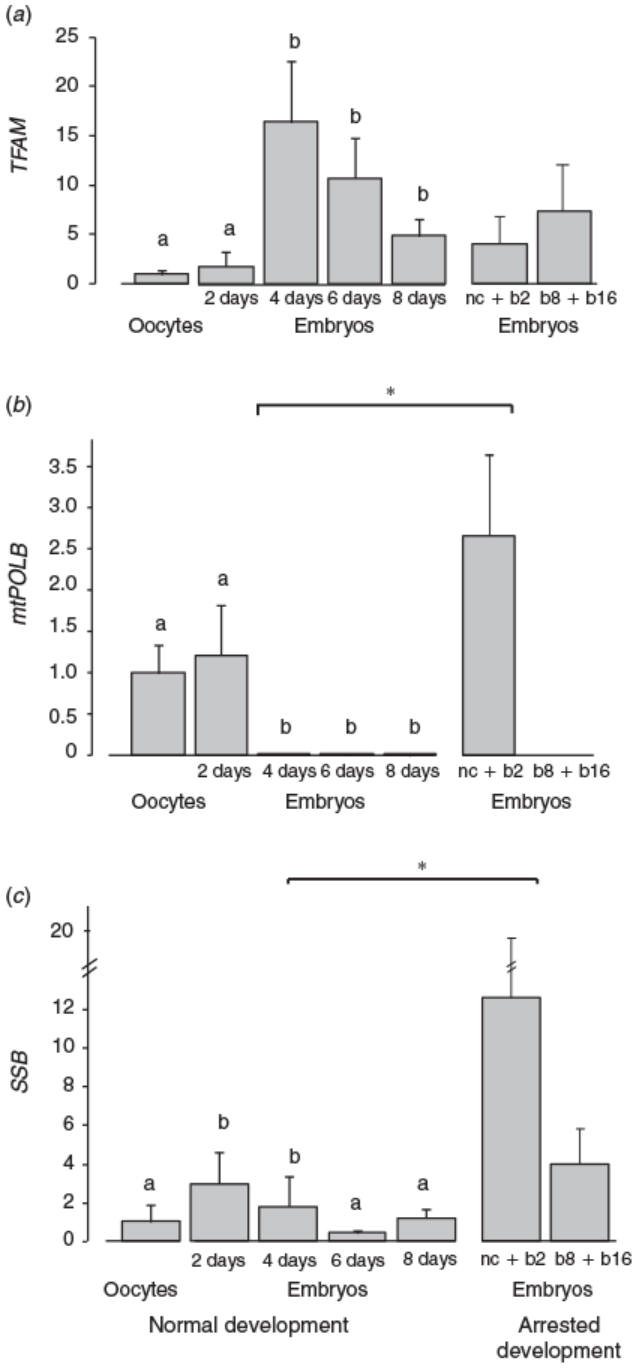

Fig. 3. Normalised relative gene expression (mean ± s.e.m.) for genes involved in mitochondrial replication, namely (*a*) mitochondrial transcription factor (*TFAM*), (*b*) mtDNA polymerase γ subunit B (*mtPOLB*) and (*c*) single-stranded DNA binding protein (*SSB*), in equine oocytes ($n = 8$) and normally developing *in vitro*-produced (IVP) horse embryos (2, 4, 6 and 8 days after intracytoplasmic sperm injection; $n = 8$ in each group; left panels) and arrested IVP embryos (uncleaved (nc; $n = 5$) + blocked at the 2-cell stage (b2; $n = 5$); blocked at the 8-cell stage (b8; $n = 2$) + blocked at the >16-cell stage (b16; $n = 3$); right panels). Within the 'normal development' group, different letters above columns indicate significant differences ($P < 0.05$). The groups marked with asterisks differ significantly (*SSB*, $P = 0.051$; *mtPOLB*, $P = 0.045$).

mtDNA quantity and gene expression in arrested in vitro produced embryos

To study a possible contribution of mitochondrial quantity and replication to failure of *in vitro* development in arrested embryos: uncleaved zygotes (n=5) and embryos blocked at the 2-cell (n=5), 8-cell (n=2) and ≥16-cell (n=3) stages were evaluated. Due to the low numbers of arrested embryos collected, the groups 'uncleaved' and 'blocked at 2-cell stage' were combined for statistical analysis, as were the groups 'blocked at 8 cell stage' and 'blocked at ≥16-cell stage'.

Surprisingly, mitochondrial copy number in zygotes that failed to cleave or arrested at the 2-cell stage tended to be higher ($2.4 \pm 0.6 \times 10^6$), rather than the expected lower, compared to oocytes ($0.3 \pm 0.1 \times 10^6$) and developing embryos at day 2 after ICSI ($0.45 \pm 0.1 \times 10^6$; Fig. 2A). Similarly, mtDNA/tDNA ratio in uncleaved zygotes and embryos blocked at the 2-cell stage was not lower than in oocytes or apparently normal embryos 2 days after ICSI (Fig. 2C).

Gene expression data from the groups 'uncleaved' and 'blocked at 2-cell stage' were combined for statistical analysis as were data from the groups 'blocked at 8-cell stage' and blocked at ≥16-cell stage', because the amplification products of some of these samples were undetectable. Overall, IVEP embryos that arrested during development showed, or tended to show, higher normalized relative gene expression than normal IVP embryos for *mtPOLB* ($P = 0.045$) and *SSB* ($P = 0.051$) respectively (Fig. 3B and C).

Discussion

This study demonstrated that mtDNA replication in equine embryos is initiated at the time of blastocyst formation and expansion. This was preceded by an increase in gene expression for *TFAM* at day four after fertilization but, unexpectedly, not by an increase in expression of mtPOLB or SSB. That mtDNA replication onsets at the time of blastocyst

formation was demonstrated by relatively stable mtDNA copy numbers up to this point, followed by a marked increase in both mtDNA copy number and the mtDNA/tDNA ratio immediately after blastocyst formation in both *in vivo* and IVEP embryos. This indicates a similar time of onset of mtDNA replication, and/or the time at which mtDNA replication outpaces any underlying mtDNA degradation, to that reported previously in porcine and bovine embryos. In the latter species, an initial increase in mtDNA copy number at the time of blastocyst formation was followed by a near exponential increase during expansion and hatching (Facucho-Oliviera *et al*., 2007; May-Panloup *et al*., 2007; Spikings *et al.,* 2007). In the current study, mtDNA copy number was similar in *in vivo* and IVEP embryos at the morula and early blastocyst stages, and it was only following blastocyst expansion that *in vivo* embryos diverged, with a much more rapid increase in both absolute mtDNA copy number and in tDNA quantity (i.e. cell number). This suggests that the lower mtDNA copy number in IVEP, compared to *in vivo*, blastocysts on day 7-8 after fertilization is primarily a function of the more rapid increase in cell number (predominantly trophectoderm cells) that the latter undergo at the time of blastocyst expansion (Tremoleda *et al.*, 2003; Rambags *et al.,* 2005). That is, the difference does not seem to be due to an initial deficit in mtDNA copy number, e.g. as a result of increased mtDNA degradation (Rambags *et al*., 2014) during *in vitro* oocyte maturation or reduced replication because of lower expression of components of the mtDNA replication machinery (Hendriks *et al*., 2015). However, this finding may in part depend on the status of the oocytes used for IVEP (e.g. age of the mare from which the oocyte was recovered), since Rambags *et al.* (2014) reported a drop in mtDNA copy number during *in vitro* oocyte maturation in oocytes from old (>12 years) but not young mares; in the current study the age of the oocyte donors was not known.

We monitored mtDNA copy number throughout early equine embryo development *in vitro*, and found the mean values to be relatively stable up to the time of blastocyst formation, despite considerable variability between individual oocytes and embryos, as reported in other species (May-Panloup *et al.,* 2007; Hashimoto et al., 2017). The major increase in mtDNA copy number was observed between days 6 and 8 after ICSI, coincident with the onset of blastocyst formation and formation of the trophectoderm cell lineage. We also attempted to examine the effect of cell number by measuring total DNA (tDNA) levels; however, the enormous number of mtDNA copies in equine oocytes (mean exceeds 10^6; Rambags *et al*., 2014) and early embryos means that mtDNA accounts for a large proportion of the total DNA (May-Panloup *et al.,* 2007), and in part explains why tDNA quantity in IVP embryos did not increase significantly until relatively late (6-8 days after ICSI) even though cell number should double on a daily basis (Grondahl and Hyttel, 1996).

One unexpected observation during the current study was that failure of oocytes to cleave and embark on embryo development following fertilization by ICSI was not associated with a reduced mtDNA copy number, as had previously been reported for human (Reynier *et al*., 2001) and porcine oocytes (El Shourbagy *et al.,* 2006). The reasons for this discrepancy are not clear but may relate to reports that, while mtDNA copy number is relatively constant between oocyte maturation and blastocyst formation, this stability masks a single mtDNA replication event that takes place in the period between fertilization and the first cleavage division (McConnel and Petrie, 2004). McConnel and Petrie (2004) further suggested that environmental conditions that disturb the normal regulation of this replication event, which they mimicked by including homocysteine (a toxic non-protein amino acid that alters the dynamics of mitochondrial turnover, probably by interfering with post-transcriptional events) in the medium, could result in an increase in mtDNA copy

number (i.e. synthesis outpacing degradation) during this window of replication.The biological function of this early zygotic mtDNA replication event has been proposed to relate to an additional opportunity to remove paternal and/or defective mtDNA copies by mitophagy ("kidnapping" of mitochondria by autophagosomes and subsequent degradation in lysosomes, to prevent mitochondrial heteroplasmy; Chiaratti *et al*., 2018). Mitochondria are maternally inherited and while paternally derived mitochondria enter the oocyte in the sperm mid-piece during fertilization, they are immediately tagged with ubiquitin ready for removal during the early cleavage divisions. Mitophagy is also involved in mitochondrial quality control and turnover, and disturbance in this process can lead to an undesirably high mitochondrial numbers, suppress embryonic development and increase the risk of the zygote harbouring mitochondria with defective mtDNA that could give rise to mitochondrial diseases (Song *et al*., 2014; Chiaratti *et al*., 2018). In short, in adverse environmental conditions may present an opportunity to untowardly affect zygote mtDNA copy number. While speculative, possibility that the normal process of mitophagy and an early mtDNA replication event is disturbed by sub-optimal *in vitro* conditions for supporting immediate post-fertilization equine embryo development could explain the unexpectedly high mtDNA copy numbers (and mtDNA:tDNA ratio) observed in equine zygotes that either failed to cleave or arrested at the 2-cell stage. The potential for an early mtDNA replication event is supported by the presence of abundant mRNA transcripts for *TFAM, mtPOLB* and *SSB*, important components of the mtDNA replication machinery, in oocytes and 2-cell stage embryos.

In several species including the mouse, cow and pig, the onset of mitochondrial replication in the early embryo is preceded by up-regulation of genes for components of the mitochondrial replication machinery, such as *TFAM*, mt*POLB* and *SSB* (Wang *et al.*, 2009; St John *et al.*, 2010; Cagnone *et al.*, 2016), at some point between embryonic

genome activation and compaction of the developing morula. In the current study, *TFAM* increased at day 4 of *in vitro* embryo culture, corresponding to the expected time of equine embryonic genome activation (Grondahl and Hyttel, 1996), and preceding the increase in mtDNA copy number. Thereafter, *TFAM* expression tended to decrease in a fashion similar to that described in porcine embryos (Spikings *et al*., 2007). What was less expected was the relatively high *mtPOLB* and *SSB* expression present in equine oocytes and zygotes 2 days after ICSI, including those that failed to develop further (Fig. 3). As discussed above, these presumably represent maternal transcripts either remaining from the intense period of mtDNA replication and stabilisation during oocyte growth and maturation, or involved in the mtDNA replication event proposed to take place between fertilization and the first cell cleavage division (McConnel and Petrie, 2004). The subsequent drop in *mtPOLB* expression 6 days after ICSI could be explained by the global degradation of maternal mRNA transcripts that occurs at the time of embryonic genome activation (St. John *et al.*, 2010). However, the absence of a more obvious upregulation of *mtPOLB* and *SSB* transcription at the time of onset of mtDNA replication, as described in mouse, pig, cattle and sheep embryos (Bowles *et al.*, 2007; May-Panloup *et al.*, 2007) was unexpected. This may in part reflect a masking effect of the very high transcript numbers persisting from the early post-fertilization mtDNA replication event.

In conclusion, mitochondrial replication commences in equine *in vivo* and IVEP embryos just prior to blastocyst expansion. This is preceded by an increase in *TFAM*, but not *mtPOLB* or *SSB* expression. Further research should focus in greater detail on the period in which paternal and defective mitochondria are removed by mitophagy, the period in which mitochondrial replication begins (between D6 and D8) *in vivo* and *in vitro*, and on other aspects of the establishment of the mitochondrial replication machinery. Differences between mitochondrial DNA regulation in inner cell mass cells versus trophectoderm cells

could help establish in which cells mtDNA replication primarily occurs, and could be used as a marker to help improve IVEP conditions in the future.

Acknowledgements

The authors thank Leni van Tol for advice on oocyte recovery and *in vitro* maturation, Gabriella Crotti, Paola Turini, Massimo Iazzi for technical support with the recovery of oocytes. Zerbini e Ragazzi for the supply of equine ovaries, Manon Vos-Loohuis for assistance with PCR product sequencing, Jan van de Broek for assistance with statistical analyses and Bernard Roelen for his support and comments during this research project. Part of this work was supported by was supported by Grant n. 26096200 (project Ex Ovo Omnia) from Regione Sardegna and Regione Lombardia to CG.

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