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Embryo Mitochondrial DNA Depletion Is Reversed During Early Embryogenesis in Cattle[1]

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ABSTRACT

The extensive replication of mitochondria during oogenesis and the wide variability in mitochondrial DNA (mtDNA) copy numbers present in fully grown oocytes indicate that mtDNA amount may play an important role during early embryogenesis. Using bovine oocytes derived from follicles of different sizes to study the influence of mtDNA content on development, we showed that oocytes obtained from small follicles, known to be less competent in developing into blastocysts, contain less mtDNA than those originating from larger follicles. However, because of the high variability in copy number, a more accurate approach was examined in which parthenogenetic one-cell embryos were biopsied to measure their mtDNA content and then cultured to assess development capacity. Contrasting with previous findings, mtDNA copy number in biopsies was not different between competent and incompetent embryos, indicating that mtDNA content is not related to early developmental competence. To further examine the importance of mtDNA on development, one-cell embryos were partially depleted of their mtDNA (64% ± 4.1% less) by centrifugation followed by the removal of the mitochondrial-enriched cytoplasmic fraction. Surprisingly, depleted embryos developed normally into blastocysts, which contained mtDNA copy numbers similar to nonmanipulated controls. Development in depleted embryos was accompanied by an increase in the expression of genes (*TFAM* and *NRF1*) controlling mtDNA replication and transcription, indicating an intrinsic ability to restore the content of mtDNA at the blastocyst stage. Therefore, we concluded that competent bovine embryos are able to regulate their mtDNA content at the blastocyst stage regardless of the copy numbers accumulated during oogenesis.

bovine, developmental biology, early development, embryo, gamete biology, mitochondria, mtDNA, oocyte development, preimplantation

INTRODUCTION

The mammalian oocyte relies heavily on components stored in the cytoplasm during oogenesis to initiate development and to develop into a healthy blastocyst. Thus, bovine oocytes derived from smaller follicles are smaller in size [1, 2], contain fewer amounts of stored components [2–4], and show a lower developmental competence [1, 5]. The components stored in the cytoplasm (e.g., mRNAs, proteins, and energetic substrates) are known to be critical in supporting the initial stages of development, when the embryo itself shows limited transcriptional activity (reviewed by Picton et al. [6] and Meirelles et al. [7]). Similarly, the number of mitochondria increases sharply during oogenesis, culminating in mature oocytes containing hundreds of thousands to more than a million of these organelles. However, although previous studies have indicated that mitochondria play an important role in development, it remains unclear whether the number of mitochondria inherited in the mature oocyte at the time of ovulation is correlated with its competence to reach the blastocyst stage (reviewed by Dumollard et al. [8]).

Mitochondria are organelles that play an essential role in cellular energetic metabolism, homeostasis, and death. In mammals, they are normally inherited exclusively from the oocyte (reviewed by Birky [9]) and are responsible for generating, through the oxidative phosphorylation (OXPHOS) pathway, most of the ATP necessary for energy-dependent biological processes. During early embryo development, mitochondria are undifferentiated and produce low levels of ATP [10, 11]. At the time of embryonic genome activation, mitochondria progressively undergo functional and structural changes and also generate higher levels of ATP to supply the increasing energy demands of the embryo that result from RNA and protein synthesis and blastocoel formation [10, 11]. However, in spite of these roles, the number of mitochondria seems to remain constant through the preimplantation period. Thus, mitochondrial number in oocytes must be sufficient to populate each blastomere and supply the ATP required to form viable blastocysts (reviewed by Dumollard et al. [8]).

Mitochondrial function depends on an orchestrated communication between nuclear DNA (nDNA) and several copies of the ~16.5-kb DNA contained within the mitochondrion

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(mitochondrial DNA, or mtDNA). Among the ~80 peptides involved in OXPHOS, 13 are encoded by the mtDNA, and the remaining peptides are encoded by nDNA. Moreover, nDNA encodes transcription factors that coordinate the expression of these peptides and other factors that regulate mtDNA replication, transcription, and translation. On the other hand, in spite of being regulated by genes contained within the nucleus, the replication of mtDNA does not depend on the cell cycle (reviewed by Shadel and Clayton [12]). Indeed, it is affected by the energetic requirements of the cell, because the amount of mtDNA is strongly associated with mitochondrial function [13, 14]. Thus, the number of copies of mtDNA can vary from hundreds to thousands among different cell types. For instance, the oocyte contains the largest cellular amount of mtDNA of any cell in the organism [15]. Large amounts of mitochondria are accumulated during oogenesis while the follicle grows [16, 17], highlighting the potential importance of the quantity of the mtDNA for the early embryo (reviewed by Smith et al. [18] and May-Panloup et al. [19]).

In spite of the large amounts of mtDNA present at the time of ovulation [15], mtDNA copy numbers vary considerably between individual oocytes [20–24]. Although it is not known what determines such variation or its effect on early development, numerous authors have linked mtDNA copy number to fertility in several species [20, 23, 25–27]. Thus, to verify the hypothesis that oocytes containing lower amounts of mtDNA are less competent to support further embryo development than those containing higher amounts, we 1) quantified the amount of mtDNA in bovine oocytes known to have different developmental competence, 2) applied a biopsy-based approach to quantify the amount of mtDNA before embryo culture, and 3) depleted one-cell-stage bovine embryos of mitochondria to assess the effects on embryo development and the control of mtDNA replication and transcription.

MATERIALS AND METHODS

All chemicals and reagents used were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise stated. In vitro experimental procedures were done in humidified incubators maintained at 38.5°C in air with 5% CO_2. All experiments were repeated at least three times, and samples were measured in replicates. Cultured embryos were assessed for developmental rates at Day 3 (cleavage, at 72 h after parthenogenetic activation [72 hpa]), Day 7 (blastocyst, at 168 hpa), and Day 9 (blastocyst, at 216 hpa). Cleavage and blastocyst rates were reported in relation to the presumptive embryos placed in culture.

Ethical Considerations

The present study was approved by the Animal Experimentation Ethics Committee of the University of Campinas. The experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals (Society for the Study of Reproduction).

Experiment 1

We sought to compare the amount of mtDNA among oocytes from follicles of different sizes. Oocytes were obtained postmortem from the ovaries of crossbred cows (*Bos indicus*) slaughtered at a local slaughterhouse. Ovaries were transported in 0.9% saline solution at 25°C–30°C to the laboratory, and follicles were individually dissected and measured in diameter. Only cumulus-oocyte complexes (COCs) with layers of several compacted cumulus cells and homogeneous cytoplasm were selected from follicles with diameters between ≥1 and <8 mm. Oocytes were denuded of cumulus cells by gentle vortexing in PBS plus 0.1% polyvinyl-pyrrolidone (PVP). The whole oocyte, regardless of its size, was placed in 0.2 ml of polystyrene microtubes containing 1 μl of PBS plus 0.1% PVP, snap frozen in liquid nitrogen, and stored at −20°C until use. Three experimental groups were considered based only on follicle diameter (≥1 to <3 mm, ≥3 to <6 mm, and ≥6 to <8 mm). A minimum of 15 oocytes were sampled for each group.

Experiment 2

Our objective in this experiment was to measure the amount of mtDNA estimated by a biopsy-based approach at the one-cell stage and compare it among parthenogenetic embryos differing in their developmental rates.

In vitro parthenogenetic embryo production. Follicles with diameters between ≥3 and <6 mm were aspirated using an 18-gauge needle attached to a 20-ml syringe. Recovered COCs were selected and washed in HEPES-buffered tissue culture medium-199 (TCM-199; GIBCO BRL, Grand Island, NY) plus 10% fetal bovine serum (FBS) heat inactivated (55°C for 30 min) plus 22 μg/ml sodium pyruvate plus 83.4 μg/ml amikacin sulfate. Groups of 10–15 COCs were placed in drops of 90 μl of bicarbonate-buffered TCM-199 plus 10% FBS plus 0.5 μg/ml follicle-stimulating hormone (Folltropin-V; Bioniche Animal Health, Belleville, ON, Canada) plus 50 μg/ml human chorionic gonadotropin (Vetecor; Lab Calier, Barcelona, Spain) plus 1 μg/ml estradiol plus 22 μg/ml sodium pyruvate plus 83.4 μg/ml amikacin sulfate under mineral oil for 20–21 h for in vitro maturation (IVM).

After IVM, COCs were denuded of cumulus cells by gentle pipetting in 0.5% hyaluronidase solution and selected based on the presence of the first polar body. Selected oocytes were chemically activated (at 26 h after IVM) by incubation in 5 μM ionomycin in HEPES-buffered TCM-199 plus 0.1% bovine serum albumin (BSA; fatty acid-free) plus 22 μg/ml sodium pyruvate plus 83.4 μg/ml amikacin sulfate for 5 min, followed by incubation in HEPES-buffered TCM-199 plus 3% BSA for 1 min and in 2 mM 6-dimethylaminopurine diluted in modified synthetic oviduct fluid (mSOF) [28] plus 2% FBS plus 0.6% BSA (mSOF(+)) for 3 h. Finally, activated oocytes were washed in mSOF(+) and were cultured in vitro in groups of 20–25 in mSOF(+) until use.

Cytoplasmic biopsies of parthenogenetic one-cell embryos. Microsurgery was performed using an inverted microscope (Leica DMI RB; Leica, Wetzlar, Germany) equipped with micromanipulators and microinjectors (Narishige, Tokyo, Japan). At first, presumptive parthenogenetic embryos (at the one-cell stage) were incubated in groups of 15–20 in mSOF(+) plus 10 μg/ml Hoechst 33342 plus 7.5 μg/ml cytochalasin B for 15 min. For microsurgery, the group was transferred to a 400-μl drop of PBS plus 10% FBS plus 22 μg/ml sodium pyruvate plus 83.4 μg/ml amikacin sulfate plus 7.5 μg/ml cytochalasin B under mineral oil in a plastic dish. Before removal of a cytoplasmic biopsy, the diameter of each one-cell embryo was measured (embryo volume = embryo radius^3 × 4/3 × π), and the pronuclei were located by a quick exposure to ultraviolet light (350-nm excitation/450-nm emission). Using a 15-μm (internal diameter) glass pipette (Eppendorf, Hamburg, Germany), a cytoplasmic biopsy equivalent to ~5% one-cell embryo volume (biopsy volume = glass pipette radius^2 × biopsy length × π) was removed without removing pronuclei. This cytoplasmic biopsy was washed in PBS plus 0.1% PVP, placed in 0.2-ml polystyrene microtubes containing 1 μl of PBS plus 0.1% PVP, snap frozen in liquid nitrogen, and stored at −20°C until use. Biopsied one-cell embryos were washed in PBS plus 10% FBS plus 22 μg/ml sodium pyruvate plus 83.4 μg/ml amikacin sulfate and were individually cultured in a 10-μl drop of mSOF(+) for 7 days under tension of 5% O_2, 5% CO_2, and 90% N_2 [29] to assess developmental rates. A total of 330 biopsied one-cell embryos were used in this experiment. To validate the approach, a portion of these biopsied one-cell embryos (10 per repetition) were, instead of cultured, sampled and stored for molecular analysis (mtDNA copy number). Whole one-cell embryos were washed in PBS plus 0.1% PVP, placed in 0.2-ml polystyrene microtubes containing 1 μl of PBS plus 0.1% PVP, snap frozen in liquid nitrogen and stored at −20°C until use.

Experiment 3

Our objective in this experiment was to partially deplete parthenogenetic one-cell embryos of mitochondria to assess the relationship between mtDNA copy number and developmental rate, the number of cells per blastocyst, the amount of mtDNA, and the control of mtDNA replication and transcription. Parthenogenetic embryo production as well as the equipment and conditions for micromanipulation were as described for experiment 2. However, before micromanipulation, one-cell embryos were incubated in mSOF(+) plus 7.5 μg/ml cytochalasin B for 30 min and then centrifuged in PBS plus 7.5 μg/ml cytochalasin B at 10 000 × *g* for 15 min [30]. This results in concentration of the mitochondria at one pole of the embryo, producing a mitochondrial-enriched cytoplasmic fraction. Using micromanipulation, this fraction was partially removed and discarded to partially deplete one-cell embryos of their mitochondria (depleted [DE] group). Embryo micromanipulation was carefully performed to remove similar proportions of cytoplasm among embryos. The pronuclei were located as described in experiment 2 to prevent removal during micromanipulation. To better characterize this method, some one-cell embryos were stained with 0.5 μM MitoTracker CMXRos (Molecular Probes, Eugene, OR) for mitochondria labeling for 30 min before micromanipulation.

TABLE 1. Number of copies of mtDNA in oocytes obtained from follicles differing in size.

	Follicle diameter (mm)		
Parameter	≥1 to <3	≥3 to <6	≥6 to <8
No. of follicles	25	38	15
Mean ± SEM	$1.09 \times 10^{6b} \pm 6.61 \times 10^{4}$	$1.37 \times 10^{6a} \pm 6.20 \times 10^{4}$	$1.37 \times 10^{6a} \pm 13.08 \times 10^{4}$
Range	$3.92 \times 10^{5} - 1.72 \times 10^{6}$	$4.36 \times 10^{5} - 2.06 \times 10^{6}$	$1.70 \times 10^{4} - 1.89 \times 10^{6}$
CV	30%	28%	37%

[a,b] Means with different superscript letters within a row denote a significant difference among experimental groups ($P < 0.05$).

MitoTracker staining was visualized under fluorescence at 579-nm excitation/599-nm emission.

After micromanipulation, one-cell embryos were washed in PBS plus 10% FBS plus 22 μg/ml sodium pyruvate plus 83.4 μg/ml amikacin sulfate and were cocultured in vitro in groups of 20–25 with a monolayer of granulosa cells in 90 μl of mSOF(+) for 9 days under mineral oil. For controls in this experiment, three other experimental groups were considered: 1) one-cell embryos that were only centrifuged and then cultured (centrifuged control [CC] group); 2) one-cell embryos that were not centrifuged but had a cytoplasmic portion removed similar to that removed from the DE group (6%–8% embryo volume; micromanipulated control [MC] group), and 3) one-cell embryos that were cultured immediately without centrifugation or micromanipulation (control [CO] group). A minimum of 15 embryos per experimental group were randomly sampled at Day 0 (at ~7 hpa), Day 3 (at 72 hpa), Day 6 (at 144 hpa), and Day 9 (at 216 hpa) for molecular analysis. At Day 0, 15 one-cell embryos were sampled and used for mtDNA quantification. At Days 3 (4- to 16-cell stages), 6 (early blastocyst stage), and 9 (hatching or hatched blastocyst stages), 15 embryos were used for quantification of the amounts of both mtDNA and mRNA transcripts. Only morphologically normal embryos that reached the expected developmental stage at each time were chosen. The whole one-cell embryo was washed in PBS plus 0.1% PVP plus 1 unit/μl RNase OUT (Invitrogen, Carlsbad, CA), placed in 0.2 ml of polystyrene microtubes containing 1 μl of PBS plus 0.1% PVP plus 1 unit/μl RNase OUT, snap frozen in liquid nitrogen, and stored at −80°C until use. Some of the hatching and hatched blastocysts from Day 9 were also sampled for determination of cell number. In this case, embryos were fixed in PBS plus 2% paraformaldehyde plus 0.1% PVP for 1 h and permeabilized in PBS plus 0.5% Triton X-100 plus 0.1% sodium citrate plus 0.1% PVP for 1 h. Next, embryos were stained in PBS plus 10 μg/ml Hoechst 33342 for 15 min. Finally, embryos were mounted on a glass slide, and nuclei were counted using a fluorescence microscope (Axioplan; Carl Zeiss, Zeppelinstrasse, Germany) and the AxioVs40 software (V4.6.1.0; Carl Zeiss). Cell doubling was considered as logarithm (base 2) of cell number.

Absolute Quantification of mtDNA

Sample preparation. Samples from experiments 1, 2, and 3 (only from Day 0) were treated as described by Wan et al. [31], with some modifications. Briefly, they were incubated at 55°C for 30 min in a final volume of 5 μl of lysis solution containing 2 μg/ml proteinase k (USB, Cleveland, OH) plus 1% Triton X-100 plus 1× PCR buffer (Invitrogen). Proteinase k was then heat inactivated by sample incubation at 100°C for 5 min, and the lysate was diluted in ultrapure H_2O to a final volume of 50 μl and immediately used for molecular analysis. For biopsies, the final volume of lysed solution was used without dilution.

External standard preparation. Primers (bMT3010-f: 5′-GCCCTAGAA CAGGGCTTAGT-3′ and bMT3096-r: 5′-GGAGAGGATTTGAATCTC TGG-3′; Promega, Madison, WI) were designed to amplify an 87-bp fragment of the mitochondrially encoded 16S RNA (*MT-RNR2*), which is fully conserved between *Bos taurus* and *B. indicus* mtDNAs based on sequences available at GenBank (access nos.: AY526085 and AY126697, respectively). Then, the external standard was prepared as previously reported [32], with some modifications. Briefly, part of the mtDNA was amplified by PCR in a 25-μl reaction containing 0.9 μM each primer plus 0.2 mM dinucleotide triphosphates (Invitrogen) plus 1.5 mM $MgCl_2$ plus 1× PCR buffer plus 0.04 units/μl Taq DNA Polymerase (Invitrogen) plus 5 μl of template (an oocyte prepared as described above). The PCR product was extracted using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, Piscataway, NJ), inserted into a vector (pGEM-T Vector; Promega), and cloned using *Escherichia coli* DH5α (Invitrogen) according to the manufacturer's recommendations. The plasmid DNA was purified using the FlexiPrep kit (Amersham Biosciences). To confirm the presence of the *MT-RNR2* gene fragment, the plasmid was digested with *Nde*I (Promega), and the products were separated electrophoretically. The concentration of copies of plasmid DNA was determined using a spectrophotometer (Eppendorf) and a stock solution prepared at 0.2×10^{9} copies/μl. This stock solution was stored at −20°C in single-use aliquots to be used as an external standard.

Quantitative real-time PCR. Quantification of the amount of mtDNA was performed using a quantitative real-time PCR (qPCR) method. The ABI PRISM SDS 7500 HT Real-Time PCR System (Applied Biosystems, Foster City, CA) was used for qPCR reactions. Briefly, a 20-μl PCR reaction prepared for each sample was composed of 0.9 μM of both the bMT3010-f and the bMT3096-r primers plus 0.25 μM TaqMan probe bMT3030-Fam (5′-FAM-AAGGTGGCAGAGCCCGGTAATTGC-BHQ1-3′; Promega) plus 1× TaqMan Gene Expression Master Mix (Applied Biosystems) plus 5 μl of template (standard or samples). The following cycling conditions were applied for amplification: initial denaturation at 95°C for 15 min followed by 40 cycles consisting of 95°C for 20 sec and 63°C for 1 min. The probe fluorescence was read at the end of each extension step (63°C). For each run, a standard curve was generated using five 10-fold serial-dilutions (10^3 to 10^7 copies) of the external standard. Pilot experiments were done to set up qPCR conditions so that the samples and standard had the same efficiency of amplification. Based on the standard curve values, it was possible to quantify the starting copy number of mtDNA in each sample using the SDS software (V2.3; Applied Biosystems). For biopsies, the number of copies was corrected considering variations in the volume of each micromanipulated one-cell embryo.

Relative Quantification of mtDNA and mRNA Amounts

External standard preparation for mtDNA quantification. A 345-bp DNA fragment that was nonsimilar to bovine genomic DNA was obtained from *Taenia saginata* as reported elsewhere [33–35]. This fragment was cloned and purified as described above (see absolute quantification of mtDNA), but using another vector (pET-29a kit; Novagen, Madison, WI). A stock solution of plasmid DNA plus 1 unit/μl RNase OUT was prepared at 0.25 ng/μl to be used as an external standard for mtDNA quantification.

Isolation of genomic DNA and RNA. Both genomic DNA and RNA were extracted from each individual embryo from experiment 3 using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations with modifications. In brief, a mix containing 100 μl of TRIzol reagent plus 5 μg of linear acrylamide (Ambion Inc., Austin, TX) plus 4 μl of external standard plus 5 μl of diethylene pyrocarbonate-treated H_2O was added to each sample. The extracted RNA was directly dissolved in 10 μl of DNase I solution (Invitrogen) plus 1 unit/μl RNase OUT for DNA degradation, as suggested by the manufacturer. To confirm the absence of contaminating DNA, samples were subjected to the amplification protocol with *MT-CO1* primer (see below) before reverse transcription. Finally, the RNA was immediately reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's protocol and was stored at −20°C until use. For DNA extraction, both the interphase and the organic phases of TRIzol reagent were used together with 5 μg of linear acrylamide. The extracted DNA was dissolved in 20 μl of 8 mM sodium hydroxide. For pH adjustments, 1.72 μl of 0.1 M HEPES was added, followed by 3.3 μl of ultrapure H_2O to a final volume of 25 μl, and samples were immediately used for qPCR to avoid DNA degradation.

Relative quantification of mtDNA amounts. For external standard amplification, a 20-μl reaction was prepared containing 0.2 μM of both primers plus 1× SYBR Green PCR Master Mix (Applied Biosystems) plus 2 μl of template (samples plus standard). For mtDNA amplification, the same conditions described for absolute quantification of mtDNA were used. Both mtDNA and an external standard were always run in the same PCR plate using the same conditions described for absolute quantification of mtDNA. Standard curves were generated for both mtDNA and the external standard using eight 2-fold serial dilutions of sample pools. A melting curve (loss of fluorescence at a

given temperature between 60°C and 95°C) of external standard amplification was analyzed to check for the specificity of the PCR product. Pilot experiments were done to set up PCR conditions so that the samples and standards had the same efficiency of amplification. Mitochondrial DNA amounts in each sample were corrected based on the external standard present in the same sample using the standard curve method [36].

Relative quantification of mRNA amounts. The target genes of interest belonged to two categories: nuclear encoded and mitochondrial encoded. The three nuclear-encoded genes were the mitochondrial transcription factor A (*TFAM*), the nuclear respiratory factor 1 (*NRF1*), and the histone cluster 1, H2AG (*HIST1H2AG*). The mitochondrial-encoded gene consisted of the mitochondrially encoded cytochrome *c* oxidase I (*MT-CO1*). Primers used for PCR amplification of these genes were described previously by May-Panloup et al. [21] and Vigneault et al. [37].

Before qPCR amplification, cDNA was preamplified using the TaqMan PreAmp Master Mix kit (Applied Biosystems) according to manufacturer's recommendations as follows: a 10-μl reaction was prepared containing 45 nM each primer (*TFAM*, *NRF1*, *HIST1H2AG*, and *MT-CO1*) plus 1× TaqMan PreAmp Master Mix (Applied Biosystems) plus 4 μl of template (cDNA samples), subjected to 14 thermal cycles and stored at −20°C. The linearity of amplification of all transcripts in all embryonic stages studied (Days 3, 6, and 9) was determined as suggested by the manufacturer.

Quantitative PCR for relative quantification of gene-specific mRNA transcripts was done in 20-μl reactions containing 0.2 μM (*TFAM*) or 0.15 μM (*NRF1*) or 0.1 μM (*HIST1H2AG*) or 0.2 μM (*MT-CO1*) of both primers plus 1× SYBR Green PCR Master Mix (Applied Biosystems). For each sample, preamplified cDNAs were diluted 8-fold (*TFAM* and *NRF1*) or 80-fold (*HIST1H2AG*) or 800-fold (*MT-CO1*) to be used as template. All gene-specific cDNAs amplified for a particular sample were always run in the same PCR plate. The following cycling conditions were applied for amplification: initial denaturation at 95°C for 15 min followed by 40 cycles consisting of 95°C for 20 sec, 57°C for 45 sec, and 60°C for 1 min. The SYBR Green fluorescence was read at the end of each extension step (60°C). Standard curves were generated for each gene-specific cDNA analyzed using six 4-fold serial dilutions of sample pools. A melting curve of each amplification assay used was analyzed to check the specificity of the PCR product. Target transcript amounts in each sample were determined using the standard curve method [36].

Statistical Analysis

Statistical analysis was performed using the SAS System (V8; Cary, NC). In experiment 3 (developmental rates), the experimental groups were compared using χ^2 test. The remaining data were tested for assumption of normal distribution and homogeneity of variance, and they were transformed (square root) when these criteria were not met. In experiments 1 and 2 (mtDNA in biopsies) and 3 (cell numbers/doublings and mtDNA in one-cell embryos), experimental groups were compared using one-way ANOVA, followed by Tukey or Duncan posthoc tests. In experiment 2 (approach validation), a Person correlation (r) test was performed. In experiment 3 (mtDNA and mRNAs), a cross-classification model was used considering experimental groups and days of development (Days 3, 6, and 9) as main factors. These data were analyzed using two-way ANOVA followed by Student t-posthoc test. Differences with probabilities $P < 0.05$ were considered significant. In the text, values are reported as means ± SEM. The coefficient of variation (CV) is also reported.

RESULTS

Mitochondrial DNA Copy Numbers Vary Substantially among Oocytes from All Follicle Sizes

First, we designed an experiment to determine whether the amount of mtDNA in oocytes is related to the size of the follicle from which they are recovered (Table 1). The number of copies of mtDNA in oocytes derived from small (≥1 to <3 mm) follicles was significantly lower than the amount observed in oocytes from medium (≥3 to <6 mm) and large (≥6 to <8 mm) follicles. Because it is known that bovine oocytes derived from follicles smaller than 3 mm show a lower developmental competence [1, 5], these results agree with previous interpretations that the mtDNA copy number in oocytes is related to their ability to support development. However, the variability in mtDNA copy number between oocytes in all three groups was very high and in some cases ranged more than 100-fold (1.70×10^4 to 1.89×10^6). Coefficients of variation averaged

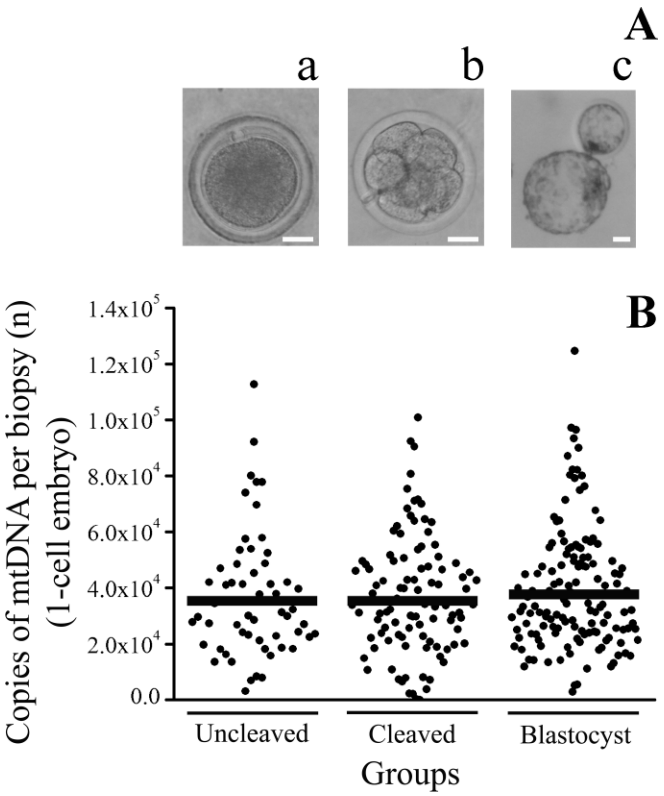

FIG. 1. Parthenogenetic embryo developmental competence is unrelated to mtDNA copy number. **A**) Photomicrographs of uncleaved (**a**), cleaved (eight-cell stage; **b**), and blastocyst (**c**) embryos. Bars = 40 μm. **B**) Content of mtDNA in cytoplasmic biopsies of embryos at the one-cell stage. The content of mtDNA in biopsies was grouped based on embryo development to the blastocyst stage. Dots indicate the number of mtDNA copies in each biopsy, and bars represent the means ($P = 0.94$).

32% among oocytes and were similar between oocytes from small, medium, and large follicle groups. Because of the wide variability among oocytes, it appears that the use of approaches similar to that used in this experiment to correlate mtDNA content and oocyte developmental competence are intrinsically prone to error. Therefore, a more accurate retrospective approach was required in which oocytes could be sampled individually to measure mtDNA content and then returned to culture to evaluate their competence for development.

Embryo Developmental Competence Is Unrelated to mtDNA Copy Number

To retrospectively relate mtDNA copy number and developmental competence, we used a biopsy-based approach to examine the amount of mtDNA in the cytoplasmic fraction at the one-cell stage and then related this with the embryo's ability to develop further in vitro. Prior to the experiment, mitochondrial distribution was assessed in one-cell embryos by staining with a mitochondrial-specific fluorochrome (Mitotracker). The distribution pattern of Mitotracker fluorescence was found to be similar among embryos within the time necessary to perform the biopsy procedure (data not shown). Moreover, a clear relationship was observed ($r = 0.68$; $P < 0.05$) between the mtDNA copy number found in the removed cytoplasmic fraction and the respective manipulated one-cell embryo. Together, these results indicate that the biopsy approach can be reliably used to determine the mtDNA content in one-cell-stage embryos. The average percentile amounts of

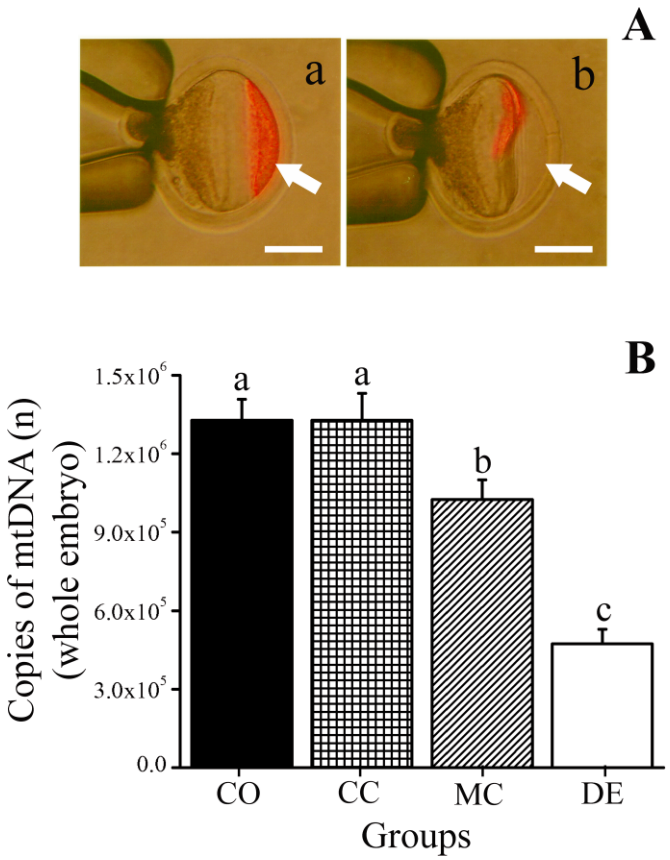

FIG. 2. Removal of the mitochondrial-enriched cytoplasmic fraction reduces the mtDNA content in parthenogenetic one-cell embryos. **A**) Merged photomicrographs obtained using light and fluorescence microscopy to visualize both the mitochondrial-enriched cytoplasmic fraction and stained mitochondria (red color; arrow) before (**a**) and after (**b**) depletion, respectively. Bars = 50 μm. **B**) Number of copies of mtDNA remaining in embryos of the experimental groups. Values are reported as means ± SEM. Bars with different letters denote a significant difference among experimental groups ($P < 0.05$).

mtDNA and cytoplasm removed from one-cell embryos using this approach were 3.1% ± 0.3% and 5.3% ± 0.1%, respectively. As observed in the oocytes used in experiment 1, analysis of biopsies from one-cell embryos showed a large variation in the number of mtDNA copies ($3.62 \times 10^4 \pm 1.25 \times 10^3$, ranging from 17 to 124 526 copies; CV, 39%), indicating that there are no significant differences in mtDNA variability between the immature oocyte and the one-cell-stage embryo. Morphological observation under light microscopy of embryos at Day 3 and Day 7 indicated normal embryo development, with blastocysts hatching from the pellucid zone at Day 7 and containing a well-defined inner cell mass (Fig. 1). Interestingly, no difference in the content of mtDNA was found between biopsies obtained from one-cell embryos that, after in vitro culture, 1) remained uncleaved ($3.50 \times 10^4 \pm 3.02 \times 10^3$), 2) cleaved but arrested after cleavage ($3.50 \times 10^4 \pm 2.12 \times 10^3$), or 3) continued development and reached the blastocyst stage ($3.80 \times 10^4 \pm 1.82 \times 10^3$; Fig. 1). These results clearly indicate that there is no relationship between mtDNA content in one-cell embryos and the capacity to develop to the blastocyst stage. In spite of this, of 296 embryos analyzed, 81% cleaved and 48% developed to blastocysts. These rates were not different ($P > 0.05$) than those obtained for nonmanipulated control embryos that were individually cultured (75% and 44%, respectively), indicating that the biopsy procedure itself did not interfere with development. Moreover, a large and similar intragroup variation was observed in the number of copies of mtDNA in the uncleaved, cleaved, and blastocyst groups (CV, 41%, 41%, and 36%, respectively). Surprisingly, even one-cell embryos with ~90% less mtDNA than the mean one-cell embryo content (blastocyst group) were able to develop into viable blastocysts, further supporting the conclusion that the amount of mtDNA at the pronuclear stage is not related to the ability to develop to the blastocyst stage.

Mitochondrial Depletion of One-Cell Embryos Does Not Inhibit Blastocyst Development

To experimentally verify that low contents of mtDNA at the one-cell stage do not affect subsequent embryo development, one-cell embryos were centrifuged to fractionate their cytoplasm. Then, the mitochondrial-enriched cytoplasmic fraction was mechanically removed. After removal of mitochondria, depleted one-cell embryos were cultured for another 9 days to examine their ability to develop to the blastocyst stage in vitro (Fig. 2). When compared to the CO group ($1.33 \times 10^6 \pm 7.98 \times 10^4$ copies), mechanical depletion of the mitochondrial-enriched cytoplasmic fraction (DE group) led to a 64% ± 4.1% ($0.47 \times 10^6 \pm 5.48 \times 10^4$ copies) reduction in the amount of mtDNA (ranging from 33% to 90%). Embryos that were centrifuged but not micromanipulated to deplete mitochondria (CC group) were stained with Mitotracker to examine the distribution pattern of Mitotracker fluorescence in cleaving embryos at 24 h after centrifugation. Mitotracker staining was homogeneous among blastomeres of two-, three-, and four-cell-stage embryos of the CC group, indicating that the segregation caused by centrifugation at the one-cell stage was mostly reversed by the time the one-cell embryo underwent its first and second cleavage divisions (data not shown). Although cleavage rates were marginally inferior in the CO group, development to the blastocyst stage was not influenced by

TABLE 2. Developmental rates and blastocyst cell number of parthenogenetic embryos partially depleted of mitochondria in comparison to control embryo.

	Day 3	Day 7	Day 9	
Groups	Cleavage (%)*	Blastocyst (%)*	Blastocyst (%)*	Cell numbers/doublings†
CO	257/321[c] (80%)	71/177 (40%)	87/177 (49%)	174[a] ± 63/7.4 ± 0.55
CC	201/235[b] (86%)	59/142 (42%)	70/142 (49%)	202[a] ± 56/7.7 ± 0.46
MC	167/196[b] (85%)	41/99 (41%)	53/99 (54%)	139[b] ± 42/7.1 ± 0.41
DE	316/355[a] (89%)	97/227 (43%)	111/227 (49%)	121[b] ± 40/6.9 ± 0.55

* Values are reported as frequency.
† Values are reported as mean ± SEM.
[a,b,c] Means with different superscript letters within columns denote a significant difference among experimental groups ($P < 0.05$).

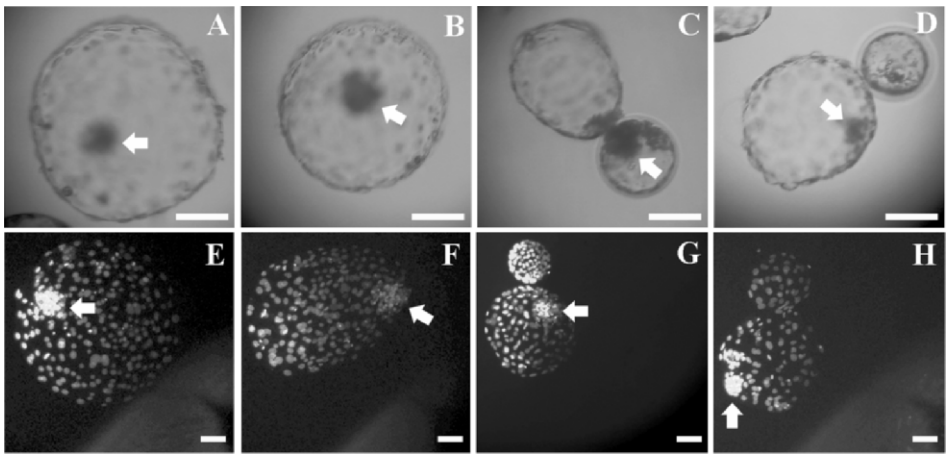

FIG. 3. Mitochondrial depletion of parthenogenetic one-cell embryo does not inhibit blastocyst development. Photomicrographs of blastocysts at Day 9 obtained using light (**A**–**D**) and fluorescence microscopy (**E**–**H**; embryos stained with Hoechst 33342). **A** and **E**) Nonmanipulated embryos. **B** and **F**) Embryos that were centrifuged at the one-cell stage. **C** and **G**) Embryos that had a cytoplasmic portion removed at the one-cell stage. **D** and **H**) Embryos that were centrifuged at the one-cell stage to remove part of the mitochondrial-enriched cytoplasmic fraction. Embryos in **A**–**D** are not necessarily the same as in **E**–**H**. Arrows indicate the inner cell mass. Bars = 100 μm.

centrifugation (CC group) or by the removal of cytoplasm (similar volume as DE) from noncentrifuged one-cell embryos (MC group; Table 2). Indeed, based on morphological observations under light microscopy, embryo development and blastocyst quality were similar among all experimental groups, indicating that the removal of cytoplasm or mitochondria from the one-cell embryo did not interfere with its ability to develop in vitro (Fig. 3). Nonetheless, cell numbers in MC and DE blastocysts were lower than in CC and CO embryos, indicating that the removal of cytoplasm decreases the number of cell divisions (cell doubling) during early development (Fig. 3 and Table 2). Together, these results confirm our previous conclusion that the amount of mtDNA at the one-cell stage is not related to the capacity to develop to the blastocyst stage.

Depleted One-Cell Embryos Replenish mtDNA by Increasing Replication

To investigate the mechanisms by which depleted one-cell embryos develop, we also investigated the amounts of mtDNA present through early development (Fig. 4). Although the amount of mtDNA of the DE group remained lower than that of all other groups at Day 3, the difference between the CO and MC controls became less apparent with development, leading to a restoration of mtDNA amount by Day 9. A more detailed analysis indicated that these control groups increased the amount of mtDNA between Days 3 and 6 and remained unchanged between Days 6 and 9. However, the amounts of mtDNA in the DE group increased both between Days 3 and 6 and between Days 6 and 9, enabling embryos in the DE group to restore their mtDNA amount to levels similar to those found in control blastocysts (CO and MC groups). These results indicate that one-cell embryos depleted of mitochondria are able to extend mtDNA amplification beyond the Day 6 blastocyst stage to assure the accumulation of sufficient copies by Day 9. Actually, when correcting for the number of cells in Day 9 blastocysts, mtDNA amounts became quite similar among all groups. Together, these results suggest that regardless of the mtDNA copy numbers present at early stages of development, competent embryos will extend mtDNA amplification to ensure that each cell in the blastocyst contains sufficient copies to enable normal development.

Transcripts Controlling mtDNA Replication Are Increased in Depleted Embryos

To investigate the molecular mechanisms by which depleted one-cell embryos are able to replenish mtDNA copies by the blastocyst stage, transcripts of factors controlling mtDNA replication and transcription were quantified and compared to mitochondrial and nuclear-encoded transcripts (Fig. 5). Between Days 3 and 6, *TFAM* transcripts increased significantly in both CO and DE groups, indicating an activation of mtDNA replication and transcription. However, whereas the control group remained unchanged between Days 6 and 9, embryos that were depleted showed a significant increase in *TFAM* transcript by Day 9, indicating that the recovery of mtDNA copy numbers by the depleted group was the result of extended upregulation of this nuclear-encoded factor involved in mtDNA replication and transcription until Day 9. Moreover, in contrast to the CO group, transcripts for *MT-CO1* also increased between Days 6 and 9 in the depleted group, confirming that *TFAM* controls both mtDNA replication and transcription. Indeed, on a per-cell basis, Day 9 blastocysts

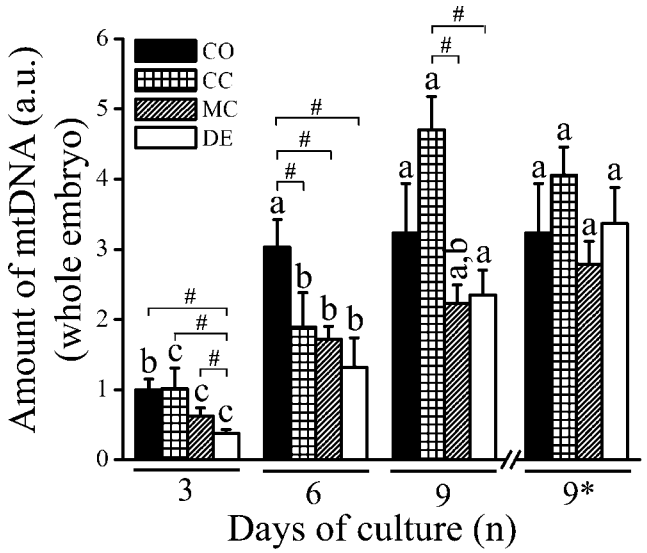

FIG. 4. Depleted parthenogenetic embryos replenish mtDNA by increasing replication. Embryos were analyzed at Day 3 (4- to 16-cell stages), Day 6 (early blastocyst stage), and Day 9 (hatching and hatched blastocyst stages). The amounts of mtDNA are expressed in relation to the CO group at Day 3. The bar labeled 9* indicates that the amount of mtDNA at Day 9 was corrected by the mean cell number of each experimental group. Values are reported as means ± SEM. Different letters over bars denote a significant difference among days (3, 6, and 9; or 3, 6, and 9*) within the experimental group ($P < 0.05$). #Difference between experimental groups within day ($P < 0.05$). a.u. indicates arbitrary units.

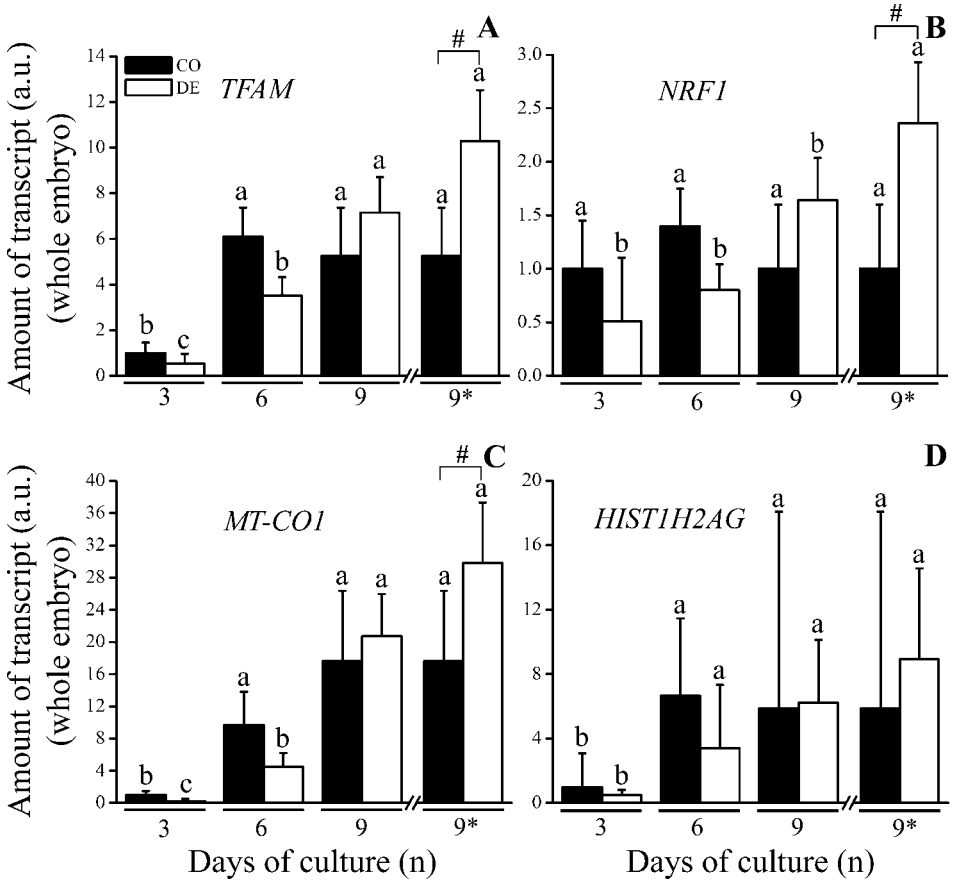

FIG. 5. Transcripts controlling mtDNA replication are increased in depleted parthenogenetic embryos. Embryos were analyzed at Day 3 (4- to 16-cell stages), Day 6 (early blastocyst stage), and Day 9 (hatching and hatched blastocyst stages). The amounts of *TFAM* (**A**), *NRF1* (**B**), *MT-CO1* (**C**), and *HIST1H2AG* (**D**) transcripts are expressed in relation to the CO group at Day 3. The bar labeled 9* indicates that transcript amounts at Day 9 were corrected by the mean cell number of each experimental group. Values are reported as means ± SEM. Bars with different letters above the bar denote a significant difference among days (3, 6, and 9; or 3, 6, and 9*) within the experimental group ($P < 0.05$). #Difference between experimental groups within day ($P < 0.05$). a.u. indicates arbitrary units.

from the DE group contained significantly more *TFAM* and *NRF1* transcripts than the controls (CO) at the same stage of development. Finally, transcripts for *HIST1H2AG*, a nuclear-encoded gene not involved in mtDNA function, did not differ between DE and CO groups at any stage of development, indicating that the transcriptional upregulation was directed specifically toward reestablishing the mtDNA copy numbers in depleted embryos. Together, these results suggest that one-cell embryos carrying low mtDNA copy numbers are able to replenish their mtDNA stocks by prolonging the expression window of nuclear-encoded genes involved in mtDNA replication and transcription.

DISCUSSION

During the preimplatation stages, the mammalian oocyte relies heavily on components stored in the cytoplasm (e.g., mitochondria and mtDNAs) to develop into a healthy blastocyst (reviewed by Dumollard et al. [8]). However, the number of mtDNA molecules accumulated in the oocyte by the end of oogenesis is widely variable (reviewed by May-Panloup et al. [19]). This, together with the fact that the quantity of mtDNA does not change in rodent embryos throughout preimplantation development [38], has led to the widespread belief that the mtDNA content in oocytes is positively correlated to developmental competence. Nonetheless, previous studies in human and animal models [20, 23, 25–27] had relied on approaches that may have prevented proper conclusions. Therefore, to test the hypothesis that threshold amounts of mtDNA in oocytes are necessary for development to the blastocyst stage, we used two original approaches in a bovine model (e.g., retrospective analysis of cytoplasm biopsy and depletion of mitochondria-enriched cytoplasmic fragments in one-cell embryos). We demonstrated that the mtDNA copy number at the one-cell stage is not related to developmental competence because competent bovine embryos have an intrinsic ability to reverse mtDNA depletion during development to the blastocyst stage.

The competence of an oocyte is defined by the ability of developing further into a blastocyst and to term. However, because of the lack of competence markers, many morphological and physiological characteristics correlated to competence have been used to predict oocyte quality. Using oocytes donated by cows previously known to differ in the developmental capacity of their oocytes, as measured by the blastocyst-formation rate, Tamassia et al. [22] investigated the role of mitochondria in oocyte competence and showed an effect of both mtDNA haplotype and oocyte ATP content. However, these authors were unable to show any relationship between mtDNA copy number in the oocyte and its ability to produce a viable embryo. They therefore proposed that because of the very large variation in oocyte mtDNA content observed both within and between animals, analysis of mtDNA amount and in vitro fertilization (IVF) of the same oocyte (e.g., a retrospective approach) would be required to resolve this question. Indeed, results from our comparison of oocytes derived from different size follicles indicate that the mtDNA content is higher in oocytes derived from larger follicles. A similar result was recently reported using rat oocytes [17]. Thus, we would expect that the better developmental capacity of oocytes derived from larger follicles [1, 5] could be due to the larger content of mtDNA. Nonetheless, because oocyte size is reduced in follicles that are less than 3 mm in diameter in cattle [1, 2], it is possible that the relationship between mtDNA and competence is not that of causality but rather due to the immaturity of oocytes that have not yet reached their full size.

Thus, in a second experiment, we developed an approach based on biopsy of activated oocytes to measure mtDNA copy number, followed by embryo culture to assess their developmental rates. Because of mitochondrial remodeling by the time the one-cell embryo underwent its first cleavage division [39], it was necessary to use parthenogenetic one-cell embryos to standardize and chronologically synchronize samples for the experimental procedures that followed. Moreover, previous studies have clearly demonstrated that the quantity of mtDNA does not change between oocytes and one-cell embryos in cattle [21, 40].

In contrast to previous experiments, our retrospective model clearly indicates that neither the mtDNA copy number nor the ranges of mtDNA amounts differed between competent and incompetent embryos. In humans, reports on oocytes derived from women who experience difficulty in producing embryos after IVF or intracytoplasmic sperm injection have described a relationship between mtDNA content and fertilization success [20, 23]. Similar results were also found in species such as porcine [26] and bovine [27]. Moreover, when the number of copies was compared in humans between women with a profile of ovarian insufficiency and those with a normal ovarian profile, the difference in mtDNA content was even higher [25]. Although these studies provide evidence of a relationship between mtDNA in unfertilized oocytes and fertilization failure, they cannot unequivocally establish that the number of mtDNA copies is the cause. Indeed, May-Panloup et al. [19] have reported that mtDNA copy number and ATP content are not correlated in the oocyte. Moreover, according to other reports [41, 42], mutations in the specific polymerase gamma gene (*POLG*) may be responsible for premature menopause. Considering the role of POLG in mtDNA replication and the similarities between premature menopause and ovarian insufficiency, May-Panloup et al. [19] suggested that a genetic failure compromising the machinery of mtDNA replication could lead to an mtDNA depletion similar to that reported in oocytes with poor developmental competence. Because mtDNA content and ATP synthesis are strongly correlated in somatic cells [13, 14], the finding that there is no correlation between these two variables in the oocyte could be due to the extraordinary amounts of mtDNA [15] and only ordinary requirements for ATP necessity during early cleavages [8, 10, 11, 19]. However, because of the sharp increase in ATP synthesis at the blastocyst stage [8, 10, 11, 19], it is likely that embryos that have the transcriptional and translational machinery to develop into healthy blastocysts can, regardless of the amount of mtDNA present at the one-cell stage, generate sufficient mtDNA copies to support the energy requirements for early development.

To better understand the mechanism by which one-cell embryos containing as much as 90% less mtDNA than the mean content develop into blastocysts, we developed an approach based on embryo centrifugation to partially deplete one-cell embryos of their mtDNA content and further culture them to assess the number of mtDNA copies and transcripts involved in the control of mtDNA replication and transcription through preimplantation development. The effect of centrifugation on stratification of the cytoplasmic components was studied by Tatham et al. [30], who found that centrifugation leads to the formation of very well-defined layers containing different cytoplasmic components. For instance, the layer in the centrifuged pole of the embryo is composed mainly of mitochondria. These mitochondria can be easily accessed and removed by micromanipulation without affecting other layers. Moreover, centrifuged embryos can be further cultured with no effect on their developmental rates.

In vitro culture of manipulated embryos allowed for experimental confirmation that one-cell embryos partially depleted of mtDNA produce healthy blastocysts with no effects on the developmental rates. Investigation of the levels of mtDNA in depleted embryos indicated that early embryos containing from 33% to 90% less mtDNA than nonmanipulated control embryos prolong the expression window of nuclear-encoded genes involved in mtDNA replication and transcription, possibly to replenish the mtDNA stores for later stages of development. As reported previously [18, 21], activation of mtDNA replication and transcription occurs at the compaction/blastulation stage of bovine embryos (between Days 3 and 6). Whereas depleted and control embryos amplified mtDNA copy numbers up to Day 6, only the depleted group continued amplification beyond Day 6, which enabled both groups to equalize mtDNA content by Day 9. During this same period (e.g., Days 6–9), a specific upregulation of factors controlling mtDNA replication (*NRF1* and *TFAM*) was seen in the depleted embryos. Similarly, in somatic cells partially depleted of their mtDNA, culture in the absence of the depleting agent leads to an upregulation of *TFAM* expression after replenishment of mtDNA levels [43]. Several mitochondrial-encoded genes within the nucleus, including OXPHOS subunits and factors involved in mtDNA replication and transcription, are transactivated by the *NRF1*. In turn, *TFAM* is a well-known regulator of mtDNA replication and transcription (reviewed by Smith et al. [18]). The role of the *NRF1* and *TFAM* during bovine embryogenesis has been suggested in a study showing that the levels of these transcripts increase concurrently with mtDNA replication [21]. Therefore, it is likely that the up-regulation of *NRF1* and *TFAM* transcripts in our depleted group accounted for the replenishment of mtDNA content and also the up-regulation of mtDNA expression (indicated by *MT-CO1* transcripts). Since mtDNA copy number is strongly correlated to the capacity of ATP synthesis in somatic cells [13, 14] and ATP synthesis is largely dependent on OXPHOS at the blastocyst stage [8, 10, 11, 19], we hypothesize that a minimum number of copies should exist in the cell to support its energetic needs. At the time of blastulation, when ATP synthesis increases sharply in bovine [8, 10, 11, 19], embryos containing fewer copies would extend their mtDNA amplification window to supply the energetic needs and enable embryo development. Meanwhile, if necessary, the energetic needs could be supplied by an increase of mitochondrial function mediated by an increase in the remaining organelles of nuclear-encoded proteins related to mtDNA transcription or OXPHOS, or an increase in the number of mitochondria [44]. However, we cannot exclude the hypothesis that the embryo could have supplied the required ATP by alternative pathways (e.g., an anaerobic pathway [45]). Taken together, these results indicate that competent embryos harbor an intrinsic ability to regulate the number of copies of mtDNA during early preimplantation development.

Here, we show that in contrast to previous studies [20, 23, 25–27], bovine one-cell embryos containing fewer copies of mtDNA develop into blastocysts, similarly to those containing a large number of copies. Moreover, embryos mechanically depleted of part of their mitochondria are able to develop normally into blastocysts and to replenish the mtDNA content by upregulating the expression of the genes involved in the mtDNA replication control. These results indicate that in spite of the wide variability in copy number, the content of mtDNA in oocytes is not related to their developmental competence. However, a failure to activate the machinery responsible for mtDNA replication could lead to developmental arrest due to an inability to replenish the mtDNA stores required around the

time of blastulation. This could be a factor in poor-quality oocytes (e.g., related to human infertility [20, 23, 25], cytoplasmic immaturity, or follicular atresia). For instance, it has been shown in humans that aging causes infertility by impairing oocyte competence (e.g., because of chromosomal abnormalities [46] and mutations in the mtDNA [47]). Mutations either in the nDNA or in the mtDNA could damage the machinery of mtDNA replication, leading to a mitochondrial dysfunction and developmental arrest after fertilization.

Although this study was carried out using bovine embryos, replication of mtDNA during early development was shown to occur also in pigs [48] and as part of a turnover event in mouse [49], suggesting that the above findings could be relevant to other mammalian species. Nonetheless, in monkey embryos, mitochondrial distribution and the control of mtDNA replication and transcription differ from what has been reported in cattle [50, 51]. Differences in the control of mtDNA replication and transcription refer mainly to an altered pattern of expression of genes involved [46]. Moreover, in vitro production of embryos has been shown to alter the control of mtDNA replication and transcription [49, 51, 52]. Therefore, further studies involving measurement of mtDNA amount will be required to determine whether the replenishment of mtDNA stores occurs in embryos produced in vivo and in species other than bovine.

In conclusion, using two original approaches, we provide evidence that mtDNA copy number is not related to the developmental competence of viable embryos obtained from fully grown bovine oocytes. This evidence is further supported by the finding that competent bovine one-cell embryos harboring fewer copies of mtDNA can reverse this condition by regulating the mtDNA replication at the blastocyst stage. Together, these findings clarify an unresolved discussion on the role of mtDNA in early development which pertains, among other practical applications, to the clinical use of mtDNA as a diagnostic tool in human and livestock fertility.

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