MARCOS ROBERTO CHIARATTI

ESTUDO DO EFEITO DA QUANTIDADE DE CÓPIAS DE DNA MITOCONDRIAL SOBRE O DESENVOLVIMENTO EMBRIONÁRIO:

Implicações na fertilidade e herança mitocondrial

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Tese apresentada ao curso de Pós-Graduação em Fisiopatologia Médica, área de concentração em Biologia Estrutural, Celular, Molecular e do Desenvolvimento, da Faculdade de Ciências Médicas, para obtenção do título de Doutor em Fisiopatologia Médica.

Orientador: Prof. Dr. Aníbal Eugênio Vercesi

Coorientador: Prof. Dr. Flávio Vieira Meirelles

CAMPINAS

Unicamp

2010

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DA FACULDADE DE CIÊNCIAS MÉDICAS DA UNICAMP

Bibliotecário: Sandra Lúcia Pereira – CRB-8ª / 6044

C43e

Chiaratti, Marcos Roberto

Estudo do efeito da quantidade de cópias de DNA mitocondrial sobre o desenvolvimento embrionário: implicações na fertilidade e herança mitocondrial / Marcos Roberto Chiaratti. Campinas, SP: [s.n.], 2010.

Orientadores: Aníbal Eugênio Vercesi, Flávio Vieira Meirelles

Tese (Doutorado) Universidade Estadual de Campinas. Faculdade de Ciências Médicas.

1. Mitocôndrias. 2. DNA mitocondrial. 3. Doenças mitocondriais. 4. Herança extracromossômica. 5. Oócitos. 6. Desenvolvimento embrionário. 7. Infertilidade feminina. I. Vercesi, Aníbal Eugênio. II. Meirelles, Flávio Vieira. III. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. IV. Título.

Título em inglês: Study of the effect of mitochondrial DNA amount on embryonic development: implications for fertility and mitochondrial inheritance

Keywords:
- Mitochondria
- DNA Mitochondrial
- Mitochondrial disease
- Extrachromosomal inheritance
- Oocytes
- Embryonic development
- Infertility, female

Titulação: Doutor em Fisiopatologia Médica
Área de concentração: Biologia Estrutural, Celular, Molecular e do Desenvolvimento

Banca examinadora:

Prof. Dr. Aníbal Eugênio Vercesi
Profa. Dra. Carmen Silvia Bertuzzo
Prof. Dr. Roger Frigério Castilho
Profa. Dra. Nadja Cristhina de Souza Pinto Lardner
Profa. Dra. Cláudia Ferreira da Rosa Sobreira

Data da defesa: 30-07-2010

Banca examinadora da tese de Doutorado

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Curso de pós-graduação em Fisiopatologia Médica da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

Data: 30/07/2010

Dedico...

Aos meus pais,

Tereza Cristina Octaviano Chiaratti e Marcos Amâncio Chiaratti,

e ao meu irmão,

Maurício Octaviano Chiaratti,

que sempre me incentivaram.

Aos meus avós,

Maria de Lourdes Alves Octaviano e Orlando Octaviano,

e ao meu tio,

Antônio Carlos Octaviano,

que também me incentivaram muito, desde meus primeiros passos.

À minha namorada,

Fernanda Altieri Ferreira,

que mesmo não tendo participado diretamente foi determinante para realização deste trabalho.

AGRADECIMENTOS

Ao professor ***Dr. Aníbal Eugênio Vercesi****, pela oportunidade, confiança e incentivo.*

Ao professor ***Dr. Flávio Vieira Meirelles****, que desde os primórdios da minha graduação tem me contagiado com o seu amor pela ciência, por todo seu apoio e estímulo.*

Ao professor ***Dr. Lawrence Charles Smith****, pela paciência, pelos ensinamentos e pela disponibilidade.*

À professora ***Dra. Joanna Poulton****, que me recebeu de braços abertos em Oxford, pelos ensinamentos e incentivos constantes.*

À ***Dra. Christina Ramires Ferreira, Dra. Karina Gotardello Zecchin, Mariana Pinheiros Fernandes****, e* ***Roberta Machado Ferreira****, pelo convívio, ensinamentos e oportunidade de trabalharmos juntos.*

Ao ***Dr. Moysés dos Santos Miranda*** *e* ***Fabiana Fernandes Bressan****, por toda a ajuda durante os experimentos e pela amizade.*

Aos ***Dr. Alexandre Rodrigues Caetano, Antonio Joaquim Rossini, Antônio Augusto Mendes Maia, Cláudia Lima Verde Leal, José Bento Sterman Ferraz, Júlio César de Carvalho Balieiro****, pelos incentivos.*

Aos amigos que conviveram comigo e que me incentivaram durante todo este período: ***Alexandre, Ana Catarina, Ângela, Brett, Bruno, Carina, Carol, Clara, Dani, Elizabeth, Felipe, Fernando, Flavinho, Franklin, Giovana, Heidge, Henderson, Isabele, Zé Rodrigo, Johanna, Juliana, Juliano, Karl, Kátia, Kirk, Kivia, Léo, Lindsay, Ligia, Lorna, Loro, Márcia, Minos, Nilton, Pati, Paula, Paulo, Pedrinho, Peninha, Rafael, Raquel, Rúbia, Simone, Sylvia, Tiago*** *e* ***Weruska****.*

Aos ***membros da banca*** *pela disponibilidade e valiosa discussão.*

À ***Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP),*** *ao* ***Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq)*** *e à* ***Fundação de Desenvolvimento da UNICAMP (FUNCAMP)*** *pelo apoio financeiro.*

O presente trabalho foi desenvolvido no Laboratório de Morfofisiologia Molecular e Desenvolvimento (LMMD), Departamento de Ciências Básicas (ZAB), Faculdade de Zootecnia e Engenharia de Alimento (FZEA), Universidade de São Paulo (USP), sob orientação do professor Dr. Aníbal Eugênio Vercesi e coorientação do professor Dr. Flávio Vieira Meirelles, na vigência de auxílios científicos concedidos pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) e Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq).

RESUMO

O DNA mitocondrial (mtDNA) dos mamíferos é composto por cerca de 16.500 pares de bases, tem herança exclusivamente materna, e codifica 13 polipeptídios essenciais para a função mitocondrial. Centenas a milhares de cópias de mtDNA estão presentes nas células somáticas dependendo da necessidade energética do tecido. No entanto, oócitos contêm mais de 150.000 cópias, no mínimo uma ordem de magnitude maior que a quantidade presente na maioria das células somáticas. Além disso, uma vez que o mtDNA não é replicado durante o desenvolvimento inicial, a quantidade de mtDNA por célula diminui a cada ciclo celular. Portanto, o número de cópias presentes no oócito deve ser suficiente para atender às necessidade energéticas das células embrionárias até que a replicação do mtDNA seja restabelecida. Considerando que há uma grande variabilidade da quantidade de mtDNA entre oócitos e que alguns trabalhos têm relacionado infertilidade e cópias de mtDNA no oócito, a quantidade de mtDNA poderia ser utilizada para selecionar embriões mais competentes a se desenvolverem. Para testar esta hipótese utilizou-se como modelo experimental o bovino uma vez que o desenvolvimento embrionário desta espécie é muito mais similar ao do humano que o de camundongo. Para tanto, em um primeiro experimento foram utilizados oócitos bovinos provenientes de folículos de diferentes tamanhos. Oócitos oriundos de folículos pequenos, os quais são sabidamente menos competentes a se desenvolverem a blastocisto, continham menos mtDNA comparado com oócitos oriundos de folículos maiores. No entanto, devido a grande variabilidade do número de cópias, num segundo experimento embriões partenogenéticos no estádio de uma célula sofreram biópsia para se determinar o conteúdo de mtDNA antes de serem cultivados para acessar a capacidade de desenvolvimento. Em contraste com achados prévios, o número de cópias de mtDNA nas biópsias não diferiu entre embriões competentes e incompetentes, indicando que o conteúdo de mtDNA não está relacionado com a competência de desenvolvimento a blastocisto. Para confirmar este achado, embriões no estádio de uma célula foram depletados em mais de 60% do seu conteúdo de mtDNA por centrifugação seguido da remoção de parte da fração citoplasmática rica em mitocôndrias.

Surpreendentemente, os embriões depletados desenvolveram-se normalmente a blastocisto, os quais continham número de cópias de mtDNA similar a controles não manipulados. O desenvolvimento dos embriões depletados foi acompanhado por um aumento na expressão de genes (*TFAM* e *NRF1*) que controlam a replicação e transcrição do mtDNA, indicando uma habilidade intrínseca do embrião bovino em restaurar o conteúdo de mtDNA no estádio de blastocisto. Em conclusão, embriões bovinos competentes são capazes de regular o conteúdo de mtDNA no estádio de blastocisto independentemente do número de cópias presente no oócito. Estes achados contrariam o que foi descrito em camundongos, ressaltando a necessidade de estudos com espécies mais semelhantes ao homem antes do uso clínico do mtDNA como ferramenta para o diagnóstico de fertilidade em mulheres. Além disso, este trabalho tem implicação na manipulação da herança mitocondrial e, portanto, na prevenção da transmissão de sérias patologias causadas por mutações no mtDNA.

ABSTRACT

The mammalian mitochondrial DNA (mtDNA) is composed by only about 16,500 base pairs, is exclusively inherited from the mother, and encodes 13 polypeptides essential for mitochondrial function. Hundreds to thousands mtDNA copies are found in somatic cells depending on the energetic requirement of the tissue. However, oocytes contain more than 150,000 copies, at least an order of magnitude greater than most somatic cells. Moreover, since replication of mtDNA is downregulated during early development, the mtDNA content per cell decreases after each cell cycle. Therefore, mtDNA copy number in oocytes should be enough to support the energetic requirement of embryonic cells until mtDNA replication to be restablished. Considering there is a wide variability of mtDNA copy number among oocytes and there are reports showing a link between infertility and oocyte mtDNA copy number, the content of mtDNA could be used to select embryos more competent to develop. To test this hypothesis we used the bovine as an experimental model since its embryonic development is more similar to human than the murine is. Therefore, in a first experiment bovine oocytes derived from follicles of different sizes were used. Oocytes obtained from small follicles, known to be less competent to develop into blastocysts, contained less mtDNA than those originated from larger follicles. However, due to the high variability in copy number, in a second experiment 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 over than 60% of their mtDNA 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 non-manipulated 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. In conclusion, competent bovine embryos are able to regulate their mtDNA content at the blastocyst stage regardless of the copy numbers present in oocytes. These findings are in disagreement with that reported for mice, highlighting the need for studies using species more similar to human before the clinical use of mtDNA as a diagnostic tool in woman fertility. Moreover, these findings are important to manipulate mitochondrial inheritance and, therefore, to prevent transmission of important disorders caused by mtDNA mutations.

LISTA DE ABREVIAÇÕES

ATP	Adenosina tri-fosfato
CVS	Amostragem das vilosidades coriônicas
FIV	Fecundação *in vitro*
H^+	Hidrogênio
HIST1H2AG	Histona grupo 1, H2AG
ICSI	Injeção intracitoplasmática de espermatozóide
mRNA	Ácido ribonucléico mensageiro
MT-CO1	Citocromo c oxidase I
mtDNA	DNA mitocondrial
NARP	Neuropatia, ataxia e retinose pigmentar
nDNA	Ácido desoxirribonucléico nuclear
NRF1	Fator respiratório nuclear 1
OXPHOS	Fosforilação oxidativa
PGC	Células primordiais germinais
PGD	Diagnóstico pré-implantação
PND	Diagnóstico pré-natal
POLG	DNA polimerase gama
POLRMT	RNA polimerase mitocondrial
PPARG	Co-ativador PPAR gama 1
RNA	Ácido ribonucléico
rRNA	Ácido ribonucléico ribossômico
ROS	Espécies reativas de oxigênio
TFAM	Fator de transcrição mitocondrial A
TFB1M	Fator de transcrição mitocondrial B1
TFB2M	Fator de transcrição mitocondrial B2
tRNA	Ácido ribonucléico transportador

LISTA DE FIGURAS

Página

SUMÁRIO

1- INTRODUÇÃO

Defeitos no oócito, o gameta feminino, são um dos principais fatores determinantes de infertilidade na mulher (1, 2). Isto ocorre porque o oócito é responsável por suprir diversos componentes (e.g. RNAs, proteínas, organelas e substratos energéticos) ao embrião enquanto este é incapaz de sintetizá-los (3-7). Estes componentes são sintetizados e armazenados no oócito durante a foliculogênese, e tem efeito direto sobre a competência de desenvolvimento do gameta (3-7). Uma vez que os oócitos possuem uma enorme quantidade de mitocôndrias, é provável que estas desempenhem uma função importante durante a embriogênese (8-10).

A mitocôndria é uma organela especializada na síntese de energia na forma de adenosina tri-fosfato (ATP), a qual é necessária para a maioria dos eventos que ocorrem no interior da célula (11). A função mitocondrial é coordenada por dois genomas, o nuclear (nDNA) e o mitocondrial (mtDNA) (12-15). Múltiplas cópias de mtDNA estão presentes no interior da célula, as quais estão envolvidas, exclusivamente, na codificação de polipeptídios que compõem a maquinaria de síntese de ATP na mitocôndria (8). Além disso, o número de cópias de mtDNA está diretamente relacionado com a capacidade de síntese de ATP (8) e mutações no mtDNA podem comprometer a síntese de ATP mitocondrial (12, 16-18).

O número de cópias de mtDNA varia muito entre oócitos, mesmo quando provenientes de uma mesma mulher (8, 19-22). Além disso, alguns trabalhos têm relacionado quantidade de mtDNA no oócito e infertilidade em diversas espécies (8, 19-21, 23, 24). Mutações no mtDNA podem comprometer a fertilidade do oócito (25), além de serem responsáveis por importantes patologias durante a vida adulta (12, 26, 27). Todavia, pouco se conhece a respeito da herança mitocondrial e da relação entre mtDNA e fertilidade (8). Neste sentido, novos estudos que abordem o assunto são necessários. Por exemplo, se o número de cópias de mtDNA estiver relacionado com a competência de desenvolvimento do oócito, o mtDNA poderia ser utilizado como parâmetro de seleção de oócitos competentes (8). Além disso, o melhor compreendimento do assunto permitiria o desenvolvimento de técnicas

para a correção de defeitos do oócito (28-35) e até mesmo prevenir a transmissão de doenças mitocondriais causadas por mutações no mtDNA (27, 36-38).

2- REVISÃO DE LITERATURA

A Revisão de Literatura apresentada a seguir foi submetida para publicação em dois periódicos especializados. A convite do comitê editorial do periódico *Mitochondrion*, parte do texto foi enviado para publicação no mesmo periódico, e parte foi publicado no periódico *Plos Genetics* (vide Apêndice 1).

2.1- Bioenergética mitocondrial

A mitocôndria, organela presente no citoplasma das células eucarióticas, é conhecida como o "motor" da célula devido ao seu papel no metabolismo oxidativo energético. A maior parte da energia necessária para os eventos celulares é produzida na mitocôndria na forma de ATP (11). O estabelecimento na célula de uma organela especializada na síntese de ATP é considerado evento chave no curso evolutivo das espécies, possibilitando o surgimento de organismos altamente complexos como os mamíferos (39). A síntese de ATP na mitocôndria é realizada por um processo conhecido como fosforilação oxidativa (OXPHOS). Por meio deste processo, elétrons oriundos, principalmente, da oxidação de carboidratos são doados por carreadores específicos a uma cadeia de moléculas organizada na membrana mitocondrial interna como quatro complexos enzimáticos (complexos I, II, III e IV). O fluxo de elétrons por estes complexos libera energia, a qual é utilizada para armazenar prótons (H^+) oriundos da matriz mitocondrial no espaço inter-membranas. Isto gera um gradiente de H^+ na mitocôndria, o qual pode ser convertido em ATP através do retorno dos H^+ para a matriz (11). Este evento é regulado por um quinto complexo enzimático (complexo V ou ATP sintase) também localizado na membrana mitocondrial interna que converte a energia gerada pela despolarização mitocondrial em ATP (11). Desta forma, para cada mol de glicose, 26 moles de ATP são gerados pela OXPHOS em comparação a apenas dois moles gerados pela glicólise. Assim, o ATP sintetizado na mitocôndria é responsável por suportar a grande maioria dos processos dependentes de energia na célula (11).

2.2- Regulação da função mitocondrial

A mitocôndria é composta por aproximadamente 1.500 diferentes polipeptídios, sendo a maioria codificada pelo nDNA (12). No entanto, dos cerca de 80 polipeptídios envolvidos na OXPHOS, 13 são codificados na mitocôndria pelo mtDNA e compõem parte dos complexos enzimáticos I, III, IV e V. O mtDNA também abriga 22 RNAs transportadores (tRNAs) e 2 RNAs ribossômicos (rRNAs)

que estão envolvidos no processo de tradução dos RNAs mensageiros (mRNAs) mitocondriais (13). Devido à ausência de íntrons e de sequências não traduzidas nas extremidades 5'e 3' do DNA, o mtDNA dos mamíferos contém somente cerca de 16.500 pares de bases (13). Ainda, a molécula de mtDNA possui formato circular e é organizada em um complexo núcleo-protéico conhecido como nucleóide (40). Diferente do nDNA, existem centenas a milhares de cópias de mtDNA em cada célula e o número de cópias varia dependendo do metabolismo e do tipo celular. Células de tecidos com elevada exigência energética (e.g. neurônios e células musculares) contém mais moléculas de mtDNA do que células de tecidos com menor exigência (e.g. leucócitos e monócitos) (12).

De acordo com a teoria endossimbiótica, as mitocôndrias são descendentes evolutivos de uma célula procariótica aeróbia (39). Esta teoria propõe que as mitocôndrias tornaram-se especializadas na síntese de energia após simbiose com uma célula anaeróbia. Este evento simbiótico ocorreu provavelmente a 1,5 bilhões de anos. A dupla membrana mitocondrial, o genoma circular, a prevalência de um conjunto completo de tRNAs e rRNAs e o código genético parcialmente degenerado são fósseis moleculares que suportam esta teoria (41). Durante a evolução, provavelmente para ser mais eficiente e para controlar a replicação e função mitocondrial, a maior parte dos genes mitocondriais foram aparentemente transferidos para o nDNA. Isso inclui cerca de 70 polipeptídios envolvidos na OXPHOS bem como fatores responsáveis por regular a replicação, transcrição e parte da tradução do mtDNA (12, 13).

Uma vez que a função mitocondrial depende de polipeptídios codificados tanto pelo nDNA como pelo mtDNA (12), a expressão de subunidades codificadas por ambos os genomas deve ser estreitamente coordenada (14, 15). Diversos fatores regulam o metabolismo da célula, mas o mais conhecido é o coativador PPAR gama 1 (PPARG) que é codificado pelo nDNA (42-44). A nível nuclear, o PPARG regula a expressão dos fatores de respiração nuclear 1 e 2 (NRF1 e NRF2).

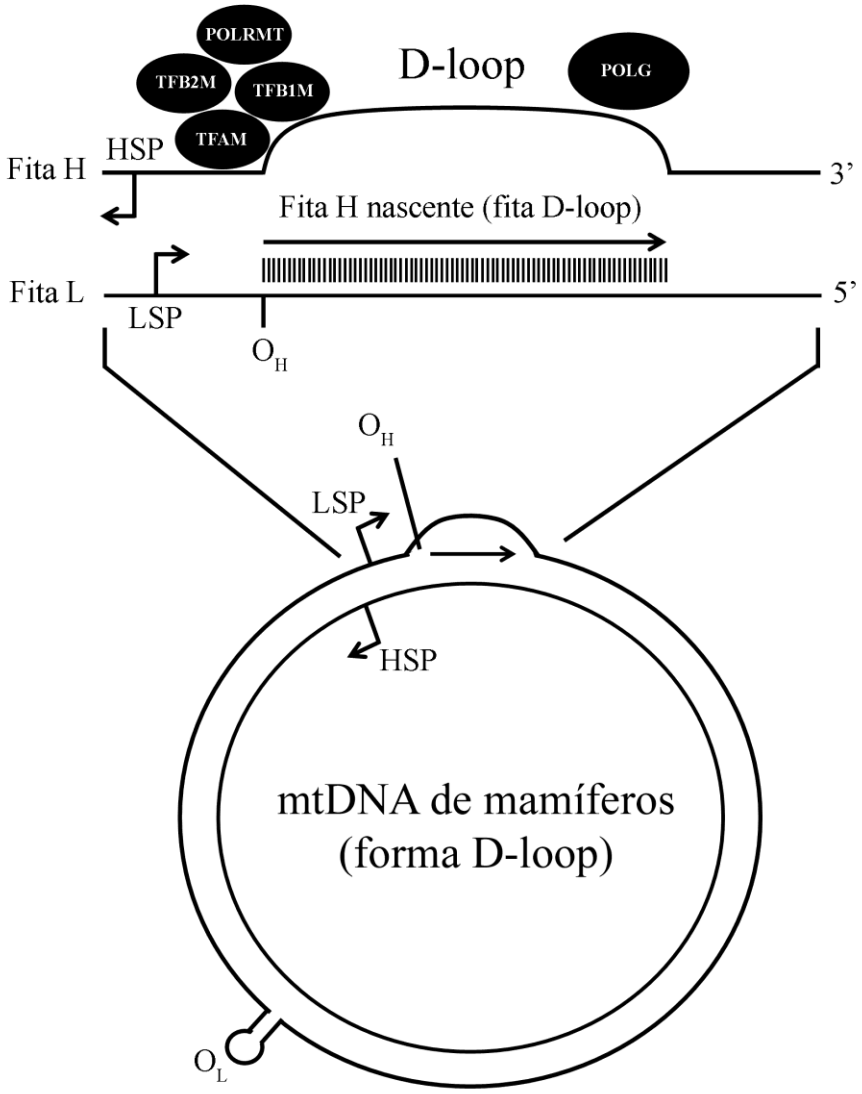

Figura 1. Representação esquemática do DNA mitocondrial (mtDNA) de mamíferos e os fatores nucleares responsáveis pelo controle da replicação e transcrição mitocondrial. A região regulatória do mtDNA (D-loop) foi expandida para ressaltar como a replicação da molécula é iniciada e os fatores nucleares envolvidos neste evento (TFAM, TFB1M, TFB2M, POLRMT, POLG etc). A transcrição das fitas pesada (H) e leve (L) inicia-se a partir dos promotores de replicação das mesmas (HSP e LSP, respectivamente). A síntese da fita pesada inicia-se na sua origem de replicação (O_H) e dispara a replicação da fita leve ao passar pela origem de replicação da mesma (O_L) (adaptado de (13, 45)).

Por sua vez, o NRF1 e o NRF2 coordenam, também no núcleo, a expressão de polipeptídios da OXPHOS bem como de fatores que controlam a replicação, transcrição e tradução do mtDNA (42-45). Em nível mitocondrial tanto a replicação como a transcrição do mtDNA são reguladas por fatores importados pela mitocôndria que interagem com uma região específica do mtDNA conhecida por D-loop (13, 42-46). Nesta região estão localizados os sítios promotores de transcrição (HSP e LSP) da fita pesada (H) e da fita leve (L) do mtDNA e a origem de replicação da fita pesada (O_H) (Figura 1). Fatores como o fator de transcrição mitocondrial A (TFAM), fatores de transcrição mitocondrial B1 e B2 (TFB1M e TFB2M) e a RNA polimerase mitocondrial (POLRMT) interagem com a D-loop e são responsáveis por iniciarem a transcrição bidirecional do mtDNA (13, 45-47). Estes mesmos fatores também regulam a replicação do mtDNA, pois a fita crescente de RNA serve como iniciador de replicação da fita pesada do mtDNA (13, 46, 47). A fita leve, por sua vez, tem sua replicação iniciada quando a replicação da fita pesada atinge a sua origem de replicação (O_L) permitindo assim o acesso dos fatores de replicação (Figura 1) (13, 46, 47). A replicação do mtDNA é conduzida por uma DNA polimerase específica (POLG) que também é codificada pelo núcleo. Embora existam evidências de que a replicação da molécula de mtDNA ocorra de forma diferente ("*Strand-coupled model*"[1]), o modelo "*Strand-displacement model*"[2] descrito acima é o mais aceito (Figura 1) (13, 46-52). Com relação à transcrição, ambas as fitas do mtDNA são transcritas em duas fitas de RNA policistrônico que então são clivadas em mRNAs, tRNAs e rRNAs (13, 15, 45, 46). Os tRNAs e rRNAs são utilizados exclusivamente na tradução dos mRNAs codificados pelo mtDNA (13, 46). Apesar do controle nuclear da replicação e transcrição do mtDNA, estes eventos são independentes do ciclo celular e ocorrem de forma contínua na célula, mesmo em células recém divididas (53).

[1]Modelo de fitas acopladas; [2]Modelo de fitas desacopladas

2.3- Herança mitocondrial

A herança mitocondrial nos mamíferos é exclusivamente uniparental ou materna uma vez que as mitocôndrias são herdadas unicamente da mãe (26, 54-56). Isto ocorre porque o oócito contribui com uma quantidade muito maior de mitocôndrias que o espermatozóide (100.000 mitocôndrias versus 100, respectivamente) (57). Além disso, as poucas mitocôndrias paternas carregadas para dentro do oócito pelo espermatozóide estão marcadas com ubiquitina para serem destruídas por proteólise durante os estádios iniciais do desenvolvimento embrionário (58, 59). A eliminação do mtDNA paterno é vantajosa, uma vez que este pode ter sido danificado pelos altos níveis de espécies reativas de oxigênio (ROS) geradas no espermatozóide antes da fecundação (55). Se tais mutações fossem transmitidas para as próximas gerações, poderiam comprometer a função mitocondrial resultando em disfunção da organela e sérias patologias (vide item 2.5).

Outra importante característica da biologia mitocondrial refere-se à homogeneidade do seu genoma dentro de um mesmo indivíduo (26). Centenas a milhares de cópias de mtDNA estão presentes em cada célula nucleada e indivíduos normais são homoplásmicos, e.g. todas as moléculas de mtDNA são idênticas (60). Uma vez que as mitocôndrias são o principal sítio de reações oxidativas na célula elas também são a principal fonte de ROS (12). Devido, entre outros motivos, à proximidade a estes sítios, a taxa de substituição de nucleotídeos no mtDNA é de cinco a dez vezes maior que no nDNA (12, 61-63). Como resultado, o mtDNA é altamente polimórfico e moléculas mutantes e selvagens podem coexistir em uma mesma célula, tecido ou indivíduo (heteroplasmia) (12, 26). Assim, esperar-se-ia encontrar uma grande variação de nucleotídeos entre indivíduos e uma variação ainda maior entre espécies. No entanto, as sequências codificantes do mtDNA foram extremamente preservadas durante a evolução (12, 26, 64). Além disso, embora existam diferenças em sequências não codificantes entre indivíduos de uma mesma espécie, é raro encontrar tais diferenças dentro de um mesmo indivíduo (26). Portanto, a baixa

frequência de heteroplasmia em uma célula ou indivíduo contrasta com a alta frequência de polimorfismo no mtDNA na população (26, 27). Trabalhos pioneiros realizados em vacas heteroplásmicas para polimorfismos na D-loop (região não codificante) mostraram que a homoplasmia é restabelecida dentro de poucas gerações ou até menos, e.g. dentro de uma geração (60, 65-69). Em humanos um padrão similar de rápida segregação resultando em trocas na frequência alélica vem sendo descrito em famílias afetadas por doenças causadas por mutações no mtDNA (26, 70-74). No entanto, permanece desconhecido como é possível um mtDNA polimórfico segregar tão rápido para restabelecer o estado homoplásmico nas gerações seguintes (26, 27, 64).

Hauswirth e Laipis (10, 65-69) propuseram que a redução do número de cópias de mtDNA e subsequente proliferação clonal de um pequeno grupo de moléculas durante o crescimento do oócito poderia explicar as rápidas trocas na frequência genotípica do mtDNA entre gerações. Esta hipótese foi baseada no grande aumento, cerca de 100 vezes, do número de cópias de mtDNA durante a oogênese e/ou foliculogênese. Mais tarde, os mesmos pesquisadores propuseram um segundo mecanismo que também contribuiria para o restabelecimento da homoplasmia (10, 65-69). O mtDNA não é replicado durante os primeiros estádios do desenvolvimento pré-implantação levando as moléculas pré-existentes a segregarem entre as células embrionárias do blastocisto (10, 75-78). A massa celular interna neste estádio compreende somente algumas poucas células que irão dar origem ao embrião propriamente dito. Portanto, tanto a proliferação clonal de mtDNA no oócito em crescimento como a segregação do mtDNA entre as células do blastocisto poderiam funcionar como um gargalo genético que seleciona apenas um pequeno número de moléculas de mtDNA para colonizarem a próxima geração (55, 79-82).

As hipóteses a respeito dos mecanismos que explicariam o gargalo genético descrito inicialmente por Hauswirth e Laipis (10, 65-69) foram investigadas por Jenuth et al. (83), que criaram linhagens heteroplásmicas de camundongos para estudar as bases moleculares deste evento. Estes

pesquisadores concluíram que o principal componente da rápida segregação de polimorfismos no mtDNA seria a segregação ao acaso destas moléculas durante a oogênese (83). Neste estudo (83), a variância genotípica foi avaliada em células germinais primordiais (PGCs) e concluiu-se que a mesma é marcadamente reduzida se comparada à variação genotípica verificada em oócitos primários e maturos. Assim, uma redução intensa da quantidade de mtDNA durante o desenvolvimento embrionário inicial de 250.000 cópias por oócito para cerca de 200 cópias por PGC, seguido por uma grande expansão do número de cópias durante a oogênese para repor as 250.000 cópias no oócito, explicaria o padrão de segregação descrito (55, 79-82).

Recentemente este assunto foi retomado por alguns grupos de pesquisa que mensuraram a quantidade de mtDNA em células individuais de camundongos ao longo da oogênese e/ou foliculogênese (75, 78, 84, 85). Conforme proposto por Jenuth et al. (83), o reduzido número de cópias de mtDNA presente nas PGCs de camundongos sete dias e meio após a fecundação, parece corresponder ao número de unidades segregantes estimado a partir de análises pós-natal. Com base em uma simulação matemática realizada utilizando os dados gerados por Cree et al. (78), sugeriu-se que cerca de 70% da variação genotípica do mtDNA entre duas gerações é explicada pela partição das moléculas de mtDNA entre as células filhas antes da replicação do mtDNA ser retomada nas PGCs (78). Os outros 30% de variação seriam fruto da intensa proliferação do mtDNA durante a expansão das PGCs (78). No entanto, este resultado é controverso uma vez que outro grupo (75, 84) encontrou que a quantidade de mtDNA nas PGCs é de aproximadamente 2.000 cópias (também mensurado sete dias e meio após a fecundação) e não diminui para 200 cópias como sugerido por Jenuth et al. (83), Cree et al. (78) e Wai et al. (85). Esta discrepância de resultados pode ser devido a diferenças técnicas nos protocolos de quantificação de mtDNA e também a diferenças quanto ao momento em que a replicação do mtDNA é retomada nas PGCs entre as linhagens de camundongos utilizadas nos estudos. Os oócitos

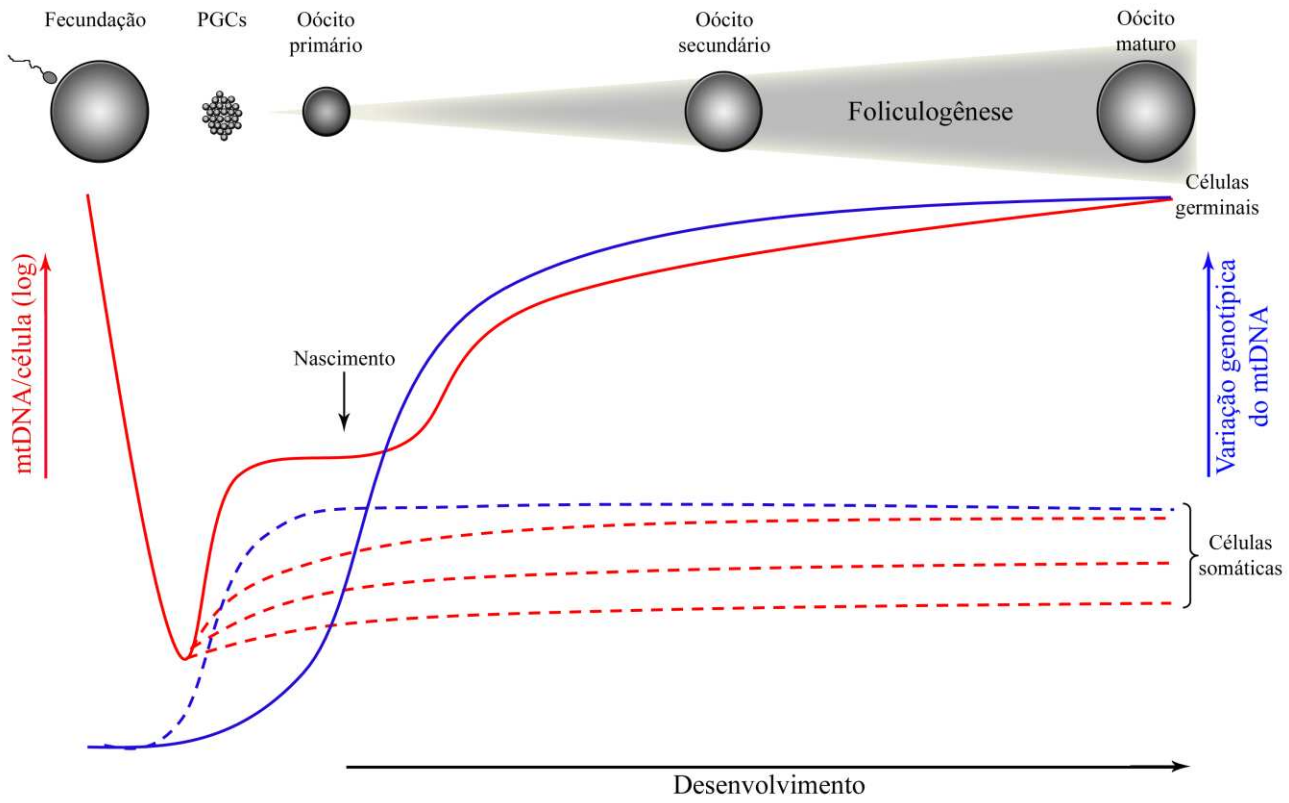

Figura 2. Quantidade de DNA mitocondrial (mtDNA) e variação genotípica ao longo do desenvolvimento de células germinativas e somáticas de mamíferos. Embora a variação genotípica do mtDNA em células somáticas aumente cedo durante o desenvolvimento devido a diferenciação celular, o mesmo só ocorre nas células germinais após o nascimento, durante a foliculogênese. Isso significa que em humanos o genótipo mitocondrial da próxima geração seja somente definido na idade adulta da mãe durante a foliculogênese que ocorre a cada ciclo de 28 dias.

dos camundongos utilizados por Cao et al. (75, 84) possuíam cerca de uma vez e meia mais mtDNA que os oócitos dos camundongos utilizados por Cree et al. (78), o que sugere que existam diferenças entre os animais utilizados. Por exemplo, a antecipação do início da replicação do mtDNA em um ou dois ciclos durante o desenvolvimento das PGCs no caso do estudo de Cao et al. (75, 84) poderia justificar tal divergência. Todavia, Wai et al. (85) revisaram seus achados iniciais de número de cópias de mtDNA e heteroplasmia durante o desenvolvimento pré e pós-implantação de camundongos (83) e concluíram que a redução da quantidade de mtDNA nas PGCs durante o desenvolvimento inicial não é suficiente para explicar as trocas nas frequências genotípicas descritas para o mtDNA (55, 79-82). Wai et al. (85) identificaram que uma sub-população de moléculas de mtDNA é preferencialmente replicada durante a foliculogênese como inicialmente sugerido por Hauswirth e Laipis (10, 65-69). De acordo com este achado (85), apenas um pequeno grupo de moléculas seria responsável por povoar o oócito e consequentemente a próxima geração. Este mecanismo, portanto, poderia ser responsável pelo rápido restabelecimento da homoplasmia mitocondrial entre gerações (Figura 2) (26, 85), no entanto, há controvérsias sobre o assunto (86). Além destes, outros mecanismos parecem influenciar a herança mitocondrial em casos em que polimorfismos estão presentes em regiões codificantes do mtDNA (vide item 2.5).

2.4- A mitocôndria e o desenvolvimento pré-implantação

O oócito depende dos diversos componentes sintetizados e armazenados durante a oogênese para ser fecundado e se desenvolver. Sabe-se que estes componentes, e.g., RNAs, proteínas, e substratos energéticos, são críticos para as primeiras fases do desenvolvimento enquanto o embrião apresenta uma capacidade limitada de transcrição do seu material genético (3-7). Da mesma forma, o número de cópias de mtDNA aumenta drasticamente durante a oogênese e foliculogênese, culminando em oócitos maturos contendo centenas de milhares a mais de um milhão de cópias de mtDNA (8, 10, 45, 75, 78, 85, 87, 88). Esta

enorme quantidade de mtDNA é maior que a de qualquer outro tipo celular existente no organismo (8, 10), o que chama a atenção para a provável importância das mitocôndrias para a fecundação e o desenvolvimento inicial (8, 45). Por exemplo, oócitos contendo um reduzido número de cópias de mtDNA poderiam apresentar um desenvolvimento inferior, ou até mesmo serem incapazes de se desenvolver após a fecundação, se comparado a oócitos com um estoque normal de cópias (8, 19-21, 23).

Nos oócitos e nos embriões, durante os primeiros estádios após a fecundação, as mitocôndrias caracterizam-se por seu pequeno tamanho, pelo formato arredondado, por apresentarem uma matriz bastante densa e pela presença de poucas cristas arranjadas de forma concêntrica (9). Estas características são indicativas do baixo estado metabólico dessas organelas durante o período, o qual é progressivamente alterado ao longo do desenvolvimento embrionário (9, 77, 88-93). As profundas mudanças morfológicas e funcionais que as mitocôndrias sofrem durante o desenvolvimento embrionário ocorrem concomitantemente com o aumento da demanda por energia por parte do embrião (94, 95). Ao longo das clivagens, os componentes herdados do oócito necessários para a sobrevivência do embrião vão se esgotando até que a partir do estádio de oito células (e.g. embrião humano e bovino), o embrião passa a sintetizar os componentes necessários para o seu próprio desenvolvimento (3, 96). Tais eventos de síntese, como transcrição e tradução, demandam grande quantidade de ATP. Além disso, outros eventos, como a formação da blastocele, também exigem grande suprimento de energia (94, 95). Assim, a quantidade de ATP produzida no embrião aumenta em função do desenvolvimento e este é em sua grande maioria sintetizado pelas mitocôndrias (9, 89, 90). No entanto, o número de cópias de mtDNA parece se manter constante durante todo o desenvolvimento pré-implantação (10, 75-78), posto que a replicação mitocondrial é somente iniciada após a implantação do embrião no útero (97). Na verdade, o número de cópias se mantém constante quando se considera o embrião como um todo. Porém, a cada clivagem as cópias presentes em uma célula embrionária

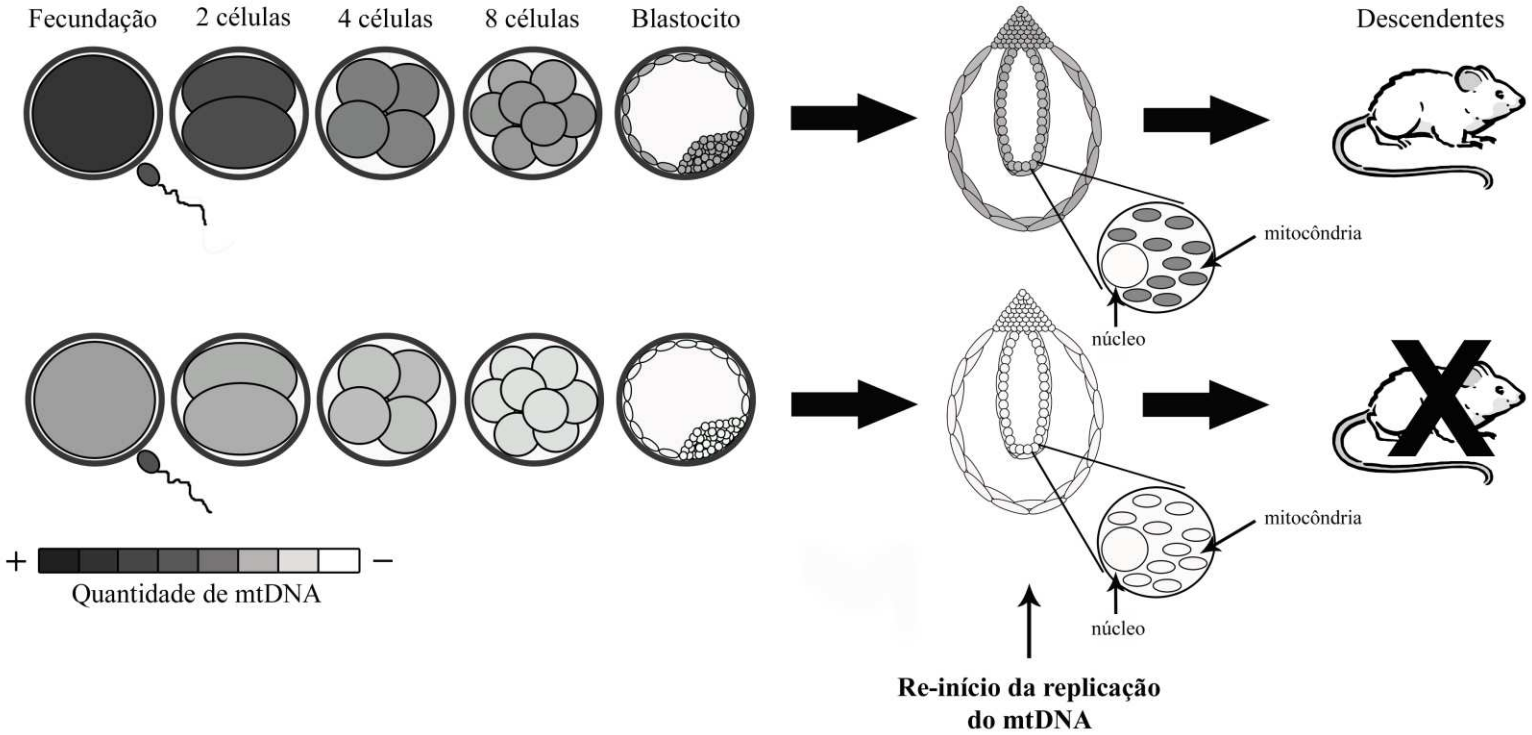

Figura 3. Modelo do efeito do número de cópias de DNA mitocondrial (mtDNA) em oócitos de camundongos sobre a progressão do desenvolvimento. Esta figura ilustra o desenvolvimento pós-fecundação de oócitos contendo mtDNA acima (parte superior da figura) e abaixo (parte inferior da figura) de um limiar teórico de cópias necessárias para o desenvolvimento. Após a fecundação, a clivagem embrionária leva a segregação do mtDNA entre as células filhas porque o mtDNA não é replicado em murinos até o estádio de ovo cilíndrico (cerca de seis dias após a fecundação). Desta forma, o número de moléculas de mtDNA por célula diminui a cada ciclo celular resultando no bloqueio do desenvolvimento se o número de cópias inicial estava abaixo do limiar. O bloqueio do desenvolvimento é provavelmente causado pela diluição das cópias de mtDNA para abaixo de um número mínimo necessário para suportar as necessidades energéticas da célula (adaptado de (98)).

(blastômero) são partilhadas entre as células filhas, resultando em redução exponencial do número de cópias por blastômero durante o desenvolvimento inicial (75, 78, 97). Isto ressalta a importância da quantidade de mtDNA herdada do oócito, visto que as cópias de mtDNA devem ser suficientes para povoar cada blastômero e suprir a energia necessária para o prosseguimento do desenvolvimento, até que a replicação do mtDNA seja retomada (Figura 3).

Apesar da grande quantidade de mtDNA presente no momento da ovulação, o número de cópias de mtDNA varia consideravelmente entre oócitos (20-22, 87, 88, 99). Embora não se saiba o que determina tal variação ou o seu efeito sobre o desenvolvimento, vários trabalhos têm relacionado cópias de mtDNA e fertilidade em diversas espécies (19-21, 23, 24). Por exemplo, Reynier et al. (20) observaram que em humanos, oócitos não fecundados devido a defeitos intrínsecos possuíam uma menor quantidade de mtDNA comparado a oócitos em que a falha na fecundação foi causada por defeitos relacionados a outros fatores. Utilizando um delineamento experimental similar, Almeida Santos et al. (21) também reportaram uma relação entre cópias de mtDNA e fecundação em humanos. May-Panloup et al. (19), por sua vez, reportaram evidências ainda mais claras de tal efeito. Neste trabalho, os autores verificaram que a quantidade de mtDNA é consideravelmente reduzida em oócitos provenientes de mulheres que sofrem de insuficiência ovariana. Este tipo de síndrome está associado a problemas no ciclo reprodutivo, oócitos de baixa qualidade, e reduzidas taxas de produção de embriões (19).

Embora existam indícios de que a depleção de cópias de mtDNA está associada a um efeito negativo na competência do oócito (19-21, 100, 101), há controvérsias sobre o assunto (8, 87, 92). Além disso, sabe-se muito pouco sobre como a quantidade de mtDNA poderia influenciar o desenvolvimento embrionário (8). Wai et al. (101) mostraram recentemente que um número mínimo de cópias de mtDNA no oócito é necessário para povoar as células embrionárias até que a replicação do mtDNA seja retomada. Caso os oócitos possuam uma quantidade de mtDNA abaixo de um mínimo (estimado em 40.000 a 50.000 cópias para

camundongos), o desenvolvimento pós-implantação seria interrompido devido a escassez de cópias nas células embrionárias. Contudo, a existência de diferenças entre espécies com relação ao desenvolvimento embrionário e função mitocondrial impede que estes resultados sejam extrapolados para os humanos antes de serem confirmados em outros modelos animais ou mesmo no homem (98). Por exemplo, em bovinos e suínos a replicação do mtDNA é retomada no estádio de blastocisto, cerca de seis dias após a fecundação (45, 88, 100). Nos camundongos o estádio de blastocisto é alcançado três dias após a fecundação, mas a replicação do mtDNA só é restabelecida seis dias após a fecundação, quando o embrião encontra-se no estádio de ovo cilíndrico (97). Embora Mtango et al. (102) não tenham estudado quando a replicação do mtDNA é retomada em primatas não humanos, eles encontraram fortes evidências de que a mesma é restabelecida em estádios anteriores ao descrito para camundongos. Estas divergências ressaltam a importância de novos estudos com outros modelos experimentais que confirmem os achados descritos para camundongos (101) e esclareçam como o número de cópias de mtDNA nos oócitos afeta a competência dos mesmos em se desenvolverem a termo e gerarem indivíduos saudáveis (19-21, 23, 24).

O estudo da relação entre mtDNA e fertilidade é de grande importância para os humanos uma vez que se comprovada tal relação, o número de cópias de mtDNA poderia ser utilizado como parâmetro de seleção de oócitos ou embriões com maior potencial de desenvolvimento (19-21, 23, 24, 98, 99, 101). Por exemplo, o número de cópias de mtDNA poderia ser facilmente determinado em casos em que o embrião sofre biópsia com o propósito de diagnóstico de mutações (vide item 2.6). Neste caso, a quantificação de mtDNA em um ou mais blastômeros em paralelo ao diagnóstico de interesse permitiria aumentar as taxas de gestação, pois somente os embriões com maior potencial de desenvolvido seriam transferidos para o útero da mulher (98, 101). Outro aspecto a ser considerado sobre a importância deste tipo de estudo é que a suplementação de oócitos, com baixo potencial de desenvolvimento, com mitocôndrias poderia

corrigir falhas intrínsecas dos mesmos (28, 31-35, 103, 104). Por exemplo, sabe-se que parte dos casos de mulheres inférteis é causado por defeitos no citoplasma de seus oócitos que resultam em embriões com reduzida capacidade de implantação uterina e desenvolvimento a termo (8, 9, 103-107). Nestes casos, a introdução nos oócitos incompetentes de uma pequena porção de citoplasma proveniente de oócitos de mulheres férteis resultou em uma considerável melhora do desenvolvimento e nascimento de várias crianças (28, 29, 32-34). Uma vez que alguns autores têm relacionado este tipo de infertilidade com mitocôndrias defeituosas (8, 9, 103-107), a introdução de mitocôndrias sadias por transferência de citoplasma foi considerada como sendo a responsável pela restauração do desenvolvimento (108-110). Contudo, existem poucos trabalhos que suportam esta hipótese (111-114). Além disso, uma elevada taxa de anormalidades cromossômicas e defeitos ao nascimento foram observados entre os bebês nascidos após transferência de citoplasma (108), o que levou tal técnica a ser proibida em vários países. Isto ressalta a necessidade de mais estudos utilizando modelos animais que esclareçam o que determina as falhas de desenvolvimento embrionário nestes casos, assim como os motivos que resultaram na alta incidência de anormalidades ao nascimento nos casos em que a transferência de citoplasma foi empregada em humanos (109, 110, 115-117).

2.5- A herança de doenças mitocondriais

Disfunção mitocondrial representa um grupo de patologias humanas em que a severidade varia de leve a letal (26, 27). Uma vez que a mitocôndria é responsável por sintetizar a grande maioria do ATP celular, os tecidos afetados por disfunções mitocondriais comumente são os que apresentam elevada exigência energética como, por exemplo, o tecido nervoso, muscular e hepático (26, 27). Posto que a função mitocondrial depende de uma comunicação articulada entre o nDNA e o mtDNA, mutações tanto em um quanto no outro genoma podem resultar em disfunção da mitocôndria (26). Embora o mtDNA codifique apenas 13 componentes da cadeia respiratória mitocondrial, a

prevalência no Reino Unido de patologias causadas por mutações no mtDNA é de 1:4.000, enquanto que a prevalência de mutações patogênicas no mtDNA é ainda maior, 1:200 (26). Até o presente momento, mais de 150 mutações patogênicas no mtDNA já foram descritas em humanos (118) e, estão associadas a diferentes patologias incluindo surdez, cegueira, cardiopatia, neuropatia, falência do fígado e diabetes (26, 119, 120).

Posto que ainda não há tratamento eficaz para doenças causadas por mutações no mtDNA (26), grande enfoque tem sido dado à prevenção da sua transmissão para as gerações seguintes (27, 36, 37). O aconselhamento genético para prevenir a transmissão de doenças mitocondriais depende tanto do entendimento da segregação do mtDNA na linha germinativa quanto das bases fisiológicas do gargalo genético (27). Todavia, a quantidade de dados provenientes de humanos referentes ao assunto é mínima (86). Embora os dados disponíveis, oriundos de drosófilas, camundongos e humanos, sejam consistentes com a hipótese de segregação ao acaso, os mesmos não excluem a possibilidade de seleção negativa de mutações no mtDNA durante o desenvolvimento da linhagem germinativa (17, 26, 64, 70, 83, 121-126).

As patologias causadas por mutações no mtDNA são herdadas exclusivamente da mãe e a transmissão para os descendentes depende dos mecanismos de seleção descritos acima (26). Em estudos com modelos animais estes mecanismos eficientemente restabeleceram a homoplasmia dentro de poucas gerações (60, 66, 83, 121, 122, 124, 125). Tais mecanismos poderiam assegurar que mutações patológicas no mtDNA não sejam transmitidas por muitas gerações, uma vez que o restabelecimento da homoplasmia nos descendentes resultaria em seleção em nível do indivíduo. Neste caso, se a mutação presente comprometesse seriamente a função mitocondrial, do tecido e do organismo como um todo, o indivíduo morreria e a mutação não seria mais transmitida (26). No entanto, estudos recentes realizados com camundongos sugerem que outros mecanismos selecionem de forma negativa mutações patogênicas presentes no mtDNA (122, 125, 127).

Utilizando um modelo com camundongos em que o mtDNA possuía rearranjos de sequências similares ao que ocorrem na síndrome de Kearns-Sayre (128), Sato et al. (125) reportaram que o nível de mtDNA mutante diminui em função do tempo nos oócitos de uma mesma fêmea.

Um segundo estudo investigou a transmissão de mutações geradas ao acaso no mtDNA em camundongos (121). Neste modelo, as fêmeas fundadoras eram homozigotas para uma mutação em um dos domínios da *POLG* (129), o que gerava elevados níveis de mutações pontuais nos seus mtDNAs. Estas fêmeas mutantes foram acasaladas com animais selvagens e transmitiram múltiplas mutações no mtDNA para os seus descendentes (em média 30 mutações por mtDNA para a primeira geração) (121). Subsequentes acasalamentos dos descendentes com animais que não possuíam a mutação na *POLG* eliminaram o alelo mutante e a partir de então os mtDNAs mutantes foram transmitidos de uma geração para a outra sem que novas mutações fossem produzidas. Desta forma foi possível estudar a segregação de diferentes mutações no mtDNA em uma mesma linhagem de camundongos (121). Ao comparar mutações neutras que não alteram a sequência das proteínas e, portanto sofrem menos seleção do que aquelas que alteram, os autores demonstraram seleção negativa de mutações deletérias num tempo consideravelmente reduzido (121). Na verdade, muitas das mutações foram eliminadas dentro de quatro gerações. Contudo, mutações em genes que codificam tRNAs e rRNAs sofreram uma pressão de seleção muito menor que mutações em genes que codificam mRNAs (121). Este resultado está de acordo com o que é observado em humanos, espécie em que a grande maioria das mutações no mtDNA relacionadas a doenças mitocondriais são causadas por mutações em tRNAs e rRNAs (64, 121). É provável que isto ocorra devido a elevada porcentagem de mtDNAs mutantes necessária para comprometer a função mitocondrial quando a mutação está localizada em um tRNA ou rRNA comparado com uma mutação localizada em um mRNA (64, 121).

Por fim, um terceiro estudo envolvendo duas mutações pontuais no mtDNA, também demonstrou seleção negativa de mutações em camundongos (122). Os autores introduziram o mtDNA mutante obtido de uma linhagem celular somática em uma linhagem de células-tronco embrionárias utilizando a tecnologia de cíbridos[3]. As duas mutações localizadas na mesma molécula de mtDNA foram caracterizadas como sendo severa e média. Quando presente em homoplasmia a mutação severa era capaz de inativar completamente o complexo I da OXPHOS, enquanto que a mutação média apenas reduzia a atividade do complexo IV em 50% (122). Inicialmente ambas as mutações eram homoplásmicas, conferindo um severo defeito na cadeia respiratória. Todavia, um dos clones da linhagem de células-tronco embrionárias tornou-se heteroplásmico devido ao surgimento de uma reversão que restituiu a sequência selvagem de aminoácidos codificada pelo gene (122). Quando esta linhagem celular foi introduzida na linhagem germinativa, os camundongos descendentes desenvolveram miopatia e cardiomiopatia sub-clínica, mas eram capazes de se reproduzir normalmente. A mutação severa foi perdida em favor da reversão dentro de quatro gerações (122). Além disso, nenhum dos descendentes apresentou um nível maior da mutação severa do que a mães deles. A análise dos oócitos destas mães revelou que a seleção provavelmente ocorreu durante a vida adulta (e.g. durante a foliculogênese) uma vez que a proporção de mtDNA mutante diminuiu nos oócitos de uma mesma fêmea em função do tempo (17, 122).

Estes estudos são consistentes com outros estudos em humanos e camundongos (83, 126), e sugerem que a seleção depende da função mitocondrial, mas não esclarecem em que estádio da oogênese e/ou foliculogênese é provável que ela ocorra. Tal seleção explicaria algumas características do padrão da herança de mutações no mtDNA que antes eram difíceis de serem explicados (26, 27, 36, 64). Além disso, a hipótese de seleção negativa rigorosa de mutações detrimentais no mtDNA durante a oogênese e/ou foliculogênese é consistente com os mecanismos já descritos de exclusão de mitocôndrias paternas (58, 59). Mas, qual seria a base desta seleção? A diluição

[3]Traduzido do inglês (cybrids): citoplasma híbrido (e.g. mtDNA).

do mtDNA para poucas cópias por célula durante o período do desenvolvimento inicial em que a replicação do mtDNA é inativa (75, 78, 97, 98, 101) poderia maximizar o efeito de mutações no mtDNA no fenótipo celular, o que por sua vez tornaria possível a seleção em nível celular (98, 101). Por exemplo, durante o período em que as PGCs migram para as gônadas e se diferenciam em oogônias, as células com altos níveis de mutações poderiam migrar e dividir-se mais lentamente, consequentemente sendo diluídas por PGCs com mtDNA mais conservado e, portanto mais eficientes na geração de energia (64, 98, 101). Por outro lado, somente cerca de 30% das oogônias estabelecidas durante a vida fetal atingem o estádio de oócito maturo, as demais são eliminadas por morte celular programa (apoptose) (130). Uma vez que mitocôndrias disfuncionais (devido a mutações no mtDNA) geram elevados níveis de ROS (122), este poderia ser o sinal para seleção negativa por apoptose das oogônias e/ou oócitos com elevados níveis de mtDNA mutante (17, 122).

Um segundo possível mecanismo seria seleção em nível da organela (64, 131). O número de cópias de mtDNA por organela nas células germinais parece ser limitado a poucas moléculas (e.g. uma ou duas cópias), em comparação a dez ou mais cópias presentes em células somáticas (75, 132). Desta forma, mutações em algumas moléculas de mtDNA poderiam ser distinguidas de moléculas selvagens presentes na mesma célula pelo efeito da mutação no fenótipo mitocondrial. Isto posto, mitocôndrias disfuncionais poderiam ser degradadas por mecanismos intracelulares como autofagia (64, 131). Evidências disto foram fornecidas por Twig et al. (133) ao mostrarem que mitocôndrias defeituosas possuem uma menor capacidade de fusão com as demais mitocôndrias da célula e acabam sendo autofagocitadas. Embora este evento tenha sido descrito em células somáticas, ele está também presente em células germinais (134, 135) e, portanto, poderia estar envolvido na remoção de mtDNA mutante da próxima geração. Uma segunda hipótese para a seleção em nível da organela seria a competição entre mitocôndrias normais e mitocôndrias com disfunção (64, 131). Neste caso, mitocôndrias disfuncionais seriam menos eficientes para a importação

e função enzimática de proteínas codificadas pelo nDNA que são necessárias para a replicação do mtDNA. Isto poderia resultar em uma vantagem replicativa das moléculas selvagens sobre as mutantes, consequentemente reduzindo a proporção de mtDNA mutante na linhagem germinativa e na próxima geração (64, 131). Embora Wai et al. (85) tenham reportado que uma sub-população de mtDNAs é preferencialmente replicada durante a foliculogênese para repor o mtDNA nos oócitos, não existe qualquer indício que mostre se esta sub-população é selecionada ao acaso ou se há algum mecanismo que selecione certas moléculas. Caso tal mecanismo exista, este poderia compreender a seleção de moléculas selvagens, o que explicaria o padrão observado de seleção negativa de moléculas mutantes (64, 131).

Um terceiro mecanismo possível é específico de oócitos, baseado em uma estrutura conhecida como Corpo Balbiano[4] (136). Esta estrutura corresponde a organização no oócito de mitocôndrias e retículos endoplasmáticos ao redor de complexos de Golgi, o que possibilita que determinados mRNAs sejam especificamente herdados pelas PGCs do embrião futuro (136-138). Da mesma forma, uma sub-população específica de mitocôndrias poderia compor os Corpos Balbianos e finalmente povoar as PGCs (137, 139-141), o que poderia explicar o padrão observado de seleção negativa de mutações no mtDNA (136). Em algumas espécies as mitocôndrias com o maior potencial de membrana mitocondrial são encontradas nos Corpos Balbianos (139-141), sugerindo que mitocôndrias defeituosas são excluídas de compô-los, assim evitando a transmissão de moléculas mutantes para as futuras PGCs (136). Embora este seja um possível mecanismo de seleção, pouco se conhecesse sobre o Corpo Balbiano em mamíferos (136). Além disso, os relatos de que a proporção de mtDNA mutante diminui em função do tempo nos oócitos de um mesmo animal (122, 125) faz com que este mecanismo seja bastante improvável de ser responsável pela seleção do mtDNA nos mamíferos (64, 85).

[4]Traduzido do inglês (Balbiani body)

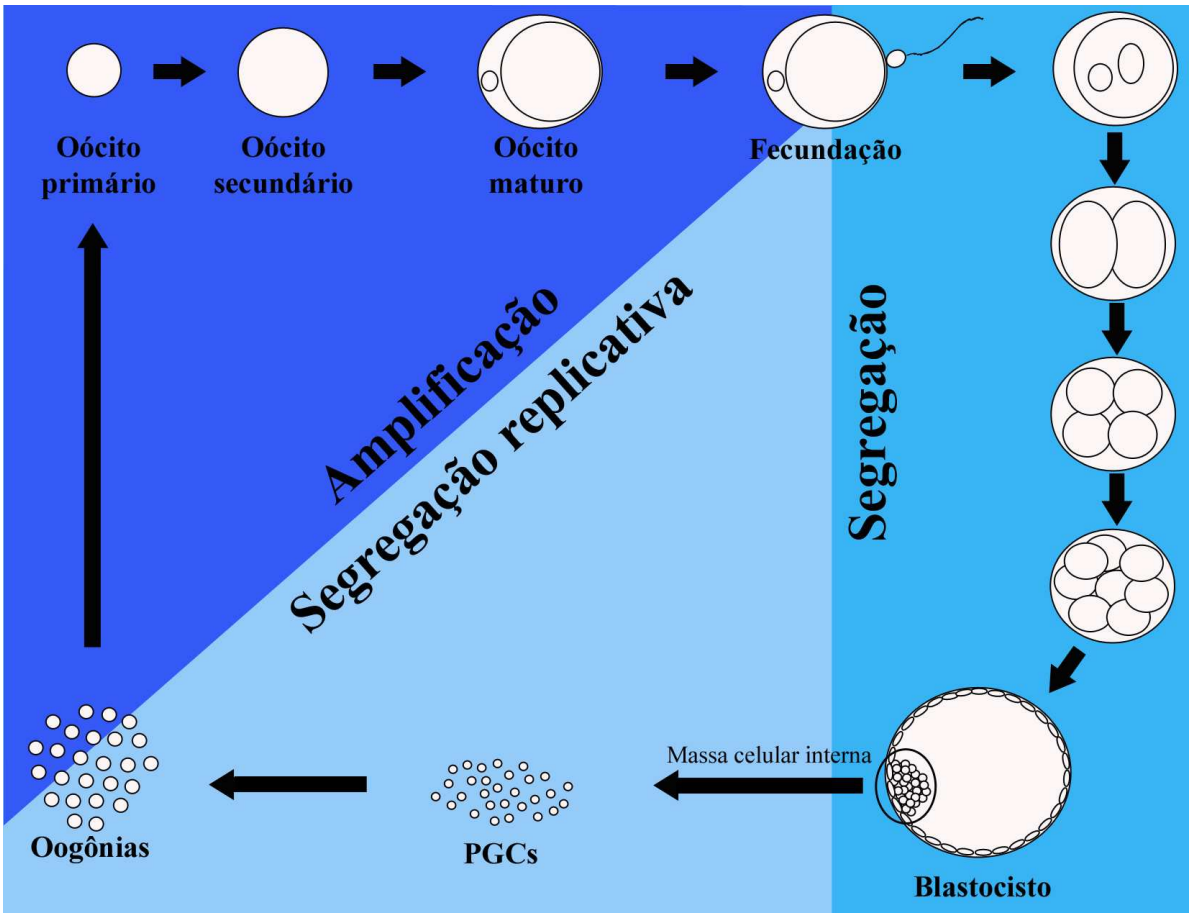

Figura 4. Ciclo do DNA mitocondrial (mtDNA) na linhagem germinativa de mamíferos. Durante o desenvolvimento embrionário inicial ("Segregação") o mtDNA é segregado entre as células filhas sem que seja replicado. Portanto, o número de cópias de mtDNA por célula diminui a cada clivagem atingindo uma quantidade mínima nas células primordiais germinais (PGCs). O próximo estádio é marcado como "Segregação replicativa", o que implica em replicação e partição ao acaso das moléculas de mtDNA entre as células filhas. O último estádio, "Amplificação", é caracterizado por amplificação exponencial da quantidade de mtDNA. Tem se sugerido que a replicação do mtDNA durante este estádio seja restrita a um sub-grupo de moléculas o que resulta em drásticas mudanças no genótipo mitocondrial no oócito. Além disso, parece ocorrer durante este estádio uma seleção negativa de mutações no mtDNA, o que tem implicações diretas sobre a herança de doenças mitocondriais.

Qualquer que seja o mecanismo, algum evento que ocorra durante a oogênese e/ou a foliculogênese parece atuar na seleção positiva de moléculas selvagens de mtDNA (Figura 4). Na verdade, os estudos de Sato et al. (125) e Fan et al. (122) sugerem que a seleção ocorra durante a vida adulta, portanto durante a foliculogênese. Por outro lado, mutações que escapam a este filtro seriam então expostas à seleção em nível do indivíduo (26). Assim, diversos mecanismos podem contribuir para o gargalo genético, prevenindo a disseminação de mutações no mtDNA (17, 26, 64).

2.6- O controle da transmissão de doenças mitocondriais

A heteroplasmia do mtDNA nos produtos de concepção tem importante implicação em todos os tipos de diagnóstico pré-natal de doenças causadas por mutações no mtDNA (26, 27, 36, 37, 142). Casais que perderam uma criança devido a doenças mitocondriais, frequentemente buscam aconselhamento genético antes de tentarem se reproduzir novamente. Apesar do entendimento de como mutações no mtDNA podem afetar a atividade mitocondrial e a função de um tecido, assim como as doenças mitocondriais são herdadas, ainda não é possível predizer com acurácia o risco de uma mulher afetada por uma mutação no mtDNA transmitir sua doença para seus descendentes (17, 26, 27, 36, 37, 64, 142).

A doação de oócitos evitaria todos os problemas associados com a presença de moléculas mutantes de mtDNA, mas há grande escassez de oócitos disponíveis para este fim (27). Um doador de oócitos também não pode ter grau de parentesco muito próximo ao casal. As irmãs da paciente, por exemplo, podem carregar a mesma mutação. Em contrapartida, o uso de oócitos provenientes das irmãs do parceiro da paciente resultaria em consanguinidade. Além disso, muitos casais fazem questão que seus filhos sejam geneticamente idênticos a eles (27, 142).

A coleta e análise de oócitos de mulheres acometidas por doenças mitocondriais têm ocasionalmente sido utilizada para predizer o risco de

recorrência da doença (142, 143). Isto envolve a quantificação do nível de mtDNA mutante em 10 a 20 oócitos não fecundados obtidos pelos mesmos procedimentos utilizados em casos de fecundação *in vitro* (FIV), e.g. estimulação do ovário e coleta dos oócitos por aspiração transvaginal (142, 144). Uma vez que os oócitos são recuperados, os níveis de mtDNA mutante são determinados para cada oócito. A desvantagem deste método é que o número de oócitos recuperados pode ser insuficiente para a realização de uma análise estatística confiável. Além disso, para se determinar a proporção de mtDNA mutante os oócitos têm que ser destruídos o que, portanto, impede a sua posterior utilização para fecundação e desenvolvimento (27, 142, 144).

Uma alternativa neste caso seria o uso de diagnóstico pré-implantação (PGD) ou diagnóstico pré-natal (PND) (27, 36, 37, 142, 144, 145). Diferente do que foi descrito acima, no caso de ambos PGD e PND, apenas uma fração do embrião ou da placenta é amostrada para determinação da proporção de mtDNA mutante no embrião ou feto, respectivamente (27, 36, 145). Portanto, o risco de desenvolvimento dos sintomas da doença mitocondrial após o nascimento é estimado a partir de uma amostra, o que permite a seleção de embriões ou fetos saudáveis para prosseguirem o desenvolvimento (27, 36, 142, 145).

O diagnóstico pré-natal é frequentemente utilizado para detectar doenças mitocondriais causadas por mutações no nDNA e, portanto herdadas de acordo com as Leis Mendelianas (146). No entanto, no caso de doenças causadas por mutações no mtDNA, existem poucos dados disponíveis para se determinar a confiabilidade do método (147-150). Posto que o nível de mtDNA mutante pode variar entre células e tecidos (27, 36, 97, 142, 144, 145, 151), não é certo que a proporção de moléculas mutantes em uma amostra (cerca de 10 mg) oriunda, por exemplo, das vilosidades coriônicas (CVS) da placenta reflita a proporção presente no feto (27, 36, 37, 142, 144, 145). Elevados ou baixos níveis de mtDNA mutante na amostra obtida por CVS indicam que a criança será provavelmente afetada ou não, respectivamente, pela doença mitocondrial. Todavia, um nível intermediário de mtDNA mutante não seria muito útil para predizer se o feto irá

desenvolver a patologia, ainda mais que a proporção de moléculas mutantes e selvagens pode variar em função do tempo (27, 36, 37, 142, 144, 145). Portanto, por meio do diagnóstico pré-natal não é possível estimar com acurácia a proporção de mtDNA mutante no feto. Dentre as diferentes doenças causadas por mutações no mtDNA, uma das únicas possíveis de ser diagnostica por PND é a NARP (neuropatia, ataxia e retinose pigmentar). Esta patologia é causada por uma mutação pontual no nucleotídeo número 8993 do mtDNA (mtDNA 8993 T>G ou T>C) (152). Isso só é possível porque no caso da NARP a proporção de mtDNA mutante e a severidade da patologia estão diretamente correlacionadas (142, 147, 153, 154). Como resultado, uma baixa proporção de moléculas mutantes indicaria que o feto tem reduzido risco de desenvolver os sintomas da doença após o nascimento (27, 36, 37, 142, 144, 145).

Uma das alternativas ao diagnóstico pré-natal seria o diagnóstico pré-implantação (Figura 5), o qual envolve a geração de embriões por técnicas relacionadas à FIV (27, 36, 37, 142, 144, 145). Neste caso, um ou dois blastômeros são removidos de cada embrião gerado para determinar a proporção de mtDNA mutante. Apenas os embriões contendo baixos níveis de mutação são transferidos para o útero e consequentemente as gestações estabelecidas não devem ser afetadas pela patologia em questão. Embora a quantidade de dados disponíveis envolvendo PGD para doenças causadas pelo mtDNA seja bastante limitada, estes indicam que o procedimento é capaz de estimar com acurácia a proporção de mtDNA mutante no embrião e, portanto o risco do recém nascido desenvolver a doença durante a sua vida (27, 36, 37, 142, 144, 145, 155, 156). Além disso, vários trabalhos recentes mostrando seleção negativa de mutações no mtDNA (121, 122, 125) têm encorajado o uso de PGD posto que a probabilidade de se encontrar um embrião com baixo risco de transmitir a doença é elevada. No caso do diagnóstico pré-implantação, o embrião humano sofre biópsia três dias após a fecundação, momento em que contém de seis a dez blastômeros (157). Uma vez que a replicação do mtDNA não é restabelecida até este momento (45, 76, 77, 88) e que todos os blastômeros são indiferenciados, a análise de um único

blastômero reflete o conteúdo do embrião como um todo com grande confiabilidade (156).

O procedimento de PGD é realizado após a estimulação ovariana controlada, o que produz múltiplos oócitos que são então fecundados por ICSI (injeção intracitoplasmática de espermatozóide) (157). A ICSI é utilizada ao invés do procedimento padrão de FIV para evitar o acúmulo de espermatozóides na zona pelúcida os quais poderiam contaminar a célula amostrada para diagnóstico (27, 36, 142, 144, 145). Este procedimento normalmente resulta em vários embriões os quais podem ser testados ao mesmo tempo, aumentando a probabilidade de que ao menos um embrião, contendo baixa proporção de moléculas mutantes, seja identificado (158). Posto que somente embriões com nenhuma ou poucas moléculas de mtDNA mutantes são transferidos para o útero da mãe, o término de gestações com altos níveis da mutação torna-se praticamente desnecessário (27, 36, 142, 144, 145).

Embora o diagnóstico pré-implantação venha sendo utilizado com sucesso para o diagnóstico de mais de 100 diferentes doenças genéticas, sendo a maioria delas devido a mutações no nDNA, este procedimento possui algumas limitações (157, 159). A mais importante delas é que o diagnóstico pré-implantação depende da produção *in vitro* de embriões, o que não garante que os embriões transferidos para o útero irão se desenvolver a termo. A probabilidade de uma paciente tornar-se gestante após a transferência de embriões sadios é de cerca de 50% (157, 159). Por esta razão, algumas pacientes necessitam de vários ciclos de tratamento até obterem uma gestação sadia.

Uma alternativa à biópsia de embriões em clivagem seria a realização de biópsia de células dois dias mais tarde durante o curso do desenvolvimento, momento em que o embrião humano encontrar-se-á no estádio de blastocisto (cinco dias após a fecundação) (142). O diagnóstico pré-implantação de embriões no estádio de blastocisto é associado com uma taxa maior de gestação e, também é vantajoso porque várias células são removidas durante a biópsia, o que resulta em um diagnóstico mais acurado (157, 159). No entanto, embriões neste estádio

ainda não foram utilizados para o propósito de doenças causadas por mutações no mtDNA devido à preocupação de que a proporção de moléculas mutantes possa variar entre as células do blastocisto (160, 161). Isto se deve ao fato de que as células removidas para o diagnóstico seria provenientes do trofectoderma, camada de células que origina os tecidos extra-embrionários. Portanto, a massa celular interna, da qual o feto se origina, não é testada, o que aumenta o risco de diagnóstico incorreto (142).

Outro problema relacionado ao diagnóstico pré-implantação envolve o efeito dos meios de cultivo de embrião sobre a fisiologia mitocondrial. Trabalhos recentes têm mostrado que o cultivo *in vitro* pode afetar a replicação do mtDNA durante o desenvolvimento embrionário inicial, resultado potencialmente na antecipação do início da replicação em algumas espécies de mamíferos (102, 162, 163). Isto poderia afetar a confiabilidade do diagnóstico e, portanto esforços devem ser dirigidos no sentido do desenvolvimento de meios de cultivo que se assemelhem ao máximo com as condições encontradas *in vivo*. Além disso, é vital que os casos de diagnóstico pré-implantação sejam acompanhados após o nascimento para confirmar a acurácia da estratégia de diagnóstico empregada (27, 36, 142, 144, 145).

Apesar dos benefícios oferecidos pelas estratégias de PND e PGD, ambas não podem fornecer uma garantia absoluta de que os descendentes não desenvolverão os sintomas da doença e a validade destas estratégias é extremamente limitada em casos em que a mutação no mtDNA não é bem correlacionada com a severidade da patologia. Estratégias alternativas que visam tratar ao invés de diagnosticar a doença incluem transferência de citoplasma e transferência de núcleo (142).

2.6.1- Transferência de citoplasma

A transferência de citoplasma foi introduzida nos humanos no final da última década do século XX com a finalidade de tratar mulheres inférteis (vide item 2.4). Durante o procedimento, 5 a 15% de citoplasma proveniente de um oócito doador

é microinjetado no citoplasma do oócito de uma paciente (28, 29, 32-34). Isto resulta na mistura de mitocôndrias de duas origens diferentes (heteroplasmia), situação esta que se mantém mesmo após o nascimento (108, 164, 165). Nas crianças geradas após transferência de citoplasma, apesar da proporção de mtDNA proveniente do oócito doador ser pequena, em alguns casos ela excede a quantidade de mtDNA introduzida (108, 164, 165). Uma vez que a heteroplasmia é uma condição que não é comumente observada em indivíduos normais, mas geralmente presente em pacientes com doenças mitocondriais (vide item 2.5), isto gerou uma grande preocupação a respeito do uso da transferência de citoplasma em humanos (31, 36). Além disso, o fato de este procedimento gerar uma criança que possui mtDNA de duas origens diferentes tem implicações éticas uma vez que ela terá três pais genéticos (166).

O uso da transferência de citoplasma com o propósito de tratar doenças mitocondriais ainda não foi testado, mas o potencial deste método em curar, ou pelo menos atenuar os sintomas da doença tem gerado grande interesse entre os pesquisadores que trabalham na área (36, 38, 142, 155). Em teoria o citoplasma oriundo dos oócitos de mulheres saudáveis poderia diluir o mtDNA mutante para baixo do limiar crítico para o desenvolvimento da patologia e assim prevenir a transmissão da mesma para a próxima geração (36, 38, 142, 155).

Para garantir que os descendentes não irão desenvolver os sintomas da doença mitocondrial, o mtDNA mutante deve ser diluído para um nível bastante baixo (36, 38, 142, 155). Isto seria particularmente importante para doenças como NARP em que a proporção de moléculas mutantes e a severidade da patologia são bem correlacionadas, assim como uma elevada proporção de moléculas mutantes é necessária para a manifestação da mesma (142). Considerando que somente 5 a 15% de citoplasma foram introduzidos por transferência de citoplasma no passado (28, 29, 32-34), talvez esta quantidade seja insuficiente para diluir as moléculas mutantes para um nível seguro que evite a transmissão da doença (36, 38, 142, 155). Estima-se que a transferência de pelo menos 50% de citoplasma ou introdução de mitocôndrias purificadas seria suficiente para prevenir

a transmissão da NARP (155). Contudo, não se sabe se é possível introduzir uma quantidade tão grande de citoplasma ou mitocôndrias no oócito, ou ainda remover parte das mitocôndrias do oócito receptor (proveniente da paciente) antes da transferência de citoplasma (36, 142). Estas questões tornam no momento a transferência de citoplasma inadequada para tratar doenças mitocondriais (36, 38, 142, 155).

2.6.2- Transferência de núcleo

A substituição completa do mtDNA mutante presente no oócito da paciente por mtDNA selvagem seria o método mais seguro para se evitar a transmissão de uma patologia mitocondrial (27, 36, 38, 142, 155). A transferência nuclear permite a troca quase que completa de mtDNAs entre duas células (167). Este procedimento consiste na remoção do núcleo de uma célula doadora e transferência do mesmo para outra célula que tenha sido previamente enucleada (168). Uma vez que a célula receptora irá fornecer todos os componentes citoplasmáticos, incluindo as mitocôndrias, em teoria todas as moléculas de mtDNA presentes na célula doadora de núcleo serão trocadas por moléculas do oócito receptor. Por isso, a transferência de núcleo é uma ferramenta com grande potencial para prevenir a transmissão de doenças mitocondriais (169). Assim como na transferência de citoplasma, uma criança gerada por este procedimento terá três pais genéticos: o casal que doar o nDNA e uma terceira pessoa que doar o mtDNA (166). No entanto, uma vez que o mtDNA doado codificará apenas polipeptídios não mutantes que irão compor a OXPHOS, a criança gerada será fenotipicamente idêntica ao casal com exceção de que ela não desenvolverá os sintomas da doença em questão (Figura 5) (27, 36, 38, 142).

A transferência de núcleo tem sido utilizada com sucesso em mamíferos por quase 30 anos (168, 170-172), mas somente na metade da última década do século passado foi utilizada como modelo para estudar a herança mitocondrial (173, 174). A partir de então, vários grupos aperfeiçoaram a técnica e confirmaram que os descendentes gerados por este procedimento herdam nenhum ou muito

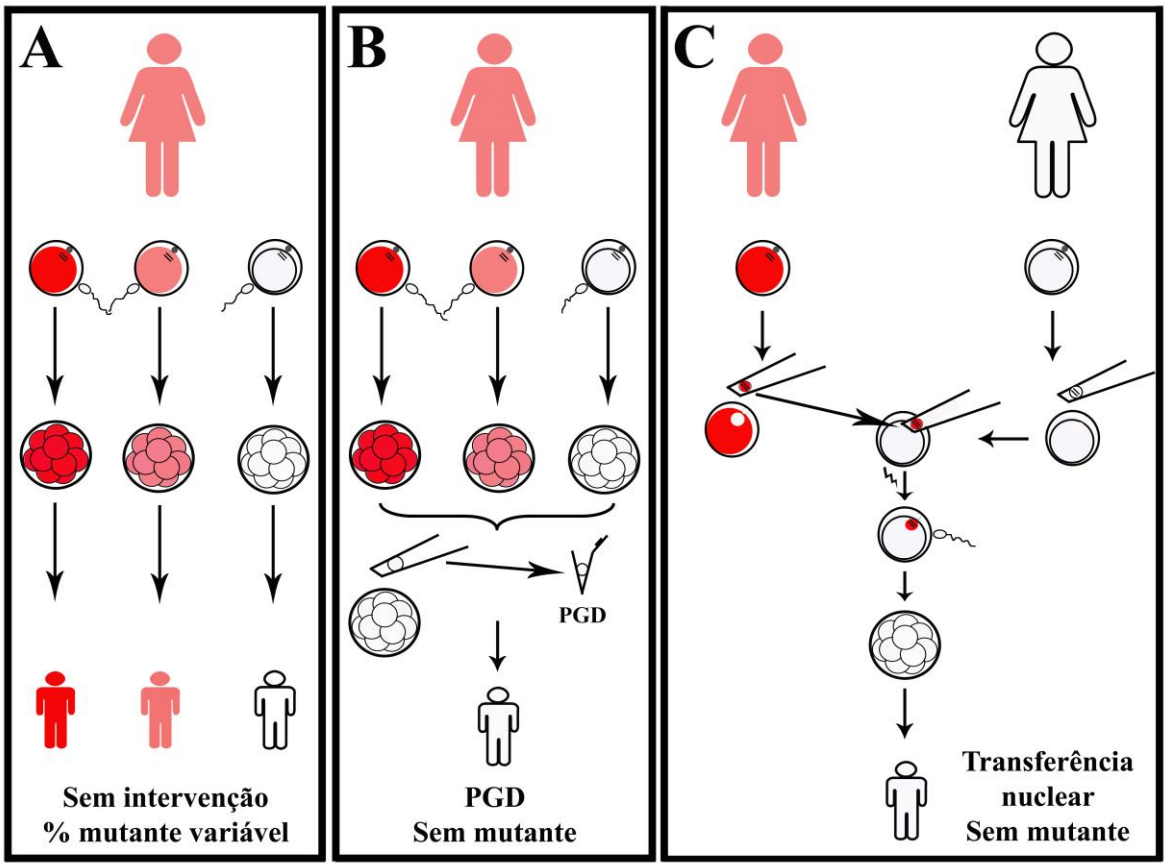

Figura 5. Duas possibilidades para reduzir o risco de transmissão de doenças causadas por mutações no DNA mitocondrial (mtDNA). Vermelho, rosa e branco representam, respectivamente, nível elevado, médio e nenhum mtDNA mutante. Painel A: se não há intervenção a mãe com a mutação terá crianças com um nível de mtDNA mutante variável. Painel B: o diagnóstico pré-implantação (PGD) envolve a retirada de uma ou duas células de embriões fecundados *in vitro* para estimar a quantidade de mtDNA mutante. Apenas embriões com reduzido risco são transferidos para o útero da mãe. Este procedimento já é oferecido por alguns países. Painel C: na transferência nuclear, os cromossomos do oócitos proveniente da mãe com a mutação (cor vermelha) são transferidos para um oócito previamente enucleado doado por uma mulher saudável (cor branca). O oócito reconstruído contendo somente mtDNA selvagem é então fecundado e transferido para o útero da mãe. Este tipo de procedimento está ainda restrito a estudos científicos.

pouco mtDNA da célula doadora de núcleo (167, 175-180). Recentemente, dois importantes passos foram dados em direção ao uso da transferência nuclear como ferramenta para prevenir a transmissão de doenças mitocondriais (181, 182). No primeiro (181), o pró-núcleo de embriões humanos foi transferido para zigotos previamente enucleados utilizando um procedimento similar ao descrito inicialmente por Meirelles e Smith (124). Os embriões reconstruídos foram cultivos *in vitro* até o estádio de blastocisto, mas então tiveram que ser descartados por razões legais. Contudo, os pesquisadores avaliaram quanto de mtDNA do zigoto doador de pró-núcleo foi transferido para o zigoto receptor. Dentre os embriões analisados, os quais continham de duas a nove células, quatro não apresentaram níveis detectáveis de mtDNA do zigoto doador, enquanto que os outros cinco apresentaram em média menos de 2% (181). O outro trabalho consistiu na transferência de núcleo entre oócitos de primatas não humanos (182). O grupo responsável por este estudo aperfeiçoou o procedimento de transferência de carioplasto[5] (124), de forma que o material genético nuclear dos oócitos fosse removido sem contaminação significativa com mitocôndrias do oócito doador de núcleo. Isto foi possível por que os pesquisadores utilizaram oócitos maturos, ao invés de zigotos, para a transferência de carioplasto (182). Nos zigotos as mitocôndrias se organizam ao redor do núcleo com a finalidade de facilitar o suprimento de energia. Todavia, oócitos maturos não contêm membrana nuclear uma vez que estão no meio da divisão meiótica e, portanto as mitocôndrias se distribuem homogeneamente no citoplasma (183, 184). A fecundação dos oócitos reconstruídos e transferência dos blastocistos produzidos para fêmeas receptoras resultou no nascimento de quatro indivíduos que não continham níveis detectáveis de mtDNA do oócito doador de núcleo (182). É muito provável que tanto no caso deste trabalho (182), quanto no caso do trabalho realizado com humanos (181), a redução da quantidade de mtDNA transferida do oócito ou zigoto doador já seria suficiente para prevenir a maioria das doenças mitocondriais em humanos. Por exemplo, a menor proporção de moléculas mutantes descrita, necessária para desencadear uma doença mitocondrial em humanos, é de cerca de 25% nos

[5]Material nuclear envolvido por membrana juntamente com um pequeno conteúdo de citoplasma

tecidos afetados (185).

Antes que a transferência nuclear se torne disponível clinicamente, muitas questões relacionadas à segurança do procedimento devem ser elucidadas (27, 36, 38, 142, 186). Por exemplo, será que o nDNA transferido é capaz de regular corretamente a replicação, tradução e transcrição do mtDNA doado pela célula receptora? Sabe-se que o nDNA e o mtDNA interagem de uma forma bastante complexa, a qual é pouco compreendida. Isto precisa ser profundamente investigado, especialmente considerando que pequenas diferenças quanto ao estádio de maturação do oócito doador e do oócito receptor podem resultar em incompatibilidade e falha de comunicação entre os genomas (110, 116, 117, 187). Além das mitocôndrias, o oócito receptor irá contribuir também com outras organelas e moléculas, e pouco se sabe sobre as consequências da mistura destes componentes com um núcleo de outra origem (27, 142). Finalmente, embora apenas poucas mitocôndrias possam ser introduzidas no oócito, existem relatos de replicação seletiva do mtDNA (180), talvez controlada pelo nDNA (188), o que poderia resultar em aumento da proporção de moléculas mutantes ao longo do desenvolvimento e até mesmo manifestação clínica da doença após o nascimento (27, 36, 38, 142).

2.7- O modelo bovino

Devido a assuntos éticos relacionados à pesquisa médica envolvendo embriões humanos, um modelo animal apropriado é necessário para o estudo da herança mitocondrial e do seu efeito sobre a competência do oócito, bem como de formas de se prevenir a transmissão de doenças mitocondriais (36, 142, 164, 181, 182). O único modelo animal de relevância para o homem são os primatas não humanos, e.g. *Callithrix jacchus* e *Macaca mulatta* (182, 189). No entanto, limitações de ordem técnica, econômica, ética etc, impedem o seu uso frequente em estudos científicos (190). Neste contexto, o bovino, por ser muito mais similar ao homem que o camundongo (191), se mostra como um interessante modelo de

pesquisa que poderia auxiliar e preceder experimentos com primatas (humanos e primatas não humanos) (192).

Primeiramente, há uma quantidade crescente de evidências de que o modelo bovino é melhor que o modelo murino para o estudo do desenvolvimento embrionário humano com relação ao momento da ativação do genoma, metabolismo intermediário, e interação com os meios de cultivo (193). Além disso, recentes trabalhos têm mostrado que a herança e a função mitocondrial diferem entre espécies durante o desenvolvimento inicial. Em segundo lugar, humanos e camundongos diferem quanto a longevidade. Evidências mostram que vertebrados que vivem por um período maior claramente apresentam uma reduzida taxa de geração de radicais livres mitocondriais, um fator determinante para a taxa de envelhecimento (194, 195). Danos oxidativos ao mtDNA também são reduzidos em vertebrados com maior longevidade do que naqueles que vivem por um tempo menor (196). Considerando estas questões, os bovinos por viverem por um período muito maior (30 anos) são muito mais adequados que camundongos (vivem por dois a quatro anos dependendo da espécie). Por fim, para se determinar a eficácia de qualquer tratamento terapêutico para doenças mitocondriais, um modelo de animal de grande porte, além de modelos murinos de heteroplasmia, para mutações patogênicas no mtDNA seria vantajoso. O modelo bovino representa um candidato apropriado neste sentido. Este modelo animal tem sido bastante utilizado para o estudo da transmissão e segregação do mtDNA (197), forneceria um grande reservatório de tipos mitocondriais (198, 199), além de respeitar as preocupações éticas referentes a caça e amostragem de espécies em risco de extinção, especialmente primatas não humanos.

3- OBJETIVOS

3.1- Objetivo Geral

Estudar o efeito do número de cópias de mtDNA presentes em oócitos sobre o desenvolvimento a blastocisto de embriões partenogenéticos cultivados *in vitro*.

3.2- Objetivos Específicos

3.2.1- Experimento I

- Determinar se a quantidade de mtDNA está relacionada com a capacidade de desenvolvimento de oócitos provenientes de folículos de tamanhos diferentes.

3.2.2- Experimento II

- Relacionar de forma retrospectiva o número de cópias de mtDNA e a competência de desenvolvimento a blastocisto de embriões no estádio de uma célula.

3.2.3- Experimento III

- Verificar se a remoção de parte das mitocôndrias de embriões no estádio de uma célula afeta o desenvolvimento a blastocisto;
- Caso os embriões depletados se desenvolvam a blastocisto, determinar o número de cópias de mtDNA ao longo do desenvolvimento (dias 0, 3, 6 e 9 após a ativação partenogenética),
- Caso os embriões depletados se desenvolvam a blastocisto, determinar a expressão de genes envolvidos no controle da replicação e transcrição do

mtDNA (*TFAM* e *NRF1*) e de genes de expressão ubíqua (*MT-CO1* e *HIST1H2AG*).

4- MATERIAL E MÉTODOS

4.1- Modelo experimental

No presente trabalho o bovino (*Bos indicus*) foi utilizado como modelo experimental ao invés do camundongo, uma vez que o mesmo tem se mostrado mais adequado como modelo do humano para o estudo da herança mitocondrial e do seu efeito sobre a competência do oócito (vide item 2.7).

Os embriões utilizados neste trabalho foram produzidos por partenogênese para padronizar e sincronizar cronologicamente o desenvolvimento dos mesmos para os procedimentos experimentais que se seguiram. Além disso, em parte dos experimentos, embriões no estádio de uma célula foram utilizados ao invés de oócitos por motivos técnicos uma vez que o número de cópias de mtDNA não varia entre oócitos e embriões no estádio de uma célula.

4.2- Delineamento experimental

O presente trabalho foi dividido em três experimentos sequenciais nos quais diferentes métodos foram utilizados para o estudo do efeito da quantidade de mtDNA sobre a competência de desenvolvimento do oócito. No primeiro, a quantidade de mtDNA foi mensurada em oócitos provenientes de folículos de diferentes tamanhos, os quais apresentam diferentes potenciais de desenvolvimento, para se determinar a existência de uma relação entre competência do gameta feminino e número de cópias de mtDNA. No segundo experimento, o potencial de desenvolvimento a blastocisto foi relacionado com a quantidade de mtDNA no início do desenvolvimento por mensuração do número de cópias de mtDNA em biópsias citoplasmáticas removidas de embriões no estádio de uma célula antes de serem cultivados *in vitro* por sete dias. Por fim, embriões também no estádio de uma célula foram depletados fisicamente de parte de suas mitocôndrias e cultivados *in vitro* por nove dias para avaliar a taxa de desenvolvimento e, o número de cópias de mtDNA e a expressão de genes envolvidos na regulação da replicação do mtDNA ao longo do desenvolvimento.

4.3- Aspectos éticos

A Comissão Ética em Experimentação Animal da Unicamp (CEEA/IB/Unicamp) considerou que não havia a necessidade de submissão do presente estudo para aprovação ética uma vez que o material biológico utilizado, células germinais obtidas de fêmeas bovinas após o abate, seria adquirido pronto no mercado (vide Anexo 1).

5- RESULTADOS

Os Resultados apresentados a seguir foram publicados no periódico *Biology of Reproduction* (vide Apêndice 2 e Anexo 2).

Title: Embryo mitochondrial DNA depletion is reversed during early embryogenesis in cattle[1]

Short title: Mitochondrial DNA and bovine embryo development

Summary sentence: The mitochondrial DNA copy number inherited by the oocyte is not related to developmental competence in cattle due to the capacity of viable embryos to regulate mitochondrial DNA replication and transcription at the blastocyst stage.

Keywords: bovine, development, oocyte, embryo, mitochondria, mtDNA, preimplantation

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[1]Supported by grants numbers 06/59074-0 and 06/03516-4 from the Fundação de Amparo à Pesquisa do Estado de São Paulo.

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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 to develop into blastocysts, contain less mtDNA than those originated from larger follicles. However, due to the high variability in copy number, a more accurate approach was examined in which parthenogenetic 1-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, 1-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 non-manipulated 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.

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 [6, 7]). Similarly, the number of mitochondria increases sharply during oogenesis, culminating in mature oocytes containing hundreds of thousands to over 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 [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 [9]) and are responsible for generating, through the oxidative phosphorilation (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 [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 (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 [12]). Indeed, it is affected by the energetic requirements of the cell, since 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 [18, 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 (i) quantified the amount of mtDNA in bovine oocytes known to have different developmental competence, (ii) applied a biopsy-based approach to quantify the amount of mtDNA before embryo culture and, (iii) depleted 1-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 post-parthenogenetic activation or 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 (CEEA/IB/UNICAMP). 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 post mortem from the ovaries of crossbred cows (*Bos indicus*) slaughtered at a local slaughterhouse. Ovaries were transported in 0.9% saline solution at 25 - 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 + 0.1% polyvinyl-pyrrolidone (PVP). The whole oocyte, regardless of its size, was placed in 0.2 ml polystyrene microtubes containing 1 µl of PBS + 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 1-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) + 10% fetal bovine serum (FBS) heat-inactivated (55°C for 30 min) + 22 µg/ml sodium pyruvate + 83.4 µg/ml amikacin sulfate. Groups of 10 to 15 COCs were placed in drops of 90 µl of bicarbonate-buffered TCM-199 + 10% FBS + 0.5 µg/ml FSH (Folltropin-V, Bioniche Animal Health, Belleville, ON, Canada) + 50 µg/ml hCG (Vetecor, Lab. Calier, Barcelona, Spain) + 1 µg/ml estradiol + 22 µg/ml sodium pyruvate + 83.4 µg/ml amikacin sulfate under mineral oil for 20 to 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 post-IVM) by incubation in 5 µM ionomycin in Hepes-buffered TCM-199 + 0.1% BSA (fatty acid-free) + 22 µg/ml sodium pyruvate + 83.4 µg/ml amikacin sulfate for 5 min, followed by incubation in Hepes-buffered TCM-199 + 3% BSA for 1 min and in 2 mM 6-dimethylaminopurine (6-DMAP) diluted in modified synthetic oviduct fluid (mSOF; [28]) + 2% FBS + 0.6% BSA (mSOF(+)) for 3 h. Finally, activated oocytes were washed in mSOF(+) and were cultured in vitro in groups of 20 to 25 in mSOF(+) until use.

Cytoplasmic biopsies of parthenogenetic 1-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 1-cell stage)

were incubated in groups of 15 to 20 in mSOF(+) + 10 µg/ml Hoechst 33342 + 7.5 µg/ml cytochalasin B for 15 min. For microsurgery, the group was transferred to a 400 µl drop of PBS + 10% FBS + 22 µg/ml sodium pyruvate + 83.4 µg/ml amikacin sulfate + 7.5 µg/ml cytochalasin B under mineral oil in a plastic dish. Before removal of a cytoplasmic biopsy, the diameter of each 1-cell embryo was measured (embryo volume = embryo radius^3 x 4/3 x 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% 1-cell embryo volume (biopsy volume = glass pipette radius^2 x biopsy length x π) was removed without removing pronuclei. This cytoplasmic biopsy was washed in PBS + 0.1% PVP, placed in 0.2 ml polystyrene microtubes containing 1 µl of PBS + 0.1% PVP, snap-frozen in liquid nitrogen and stored at -20^{o}C until use. Biopsied 1-cell embryos were washed in PBS + 10% FBS + 22 µg/ml sodium pyruvate + 83.4 µg/ml amikacin sulfate, and 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 1-cell embryos were used in this experiment. To validate the approach, a portion of these biopsied 1-cell embryos (10 per repetition) were, instead of cultured, sampled and stored for molecular analysis (mtDNA copy number). Whole 1-cell embryos were washed in PBS + 0.1% PVP, placed in 0.2 ml polystyrene microtubes containing 1 µl of PBS + 0.1% PVP, snap-frozen in liquid nitrogen and stored at -20^{o}C until use.

Experiment 3

Our objective in this experiment was to partially deplete parthenogenetic 1-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 in Experiment 2. However, before micromanipulation 1-cell embryos

were incubated in mSOF(+) + 7.5 µg/ml cytochalasin B for 30 min and then centrifuged in PBS + 7.5 µg/ml cytochalasin B at 10,000 x 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 1-cell embryos of their mitochondria (depleted or DE group). Embryo micromanipulation was carefully performed in order 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 1-cell embryos were stained with 0.5 µM MitoTracker CMXRos (Molecular Probes, Eugene, OR) for mitochondria labeling for 30 min before micromanipulation. MitoTracker staining was visualized under fluorescence at 579 nm excitation/599 nm emission.

After micromanipulation, 1-cell embryos were washed in PBS + 10% FBS + 22 µg/ml sodium pyruvate + 83.4 µg/ml amikacin sulfate and co-cultured in vitro in groups of 20 to 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) 1-cell embryos that were only centrifuged and then cultured (centrifuged control or CC group); (2) 1-cell embryos that were not centrifuged but had a cytoplasmic portion removed similar to that removed from the depleted group (DE group) (6-8% embryo volume; micromanipulated control or MC group) and, (3) 1-cell embryos that were cultured immediately without centrifugation or micromanipulation (control or 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, fifteen 1-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 1-cell embryo was washed in PBS + 0.1% PVP + 1 U/µl RNase OUT (Invitrogen,

Carlsbad, CA), placed in 0.2 ml polystyrene microtubes containing 1 µl of PBS + 0.1% PVP + 1 U/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 + 2% paraformaldehyde + 0.1% PVP for 1 h and permeabilized in PBS + 0.5% Triton X-100 + 0.1% sodium citrate + 0.1% PVP for 1 h. Next embryos were stained in PBS + 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) + 1% Triton X-100 + 1 x polymerase chain reaction (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'-GCCCTAGAACAGGGCTTAGT-3' and bMT3096-r: 5'-GGAGAGGATTTGAATCTCTGG-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 *B. taurus* and *B. indicus* mtDNAs based on sequences available at Genbank (access number: 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 of each primer + 0.2 mM dNTPs (Invitrogen) + 1.5 mM $MgCl_2$ + 1 x PCR buffer + 0.04 U/µl Taq DNA Polymerase (Invitrogen) + 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 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 NdeI (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 x 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 + 0.25 µM of the TaqMan probe bMT3030-Fam (5'-FAM-AAGGTGGCAGAGCCCGGTAATTGC-BHQ1-3'; Promega) + 1 x TaqMan Gene Expression Master Mix (Applied Biosystems) + 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 1-cell embryo.

Relative quantification of mtDNA and mRNA amounts

External standard preparation for mtDNA quantification

A 345 bp DNA fragment that was non-similar 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 + 1 U/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 + 5 µg of linear acrylamide (Ambion Inc., Austin, TX) + 4 µl of external standard + 5 µl of diethylene pyrocarbonate (DEPC)-treated H_2O was added to each sample. The extracted RNA was directly dissolved in 10 µl of DNase I solution (Invitrogen) + 1 U/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 stored at -20^{o}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 + 1 x SYBR Green PCR Master Mix (Applied Biosystems) + 2 µl of template (samples + 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- 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 pre-amplified 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 of each primer (*TFAM*,

NRF1, *HIST1H2AG* and *MT-CO1*) + 1 x TaqMan PreAmp Master Mix (Applied Biosystems) + 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 a 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 + 1 x SYBR Green PCR Master Mix (Applied Biosystems). For each sample, pre-amplified 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° for 45 sec and 60°C for 1 min. The Syber 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, 2 (mtDNA in biopsies) and 3 (cell numbers/doublings and mtDNA in 1-cell embryos), experimental groups were compared using one-way ANOVA, followed by Tukey's or Duncan's post-hoc tests. In Experiment 2 (approach validation) a Person's 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 *t*-Student's post-hoc test. Differences with probabilities (*P*) < 0.05 were considered significant. In the text, values are reported as means ± the standard error of the mean (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. Since 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 over 100 fold (1.70×10^4 to 1.89×10^6). Coefficients of variation averaged 32% among oocytes and were similar between oocytes from small, medium and large follicle groups. Due to 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 1-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 1-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 1-cell embryo. Together, these results indicate that the biopsy approach can be reliably used to determine the mtDNA content in 1-cell stage embryos. The average percentile amounts of mtDNA and cytoplasm removed from 1-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 1-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 1-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 1-cell embryos that, after in vitro culture, (i) remained uncleaved ($3.50 \times 10^4 \pm 3.02 \times 10^3$), (ii) cleaved but arrested after cleavage ($3.50 \times 10^4 \pm 2.12 \times 10^3$), or (iii) 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 1-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 non-manipulated 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 intra-group 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 1-cell embryos with ~90% less mtDNA than the mean 1-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 1-cell embryos does not inhibit blastocyst development

To experimentally verify that low contents of mtDNA at the 1-cell stage does not affect subsequent embryo development, 1-cell embryos were centrifuged to fractionate their cytoplasm. Then the mitochondrial-enriched cytoplasmic fraction was mechanically removed. After removal of mitochondria, depleted 1-cell embryos were cultured for another nine days to examine their ability to develop to the blastocyst stage in vitro (FIG. 2). When compared to non-manipulated controls (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 2-, 3- and 4-cell stage embryos of the CC group, indicating that the segregation caused by centrifugation at the 1-cell stage was mostly reversed by the time the 1-cell embryo underwent its first and second cleavage divisions (data not shown). Although cleavage rates were marginally inferior in non-manipulated controls (CO group), development to the blastocyst stage was not influenced by centrifugation (CC group) or by the removal of cytoplasm (similar volume as DE) from non-centrifuged

1-cell embryos (MC) (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 1-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 1-cell stage is not related to the capacity to develop to the blastocyst stage.

Depleted 1-cell embryos replenish mtDNA by increasing replication

To investigate the mechanisms by which depleted 1-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 non-manipulated (CO) and micromanipulated (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 1-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 1-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 up-regulation 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 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 up-regulation was directed specifically towards re-establishing the mtDNA copy numbers in depleted embryos. Together, these results suggest that 1-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 [8]). However, the number of mtDNA molecules accumulated in the oocyte by the end of oogenesis is widely variable (reviewed by [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 1-cell embryos. We demonstrated that the mtDNA copy number at the 1-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 due to 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, measured by the blastocyst formation rate, of their oocytes, 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, due to 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, since 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. Due to mitochondrial remodeling by the time the 1-cell embryo underwent its first cleavage division [39], it was necessary to use parthenogenetic 1-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 1-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 intra-cytoplasmic sperm injection (ICSI) 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 a mtDNA depletion similar to that reported in oocytes with poor developmental competence. Since 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, due to the sharply 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 developed into healthy blastocyst can, regardless of the amount of mtDNA present at the 1-cell stage, generate sufficient mtDNA copies to support the energy requirements for early development.

To better understand the mechanism by which 1-cell embryos containing as few as 90% less mtDNA than the mean content develop into blastocysts, we developed an approach based on embryo centrifugation to partially deplete 1-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 by 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 1-cell embryos partially depleted of mtDNA produce healthy blastocysts with no effect 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 non-manipulated 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 compactation/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 to 9, a specific up-regulation 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 up-regulation of *TFAM* expression following 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 [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.

Herein we show that in contrast to previous studies [20, 23, 25-27], bovine 1-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 up-regulating 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., due to 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 1-cell embryos harboring fewer copies of mtDNA can reverse this condition by regulating the mtDNA replication at the blastocyst stage. Altogether, 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.

ACKNOWLEDGMENTS

The authors thank Dr. J. P. Burgstaller and Dr. R. Steinborn for designing the primers and the probe used for mtDNA quantification. The authors gratefully acknowledge the help provided by Dr. F. H. Biase, Dr. G. K. Merighe, Dr. M. S. Miranda, Dr. P. S. Monzani, Dr. S. S. Cortezzi, J. R. Sangalli, L. G. Mesquita, P. M. Porciuncula and T. S. Machado. We also would like to thank Dr. S. C. Méo for her critical reading of the manuscript and comments.

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FIGURE LEGENDS

FIG. 1. Parthenogenetic embryo developmental competence is unrelated to mtDNA copy number. (A) Photomicrographs of (a) uncleaved, (b) cleaved (8-cell stage) and (c) blastocyst embryos. Scale bars = 40 µm. (B) Content of mtDNA in cytoplasmic biopsies of embryos at the 1-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 ($P = 0.94$).

FIG. 2. Removal of the mitochondrial-enriched cytoplasmic fraction reduces the mtDNA content in parthenogenetic 1-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) (a) before and (b) after depletion, respectively. Scale bars = 50 µm. (B) Number of copies of mtDNA remaining in embryos of the experimental groups. CO: non-manipulated embryos; CC: embryos that were centrifuged at the 1-cell stage; MC: embryos that had a cytoplasmic portion removed at the 1-cell stage; DE: embryos that were centrifuged at the 1-cell stage to remove part of the mitochondrial-enriched cytoplasmic fraction. Values are reported as means ± SEM. (a, b, c) Bars with different superscript letters denote a significant difference among experimental groups ($P < 0.05$).

FIG.3. Mitochondrial depletion of parthenogenetic 1-cell embryo does not inhibit blastocyst development. Photomicrographs of blastocysts at Day 9 obtained using (A-D) light and (E-H) fluorescence microscopy (embryos stained with Hoechst 33342). (A, E) CO: non-manipulated embryos; (B, F) CC: embryos that were centrifuged at the 1-cell stage; (C, G) MC: embryos that had a cytoplasmic portion removed at the 1-cell stage; (D, H) DE: embryos that were centrifuged at the 1-cell stage to remove part of the mitochondrial-enriched cytoplasmic fraction. Embryos

in (A-D) are not necessarily the same in (E-H). Arrows indicate the inner cell mass. Scale bars = 100 µm.

FIG. 4. Depleted parthenogenetic embryos replenish mtDNA by increasing replication. CO: non-manipulated embryos; CC: embryos that were centrifuged at the 1-cell stage; MC: embryos that had a cytoplasmic portion removed at the 1-cell stage; DE: embryos that were centrifuged at the 1-cell stage to remove part of the mitochondrial-enriched cytoplasmic fraction. Embryos were analysed 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. (9*) The amount of mtDNA at Day 9 was corrected by the mean cell number of each experimental group. Values are reported as means ± SEM. (a, b, c) Different superscript letters on 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 ($P < 0.05$).

FIG. 5. Transcripts controlling mtDNA replication are increased in depleted parthenogenetic embryos. CO: non-manipulated embryos; CC: embryos that were centrifuged at the 1-cell stage; MC: embryos that had a cytoplasmic portion removed at the 1-cell stage; DE: embryos that were centrifuged at the 1-cell stage to remove part of the mitochondrial-enriched cytoplasmic fraction. Embryos were analysed at Day 3 (4- to 16-cell stages), Day 6 (early blastocyst stage) and Day 9 (hatching and hatched blastocyst stages). The amounts of (A) *TFAM*, (B) *NRF1*, (C) *MT-CO1* and (D) *HIST1H2AG* transcripts are expressed in relation to the CO group at Day 3. (9*) Transcript amounts at Day 9 were corrected by the mean cell number of each experimental group. Values are reported as means ± SEM. (a, b, c) Bars with different superscript letters 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 ($P < 0.05$).

TABLES

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

	Follicle diameter (mm)		
	≥1 to <3	≥3 to <6	≥6 to <8
N	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	3.92×10^{5} - 1.72×10^{6}	4.36×10^{5} - 2.06×10^{6}	1.70×10^{4} - 1.89×10^{6}
CV	30%	28%	37%

($^{a, b}$) Means with different letters within row denote a significant difference among experimental groups ($P < 0.05$).

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

Groups	Day 3	Day 7	Day 9	
	Cleavage[*] N (%)	Blastocyst[*] N (%)	Blastocyst[*] N (%)	Cell numbers/doublings[#] (N)
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

([†]) CO: non-manipulated embryos; CC: embryos that were centrifuged at the 1-cell stage; MC: embryos that had a cytoplasmic portion removed at the 1-cell stage; DE: embryos that were centrifuged at the 1-cell stage to remove part of the mitochondrial-enriched cytoplasmic fraction. Values are reported as ([*]) frequency or ([#]) mean ± SEM. ([a, b, c]) Means with different superscript letters within columns denote a significant difference among experimental groups ($P < 0.05$).

FIGURES

FIG. 1

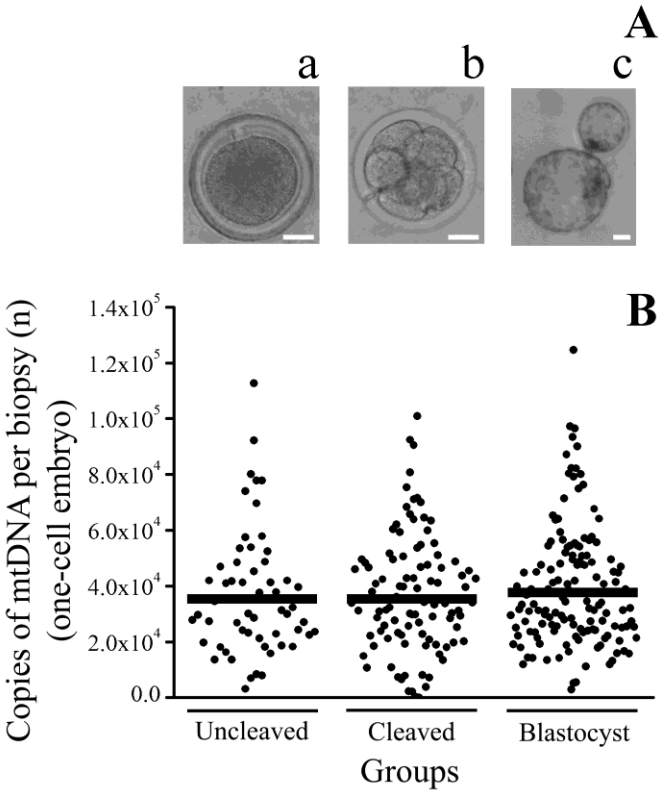


FIG. 2

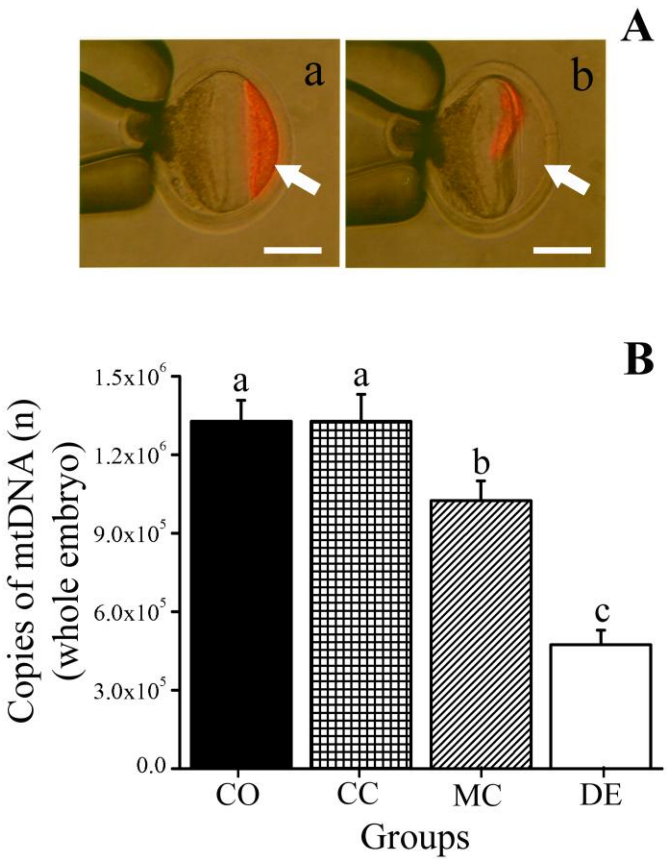

A
a
b
B
Copies of mtDNA (n)
(whole embryo)
1.5x10^6
1.2x10^6
9.0x10^5
6.0x10^5
3.0x10^5
0.0
a
a
b
c
CO
CC
MC
DE
Groups

FIG. 3

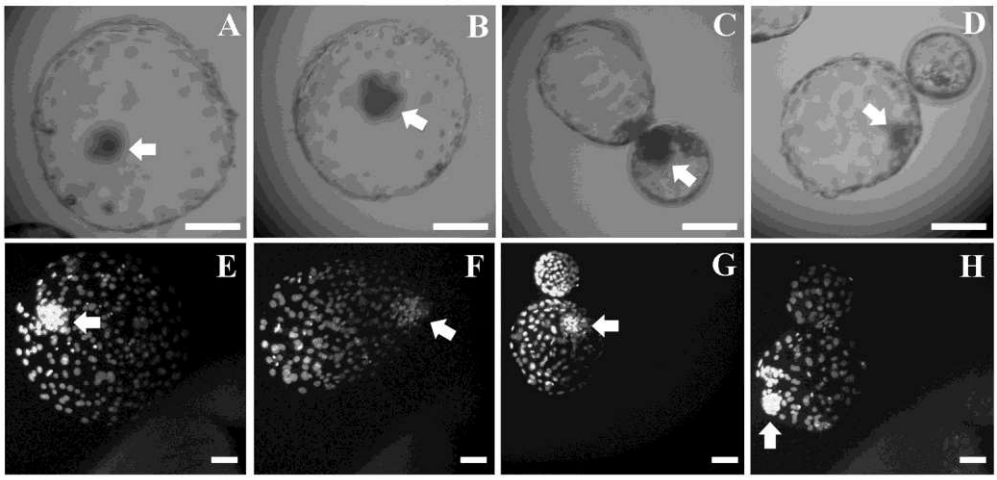


FIG. 4

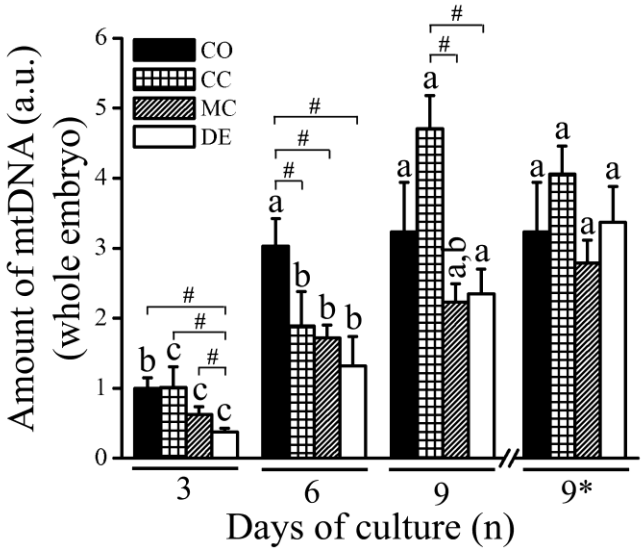
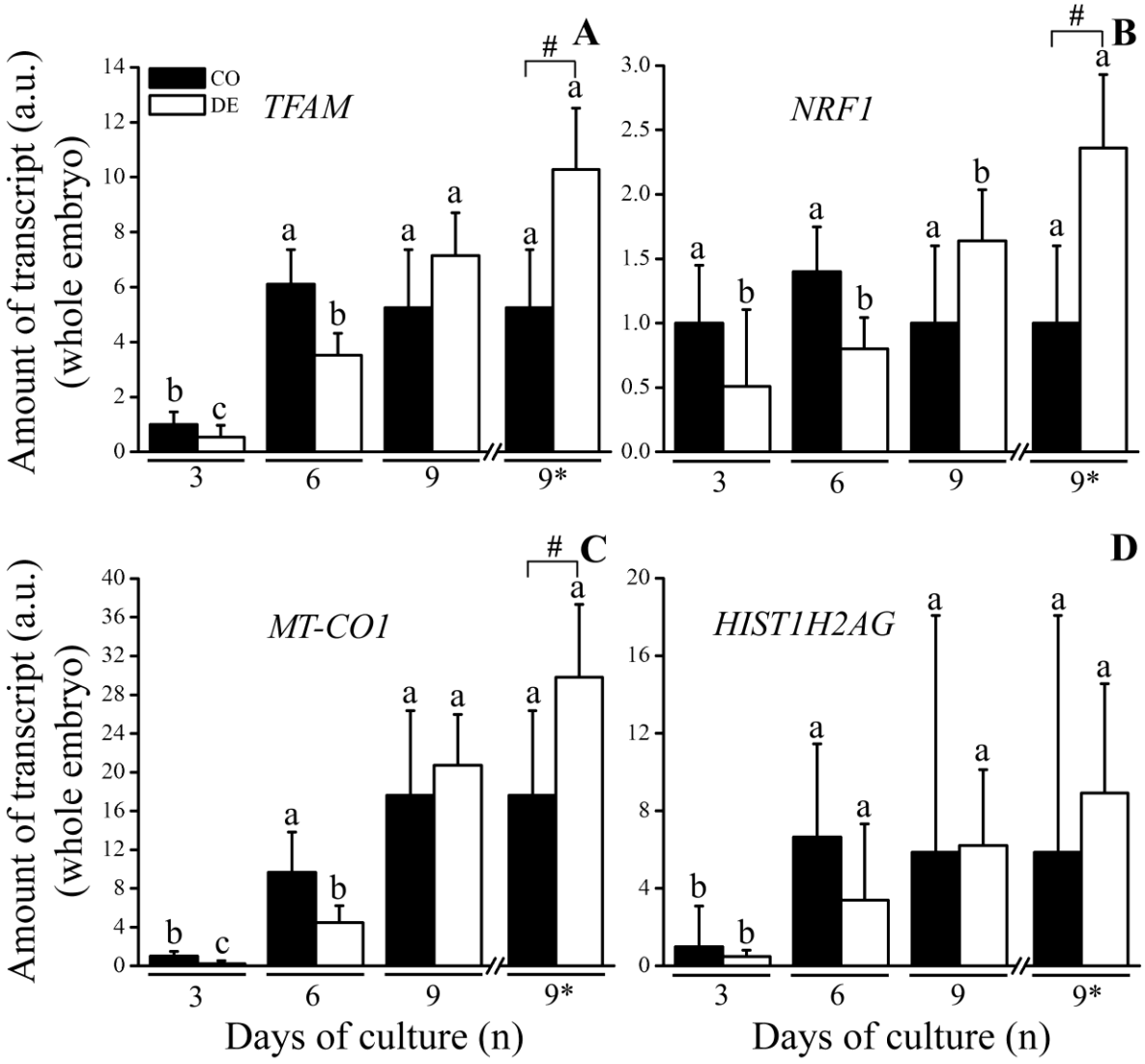

Amount of mtDNA (a.u.)
(whole embryo)
CO
CC
MC
DE
Days of culture (n)
3
6
9
9*
#
a
b
c

FIG. 5


A
B
C
D
CO
DE
TFAM
NRF1
MT-CO1
HIST1H2AG
Amount of transcript (a.u.)
(whole embryo)
Days of culture (n)
3
6
9
9*
#

6- CONCLUSÃO GERAL

6.1 Conclusões

Neste trabalho, valendo-se de duas metodologias inéditas, são fornecidas evidências de que o número de cópias de mtDNA não está relacionado com a competência de desenvolvimento de oócitos viáveis de bovinos. Estas evidências são ainda suportadas pelo achado de que o embrião é capaz de regular o número de cópias de mtDNA no estádio de blastocisto e portanto, corrigir a quantidade de mtDNA durante o desenvolvimento inicial. A relação destes achados com os descritos anteriormente em humanos é apresentada na forma de modelo hipotético (Figura 6). Estes achados contrariam o que foi descrito previamente em camundongos, ressaltando a necessidade de estudos com espécies mais semelhantes ao homem antes do uso clínico do mtDNA como ferramenta para o diagnóstico de fertilidade em mulheres (vide Apêndice 3 e Anexo 3).

Este trabalho tem implicação no controle da herança mitocondrial e, portanto, para a prevenção da transmissão de sérias patologias causadas por mutações no mtDNA. A técnica de transferência de citoplasma foi sugerida no passado como possível ferramenta para a prevenção da transmissão de doenças mitocondriais. Mas seu uso neste sentido não foi possível, dentre outros motivos, devido a limitada quantidade de citoplasma possível de se transferir para diluir o mtDNA mutante presente no oócito ou zigoto da paciente. Por meio do método de depleção mitocondrial apresentado neste trabalho seria possível remover grande parte das mitocôndrias do zigoto da paciente antes de utilizá-lo como receptor de citoplasma. Desta forma, a introdução de mtDNA selvagem proveniente de outra pessoa diluiria o mtDNA mutante para baixos níveis, potencialmente evitando a transmissão da patologia (vide Apêndice 4 e Anexo 4).

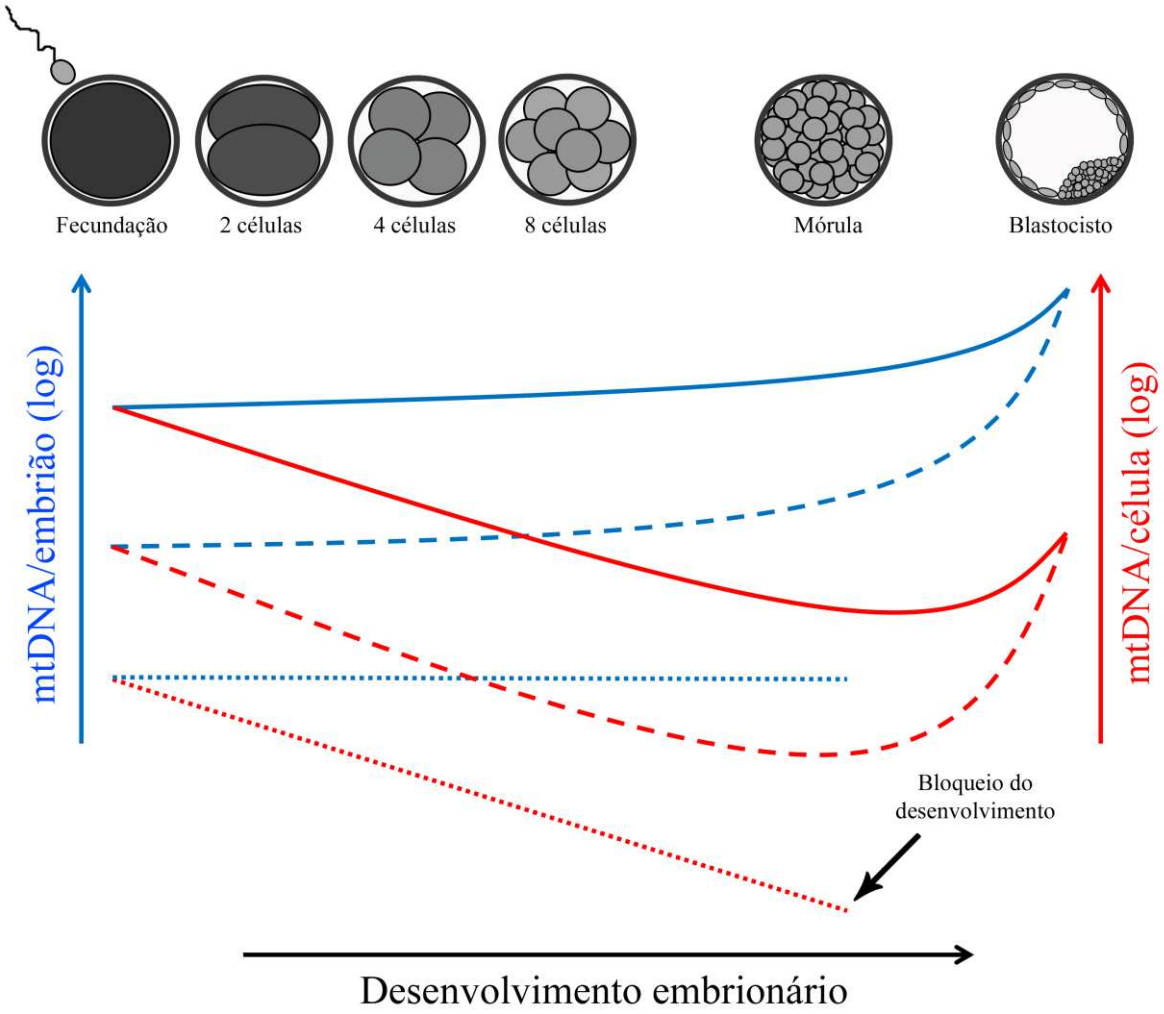


Figura 6. Modelo hipotético para o efeito da quantidade de mtDNA no oócito sobre o desenvolvimento embrionário inicial de bovinos. Em oócitos com quantidade normal de DNA mitocondrial (mtDNA; linha contínua) a replicação do mtDNA é reiniciada entre os estádios de mórula e blastocisto possivelmente para suprir a necessidade energética que aumenta bastante durante o período. Em oócitos que possuem menos cópias de mtDNA que o normal mas que se desenvolvem a blastocisto (linha tracejada), a replicação do mtDNA também é reiniciada entre os estádios de mórula e blastocisto, mas se estende por um período maior, o que resulta na restauração da quantidade de mtDNA em relação a embriões normais. Por fim, oócitos que possuem menos cópias de mtDNA que o normal e não se desenvolvem a blastocisto (linha pontuada) têm o desenvolvimento bloqueado pois i) possuem um defeito no mecanismo de replicação do mtDNA o que impede a restauração do número de cópias; ou, ii) possuem uma quantidade tão pequena de mtDNA no início do desenvolvimento que mesmo com o reinício da replicação a quantidade de mtDNA não é suficiente para suprir as necessidades energéticas do embrião.

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8- APÊNDICES

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PLoS GENETICS

Review

Transmission of Mitochondrial DNA Diseases and Ways to Prevent Them

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Abstract: Recent reports of strong selection of mitochondrial DNA (mtDNA) during transmission in animal models of mtDNA disease, and of nuclear transfer in both animal models and humans, have important scientific implications. These are directly applicable to the genetic management of mtDNA disease. The risk that a mitochondrial disorder will be transmitted is difficult to estimate due to heteroplasmy—the existence of normal and mutant mtDNA in the same individual, tissue, or cell. In addition, the mtDNA bottleneck during oogenesis frequently results in dramatic and unpredictable intergenerational fluctuations in the proportions of mutant and wild-type mtDNA. Pre-implantation genetic diagnosis (PGD) for mtDNA disease enables embryos produced by in vitro fertilization (IVF) to be screened for mtDNA mutations. Embryos determined to be at low risk (i.e., those having low mutant mtDNA load) can be preferentially transferred to the uterus with the aim of initiating unaffected pregnancies. New evidence that some types of deleterious mtDNA mutations are eliminated within a few generations suggests that women undergoing PGD have a reasonable chance of generating embryos with a lower mutant load than their own. While nuclear transfer may become an alternative approach in future, there might be more difficulties, ethical as well as technical. This Review outlines the implications of recent advances for genetic management of these potentially devastating disorders.

Introduction

One in 400 people carries pathogenic mitochondrial DNA (mtDNA) mutations [1]. These may cause epilepsy, liver failure, cardiomyopathy, or sudden death; or, more commonly, milder disorders such as age-related deafness [1] and/or diabetes [2] and loss of vision [3]. Yet, management and prevention of mtDNA diseases has lagged far behind the genetics revolution [4]. Although preimplantation genetic diagnosis (PGD) has been successfully used to prevent transmission of mtDNA disease [5,6], its use has been limited for several reasons that are developed in the following sections. Technical improvements in methods for nuclear transfer [7,8] have aroused expectations of preventing transmission of these disorders, but is this method safe?

Dose of Mutant mtDNA Determines Severity: Implications for Prenatal Genetic Diagnosis

Chorionic villus sampling (CVS, where early placental tissue is sampled with minimal impact on the foetus) has been extremely successful in preventing recurrence of Mendelian genetic diseases, but not for maternally inherited diseases, caused by mutations in the mtDNA, because of the problem of heteroplasmy [9,10]. Thousands of mtDNA copies are present in every nucleated cell. Normal individuals are homoplasmic (i.e., virtually all their mtDNA copies are identical), but individuals affected by mtDNA diseases are usually heteroplasmic: most of their tissues and cells have a mixture of both normal and mutant mtDNAs. There is also a threshold effect (tissues function normally unless the proportion of mutant mtDNA rises above a particular level) in most diseases. The level of this threshold varies with both tissue and mutation type, usually in the range 50 to 100% mutant mtDNA, but occasionally as low as 10% [11]. Hence, for many mtDNA mutants, disease might be prevented by selecting embryos or actively lowering the level of mutant mtDNA (for instance by using nuclear transfer). But this is not universally applicable, because some mtDNA diseases are commonly homoplasmic and lack a clear threshold [12].

Unique Inheritance of mtDNA: Heteroplasmy and the Mitochondrial Bottleneck

Heteroplasmy is one reason why the clinical severity of mtDNA disorders is highly variable and can progress with time. In mtDNA disease patients the level of mutant mtDNA commonly [13,14] (but not always [15]) falls in blood throughout life (perhaps as a result of selection against detrimental mutant mtDNA within a rapidly dividing population of cells [13,16]). There are a few case reports suggesting that some types of mtDNA mutant accumulate in non-dividing cells such as muscle [14,17,18], where mtDNA turnover is slow [19], and less subject to inter-cellular competition

Citation: Poulton J, Chiaratti MR, Meirelles FV, Kennedy S, Wells D, et al. (2010) Transmission of Mitochondrial DNA Diseases and Ways to Prevent Them. PLoS Genet 6(8): e1001066. doi:10.1371/journal.pgen.1001066

Editor: Anu Suomalainen, Biomedicum-Helsinki, University of Helsinki, Finland

Published August 12, 2010

Funding: This work was supported by Action Medical Research, the Wellcome Trust, the MRC and Oxford Partnership Comprehensive Biomedical Research Centre, with funding from the Department of Health's NIHR Biomedical Research Centres funding scheme. It was also supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil. The funders had no role in the preparation of or decision to publish the article.

Competing Interests: Oxford currently offers oocyte donation and oocyte sampling for mitochondrial DNA diseases. Preimplantation genetic diagnosis will be available in the near future. JP takes clinical and diagnostic referrals for the Oxford Centre in the Rare Mitochondrial Disorders Service for Adults and Children (NCG). Further information may be found on http://www.obs-gyn.ox.ac.uk/research/Poulton.

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[20]. However, this model explains by no means all of such observations [21]. The progressive change in distribution of some human mutants parallels the dynamic of apparently neutral variants in blood and spleen in an animal model [22] and underlines our inability to define the parameters determining the characteristics that we have loosely termed "detrimental." The scanty available evidence suggests that there is less segregation in somatic tissues between early embryo and birth than post-natally [10,23]. However, a major component of the germline segregation during transmission of both human [24] and mouse polymorphisms probably occurs during oogenesis [23,25], and hence during development of the mother, apparently while she was in utero herself.

Factors that affect segregation of mtDNA variants include the biological fitness of dividing cells, the mutant load, and any differences between wild-type and mutant mtDNA in the rate of replication and degradation. While accumulation of mutant mtDNA can sometimes be attributed to genetic drift [26], consistent segregation towards loss or gain of mutant mtDNA has been widely documented in human cultured cells [27–29]. Some mutant mtDNAs exhibit segregation in the opposite direction to that predicted on the basis of selection according to mitochondrial function [28,30–33]. Moreover, biased mtDNA segregation has been demonstrated in solid tissues of mice [22]. Two mouse mtDNA variants were selected in different tissues as a result of differences in genetic background [32,33], even though neither was associated with a marked functional defect [22], nor a detectable difference in mtDNA replication rate [22]. Because differences in production of reactive oxygen species (ROS) affect mtDNA copy number [34], they may contribute to segregation of heteroplasmic mutants.

Analysis of segregation of mtDNA mutants in tissue culture often uses "cybrid" technology, where mtDNA-free immortalized cells are fused with cytoplasm containing the mitochondria under investigation. Because such cells are aneuploid, some investigators dismiss this model as non-physiological [35]. However, it does indicate that several factors might underpin mtDNA segregation in cell lines, including cellular fitness, replication pausing, ROS production, and mitophagy (preferential breakdown and recycling of regions of the mitochondrial reticulum of organelles containing mutant mtDNAs) [29,36–39]. It is now increasingly possible to test the validity of such hypotheses in whole animals [32,33].

Genetic counseling of women who are carriers of mtDNA diseases is complex because the dose of mutant mtDNA transmitted to offspring may be determined by the so-called "mitochondrial bottleneck" [40,41], whereby a small number of mtDNAs become the founders for the offspring. If the number of segregating units (groups of clonal mtDNAs that co-segregate) that become the mtDNA founders of the embryo is small, then large fluctuations may occur in a single generation. Hauswirth and Laipis [42–44] suggested that two components to this may occur at different developmental stages. Firstly, there is a massive expansion from ~100 mtDNA genomes in the earliest stages of oocyte development or primordial germ cell (PGC) to 100,000 or so in the mature oocyte [42]. Mitochondrial DNA barely replicates during the early stages of development [45] and pre-existing mtDNA molecules segregate among the cells of the blastocyst [43,46–49]. This represents a second mechanism contributing to switching in the proportion of mutant mtDNAs, since mtDNAs are progressively partitioned at each cell division, ultimately producing the very few cells that will give rise to the entire embryo (the inner cell mass) [42,44]. Hence, both clonal proliferation of mtDNA in the developing oocyte and mtDNA segregation during early development contribute to the bottleneck.

Is the Bottleneck Determined by mtDNA Content in Germ Cell Development? Mouse Studies

Recent studies have carefully quantified mtDNA copy number of individual cells during mouse development [49,50–52]. As predicted [49,53], the number of mtDNA copies drops to ~200 molecules in developing PGCs until embryonic day (E) 7.5-8.5 [52], corresponding to the number of segregating units inferred from postnatal analysis [47,53]. There is, however, conflicting data suggesting that copy number does not fall to values lower than 1,000 in PGCs until E7.5 in mice [50,51]. As well as depending on technically demanding measurements of the number of mtDNAs in single cells [50,51], these models have assumed both that segregation in the germline is neutral [54] and that all mtDNA genomes have equal probability of replicating during a single round of cell division. Such assumptions may not be valid, since Wai et al. [52] showed that a sub-population of mtDNAs replicates during folliculogenesis in mice, replenishing the mtDNA content in oocytes and potentially explaining the shifts in mutant load between two generations (Figure 1). While this might explain the variance in mutant load that these authors found in oocytes [52], a more sophisticated analysis demonstrates that a larger set of biological data is needed to establish their claim [55,56].

In humans, although the meiotic division is initiated in the germline of the developing foetus during the last trimester of pregnancy, primary oocytes remain arrested in the first stage of the meiosis during the years between birth and puberty. In women of reproductive age, a group of oocytes is selected to grow and resume meiosis every cycle of ~28 days. In most cases this results in the production of a single developmentally competent oocyte. It is possible that clonal expansion of a subpopulation of mtDNA during folliculogenesis in mice (between the stages of primary and mature oocyte) may correspond to the mitochondrial bottleneck [52]. If this is correct, then the segregating unit that is the physical basis of the bottleneck might be the mitochondrial nucleoid, usually containing several mtDNAs [57], rather than a single mtDNA molecule [49]. Understanding the nature of mtDNA packaging in nucleoids would then take on a new importance for biology. On the other hand, if the mitochondrial bottleneck occurs late in germline development, what is the purpose of the dramatic reduction in mtDNA copy number reported during early development? Recent studies have suggested it serves to preserve a homoplasmic population of predominantly healthy mtDNA molecules by selecting against mtDNA mutations that damage mitochondrial function (see below).

Selection against Detrimental mtDNA Mutants in the Mouse Germline

Three studies suggest that there is selection against detrimental mtDNA mutants in the mouse germline. One group developed a mouse with mtDNA rearrangements modelling Kearns-Sayre syndrome [58] in which the level of mutant mtDNAs in a mother's oocytes fell with time [59]. Like the occasional [60] mtDNA rearrangements that are maternally transmitted [61], these mice had mtDNA duplications in addition to deletions [59].

Another group investigated the transmission of randomly generated mtDNA mutations in a mouse model of mtDNA disease [62]. In this model, there is a mutation in the proof-reading domain of the mtDNA polymerase, *PolgA*, and this generates high levels of point mutations in the mtDNA. The homozygous founder female mice were crossed with wild type and transmitted multiple

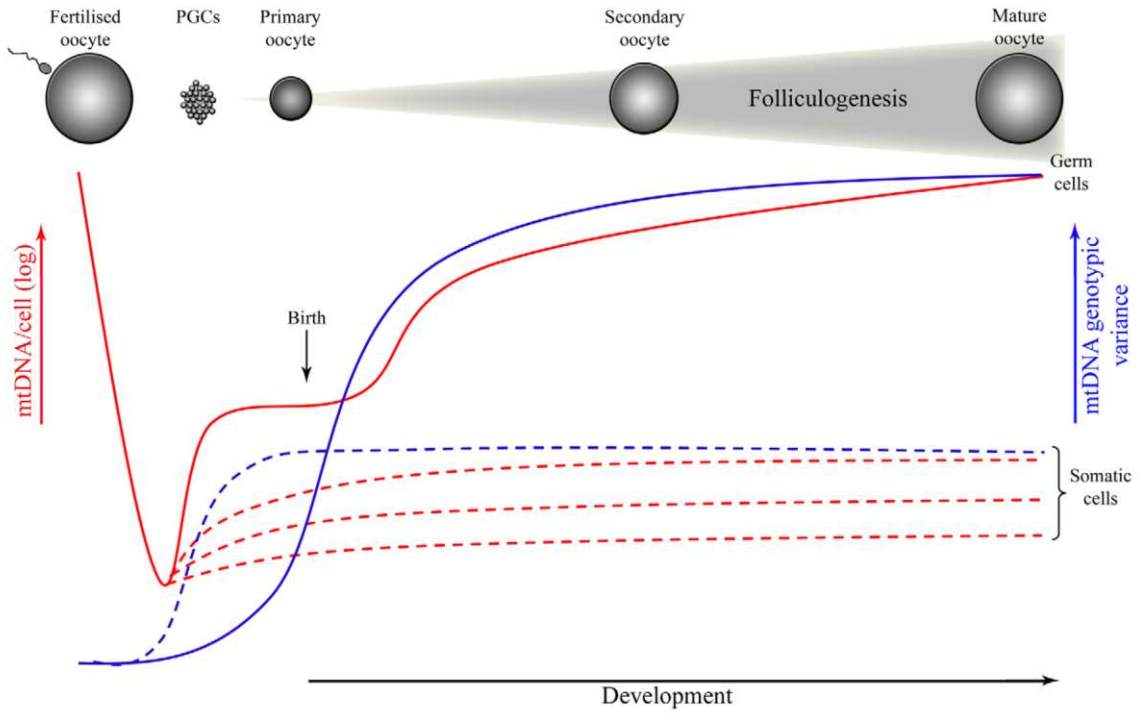

Figure 1. Mitochondrial DNA (mtDNA) copy number and genotypic variance throughout development in germ and somatic cells of mammals. Although mtDNA genotypic variance in somatic cells increases early during development due to cellular differentiation, according to recent findings this will only occur later in germ-line development, during folliculogenesis that takes place after birth. If this is correct, then the mitochondrial genotype of the next generation would be defined only during adulthood, during the folliculogenesis that occurs every cycle of 28 days in women.
doi:10.1371/journal.pgen.1001066.g001

mtDNA mutations (on average 30 mutations per first generation mouse) to their offspring who were heterozygous for the *PolgA* mutation. Subsequent backcrossing eliminated the mutant *PolgA* allele and hence mtDNA mutants were passively transmitted without generating further mutations. It was thus possible to observe and compare the segregation of multiple different mtDNA mutations in a single lineage. Neutral mutations that do not alter the protein sequence undergo less selection than those that do. Purifying selection can therefore be compared with neutral drift by the relative frequency of such mutations. Clonal selection against deleterious mutations occurred in a remarkably short time frame. Indeed, many deleterious mutations were eliminated within four generations. However, selection was stronger and occurred more rapidly against mutations in genes encoding mRNAs than tRNAs. This may be linked to the apparently high frequency of pathogenic human mtDNA mutations that are identified in tRNAs [62].

A third study, focussing on two pathogenic mtDNA point mutations, again demonstrates selection in mouse [63]. These authors introduced mutant mtDNA from a well-characterised cell line into the germline using cybrid technology and a female embryonic stem cell line. Both the more severe frameshift (insertion) mutation and the milder missense mutation were initially homoplasmic, conferring a severe respiratory chain defect. However, one of the embryonic stem (ES) cell clones became heteroplasmic because a revertant of the frameshift mutation arose; a secondary deletion of the adjacent base restored the reading frame. When this line went into the germline, the mice developed a sub-clinical myopathy and cardiomyopathy but bred normally. The frameshift mutation was lost in favour of the revertant within four generations. None of the offspring had a higher level of the frameshift mutation than their mother, and studies of oocytes showed that the selection had occurred by the time oocytes were mature. These studies are consistent with other studies on mice [52] and on humans [25,64]. The selection appears to depend on some aspect of mitochondrial function, but studies of the bottleneck have not clarified the precise mechanism or at what stage of oogenesis it is likely to have occurred. While some classic studies in humans [64,65] and in mice [53] demonstrate that level of mutant mtDNA follows a distribution that may be random [66], others are very different [23,30]. The latter are skewed towards virtual homoplasmy for both mutant and wild-type mtDNA in oocytes from individual women. One explanation would be that a single mtDNA passes the bottleneck, but there is no obvious mechanism for such an extreme situation. Alternatively this could arise because genetic drift can lead to fixation of neutral mutations [54]. While some investigators consider that the different distributions may be due to the specific mutation, we note that the skewed distributions have only been seen following super-ovulation. Furthermore, close examination of data suggest that the mean level of mutant mtDNA in the oocytes/offspring is not identical with that of the mother, so germline selection [59,62,63] is not excluded [54].

But what is the basis of the selection seen in mice and potentially in humans? Only 30% of oogonia established during fetal life develop into matured oocytes, the remainder undergoes apoptosis [67,68]. Fan et al. [63] suggested that dysfunctional mitochondria

generate high ROS levels that are the signal underlying selection against oocytes with high mtDNA mutant load by apoptosis.

A second possible mechanism for selecting against mutant mtDNA is selection at the organelle level. The number of mtDNA copies per mitochondrion in germ cells is thought to be as few as one or two molecules, in comparison to eight or so in somatic cells [57]. Thus, mutations in a few mtDNA copies can be distinguished among wild-type mtDNAs present in the same cell by the effect of mutations on mitochondrial phenotype. For instance, damaged mitochondria might be degraded by intracellular mechanisms such as autophagy or, more specifically, mitophagy [36]. Evidences of this were given by Twig et al. [69] who showed that dysfunctional mitochondria are less likely to fuse with the remaining mitochondria and are degraded by autophagy. Although this event was shown in somatic cells, autophagy is also present in germ cells and early embryos [70] and might be involved in removal of mutant mtDNA from the next generation. Another possibility for selection at the organelle level is competition between dysfunctional and normal mitochondria, where dysfunctional mitochondria might be less efficient for import and enzymatic function of the nucleus-encoded proteins that are required for mtDNA replication. This might result in an advantage of wild-type molecules to replicate over the mutant ones, thus decreasing the mutant load in germ cells and in the next generation [71]. As discussed above, Wai et al. [52] have reported that a sub-population of mtDNAs is replicated during folliculogenesis to replenish the mtDNA content in oocytes. If such a sub-population were positively selected on phenotype by an unknown mechanism, this might explain the observed pattern of selection against mtDNA mutations.

A third possible mechanism is specific to oocytes, based on a structure known as the Balbiani body or the mitochondrial cloud [72–74]. The Balbiani body comprises mitochondria and endoplasmic reticulum organized around Golgi elements [73–78] that may enable germplasm mRNAs to be specifically inherited by the PGCs in the future embryo. In the same way, a specific mitochondrial sub-population may segregate to the Balbiani bodies and ultimately populate the PGCs [73,79–81], potentially explaining the pattern of selection against severe mtDNA mutations. In some non-mammalian species mitochondria with the highest membrane potentials are found in Balbiani bodies [78,80,81], suggesting that high-quality mitochondria and mtDNAs are selected for transmission to the PGCs of the next generation. While this is an appealing mechanism for selecting against mutant mtDNAs, there is little supporting evidence and it is still controversial, even in mouse [52]. Furthermore, the Balbiani body could not explain the progressive decrease in load of mutant mtDNA in mouse oocytes of an individual female with age.

Whatever the underlying mechanism, something occurring during early oogenesis and/or folliculogenesis seems to provide a degree of selection against mutant mtDNA molecules. Studies by Sato et al. [59] and Fan et al. [63] suggest selection occurs during adult life and, therefore, during folliculogenesis, since mutant load drops in mouse oocytes as a function of time (i.e., between two litters). On the other hand, mutations that escape this filter would then be exposed to selection at the level of the individual. Thus, several mechanisms may contribute to the bottleneck and prevent dissemination of mtDNA mutations (Figure 2).

Mitochondrial DNA Bottlenecks in Human Germ Cell Development

Genetic management of patients with mtDNA disease depends on understanding both germline segregation and the physiological basis of the bottleneck. However, the published human data where oocytes are compared with load in maternal post mitotic tissues are minimal [54]. It is increasingly clear that a major component of this bottleneck has occurred by the time oocytes are mature in human controls [25], patients with mtDNA disease [64,82], and mouse models [52,53,63]. Statistical analysis of oocytes shows that in some cases the distribution of mutant mtDNA is consistent with random drift, but does not exclude the possibility of selection in the germline at an earlier stage [66]. On the other hand, a de novo mutation in a child and in oocytes appeared to be absent from the mother's other tissues [83], suggesting that it arose within the development of her germ cells. Comparison of human and mouse data suggests potentially important differences in both the type of rearrangement that is typical [59,60] and in the bottleneck size [55]. Hence, it may not be appropriate to extrapolate from the consistent selection against detrimental mtDNA mutants seen in the mouse [62] to humans.

Implications of Heteroplasmy for Genetic Management of Human Diseases

Oocyte donation would avoid all the problems associated with the presence of mutant mtDNA, but there is a shortage of oocyte donors. Pre-implantation genetic diagnosis for mitochondrial disease could be the best option for patients carrying high levels of mutant mtDNA [6]. This approach involves analyzing embryos produced by in vitro fertilization (IVF) and only transferring those determined to be at very low risk. Preimplantation genetic diagnosis is performed earlier in development (three days after fertilization) than CVS, and two cells are usually taken for mtDNA disease [6]. This is because analysis of one or two cells from an embryo containing 6–10 cells may be more representative of the whole conceptus [84], but not necessarily of the part that will become the foetus. Moreover, sampling two cells rather than only one provides a more confident result (the result from one cell can be compared against the other) and does not appear to impair pregnancy outcome [6,85].

While PGD clearly has enormous promise for women with sub-clinical levels of mtDNA mutations [6,23], it may be more complex for women carrying high mtDNA mutation loads and displaying disease symptoms [6,86]. If such women typically transmit levels of mutant mtDNA close to their own [82,87], they are likely to produce few if any disease free embryos. If, however, the level of mutant mtDNA in their oocytes were polarized to the two extremes as seen in neuropathy, ataxia and retinitis pigmentosa (NARP) [23,30], they might have a reasonable chance of usable embryos. This depends to what extent the selection against detrimental mtDNA mutants that is seen in mouse germline also occurs in humans. Nevertheless, offering PGD for certain mtDNA diseases, followed by CVS to confirm that the level of mutant mtDNA in the foetus is low, would likely have advantages over CVS alone. The main drawback of CVS for mtDNA disorders is that it is not entirely certain that the level of mutant mtDNA detectable in a single CVS sample will accurately reflect that of the foetus [10]. Indeed, such data that exist suggest that there is a degree of variation of perhaps $\pm 10\%$ in the level of neutral [88] and pathogenic variants in placenta [83]. Moreover, certain centers are now offering PGD [5,6,23].

Is Nuclear Transfer the Way Forward?

Since Dolly the sheep was created by fusing an adult somatic cell with a recipient enucleated oocyte, producing in Dolly

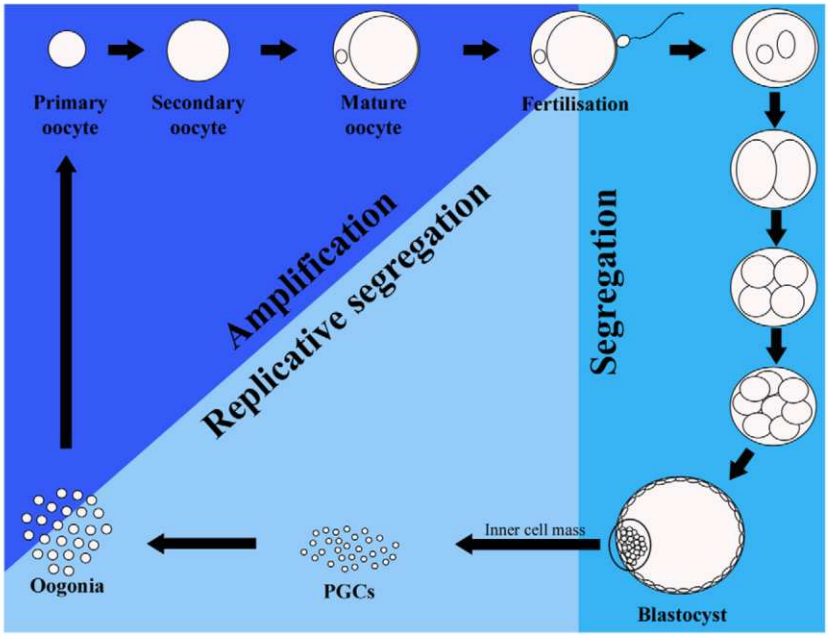

Figure 2. Mitochondrial DNA (mtDNA) cycle in the mouse germline. During early embryo development ("Segregation" on the diagram, representing the first seven to eight days after fertilization) the mtDNA is segregated among daughter cells without being replicated. The number of mtDNA copies thus decreases drastically, being lowest in primordial germ cells (PGCs). The next stage is marked "Replicative segregation," which implies random replication and partitioning of mtDNAs into daughter cells. The last stage, "Amplification," is characterized by an exponential amplification of mtDNA molecules. It has been suggested that replication of mtDNA during this stage is restricted to a sub-group of molecules leading to drastic changes in the mtDNA genotype in the mature oocyte. Yet, there seems to be during this stage a selection against mutations in the mtDNA that might occur.
doi:10.1371/journal.pgen.1001066.g002

mtDNA inherited not from the somatic cell donor but the recipient oocyte [89], researchers have contemplated altering the mitochondrial population of a human embryo using nuclear transfer. It has been possible to use nuclear transplantation at the zygote stage (pro-nuclear transfer) to partially correct respiration defects and mitochondrial diseases in mice carrying a large-scale deletion of mtDNA [90].

Recently, Tachibana et al. [8] transferred nuclei at an earlier stage; spindle-chromosomal complexes were removed from mature monkey oocytes, with minimal if any adherent mtDNA, and placed into other oocytes from which the complex had been removed. This study resulted in the generation of three healthy offspring with less than 3% of nuclear donor mtDNA [8]. More recently, Craven et al. [7] transferred pro-nuclei between human zygotes resulting in minimal carry-over of nuclear donor mtDNA and compatible onward development to the blastocyst stage in vitro. Because of the current regulations and the paucity of "spare" human embryos, this study was carried out in abnormally fertilized embryos. Disappointingly, the levels of nuclear donor mtDNA were very variable between cells of the resulting embryos (ranging from less than 0.5 to 11.4%), suggesting that mtDNA segregation might be disturbed by the procedure. This may be a consequence of using genetically abnormal embryos that would not occur in bona fide treatment cycles. But it might be because they used a drug that specifically targets the microtubule-based system (nocodazole) for organizing mitochondria in the cell. Despite this, both studies [7,8] (with their pros and cons) are of fundamental importance and hold promise for the future treatment of mtDNA diseases.

A different procedure, ooplasm donation (cytoplasm from a donor oocyte), offers an alternative [91]. Ooplasm donation has been used in humans as a treatment for poor IVF embryo development for a type of infertility that might be due to intrinsic defects of the oocyte cytoplasm. In this experimental procedure, mitochondria, cytoplasm, and associated structures from a donor oocyte are injected into a recipient unfertilised oocyte prior to IVF. Mitochondrial DNA analysis of children born following the procedure demonstrated that the contribution of donor mtDNA is small [92], but, in some cases, the proportion of donor mtDNA far exceeded the expected 10–15% [93], based on the volume of cytoplasm derived from the donor. While genetic drift might occasionally underlie such a change, experiments on bovine zygotes suggest that mitochondrial replacement can be consistently improved by centrifugation and removal of the recipient mtDNA without apparent effects on development [94,95]. Centrifugation causes mitochondria to concentrate in one of the zygote's poles [94,95], allowing removal of mitochondrion-enriched cytoplasm by micromanipulation. Doing this, it is possible to remove over 60% of recipient-zygote mtDNA before ooplasmic transfer [94]. Furthermore, the use of purified mitochondria as donor mtDNA [96–98] might decrease the mutant load to low levels, ultimately avoiding transmission of the mitochondrial disorders.

Will any of these procedures be viable alternative strategies to more conventional genetic management? Nuclear transfer sounds simple and seems effective in mice [90], monkeys [8], and in human pre-implantation embryos [7], yet there remain very many unknowns. Mitochondrial DNA encodes only a handful of

proteins, the remainder of the thousand or so proteins that go to make up the mitochondrion being encoded by the nucleus. This arrangement necessitates nucleo-mitochondrial interactions, which are as yet poorly understood. In embryos derived either by nuclear transfer or ooplasm donation, the genetic material originates from three unrelated parents (two providing the nucleus and one the mtDNA). While extreme (non-physiological) mismatch between nuclear and mitochondrial DNA has clearly deleterious effects on nucleo-mitochondrial interactions [99,100], might subtle errors in these interactions occur following nuclear transfer? The consequences of uncoupling the mitochondria and nucleus, followed by the introduction of DNA from an unrelated individual are unknown. Genetic studies of such interactions strongly suggest that major problems are unlikely [32,33]. However, backcrossing mice so that one mtDNA was substituted for another on a standardized nuclear background can alter either physical [101] or cognitive performance [102] and even the anatomy of the brain [102]. Furthermore, studies on mice suggest that mtDNA carried-over with the nuclear DNA of the donor zygote (karyoplast) may be replicated faster than that of the recipient, perhaps depending on its proximity to the nucleus [103]. Since nuclear transfer experiments in multiple species show that donor mtDNA may persist in embryos and tissues from the offspring [104], one cannot assume that the mitochondria from the "healthy" enucleated oocyte will ultimately outnumber the mutant mitochondria in the tissues of the foetus and child. Furthermore, even in the best hands, the success rate of achieving a pregnancy per egg is low and donor oocytes are scarce.

Conclusion

In conclusion, the many ethical, scientific, and pragmatic problems have been a major impediment in the genetic management of mtDNA diseases. Recent experiments on animals suggest that nuclear transplant holds future promise. Currently, the most ethical course of action may be to weigh-up the uncertainties and use new approaches such as PGD in an attempt to help these distressed families.

Acknowledgments

We thank A. Shrier and L. Macleod for corrections to the manuscript.

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APÊNDICE 2

BIOLOGY OF REPRODUCTION **82,** 76–85 (2010)
Published online before print 19 August 2009.
DOI 10.1095/biolreprod.109.077776

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.

[1]Supported by Fundação de Amparo à Pesquisa do Estado de São Paulo grant numbers 06/59074-0 and 06/03516-4.
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Received: 25 March 2009.
First decision: 21 April 2009.
Accepted: 7 August 2009.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

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

(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

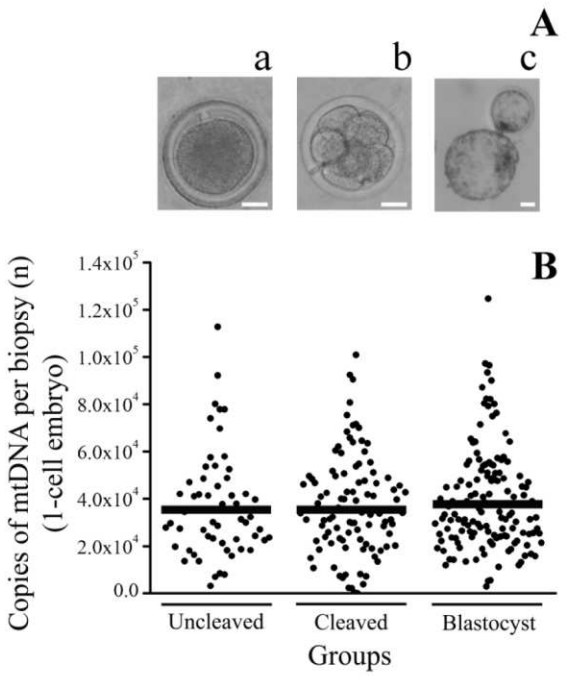

FIG. 1. Parthenogenetic embryo developmental competence is unrelated to mtDNA copy number. **A**) Photomicrographs of uncleaved (**a**), cleaved (eight-cell stage; **b**), and blastocyst (**c**) embryos. Bars = 40 µm. **B**) Content of mtDNA in cytoplasmic biopsies of embryos at the one-cell stage. The 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

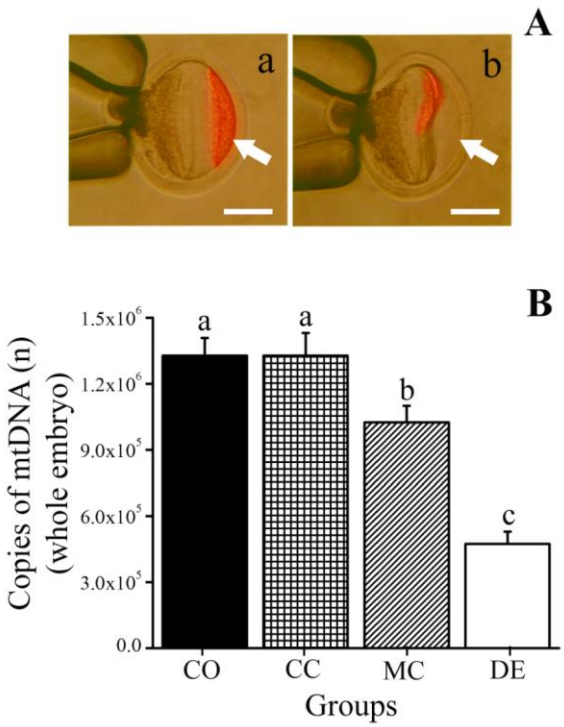

FIG. 2. Removal of the mitochondrial-enriched cytoplasmic fraction reduces the mtDNA content in parthenogenetic one-cell embryos. **A**) Merged photomicrographs obtained using light and fluorescence microscopy to visualize both the mitochondrial-enriched cytoplasmic fraction and stained mitochondria (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 ($P < 0.05$).

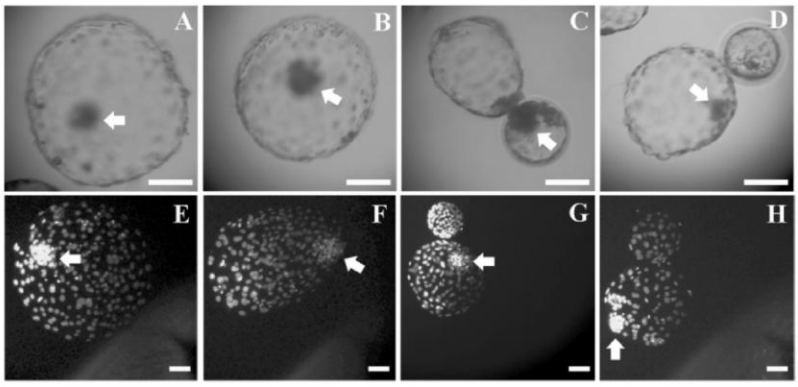

FIG. 3. Mitochondrial depletion of parthenogenetic one-cell embryo does not inhibit blastocyst development. Photomicrographs of blastocysts at Day 9 obtained using light (**A–D**) and fluorescence microscopy (**E–H**; embryos stained with Hoechst 33342). **A** and **E**) Nonmanipulated embryos. **B** 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

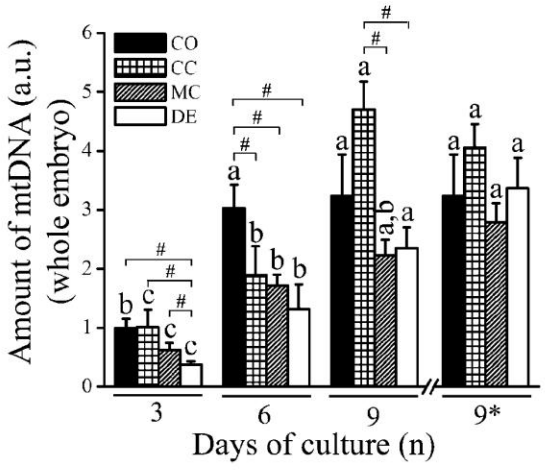

FIG. 4. Depleted parthenogenetic embryos replenish mtDNA by increasing replication. Embryos were analyzed at Day 3 (4- to 16-cell stages), Day 6 (early blastocyst stage), and Day 9 (hatching and hatched blastocyst stages). The amounts of mtDNA are expressed in relation to the CO group at Day 3. The bar 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.

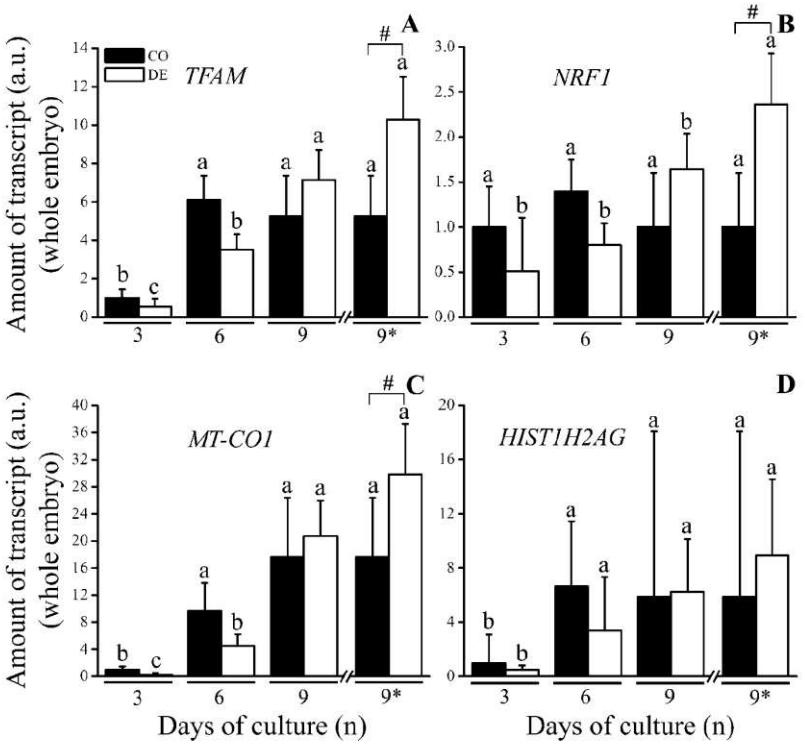

FIG. 5. Transcripts controlling mtDNA replication are increased in depleted parthenogenetic embryos. Embryos were analyzed at Day 3 (4- to 16-cell stages), Day 6 (early blastocyst stage), and Day 9 (hatching and hatched blastocyst stages). The amounts of *TFAM* (**A**), *NRF1* (**B**), *MT-CO1* (**C**), 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.

ACKNOWLEDGMENTS

The authors thank Dr. J.P. Burgstaller and Dr. R. Steinborn for designing the primers and the probe used for mtDNA quantification. The authors gratefully acknowledge the help provided by Dr. F.H. Biase, Dr. G.K. Merighe, Dr. M.S. Miranda, Dr. P.S. Monzani, Dr. S.S. Cortezzi, J.R. Sangalli, L.G. Mesquita, P.M. Porciuncula, and T.S. Machado. We also would like to thank Dr. S.C. Méo for her critical reading of the manuscript and comments.

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BIOLOGY OF REPRODUCTION **83,** 1–2 (2010)
Published online before print 10 March 2010.
DOI 10.1095/biolreprod.110.084269

Commentary

Mitochondrial DNA Copy Number, a Marker of Viability for Oocytes[1]

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Finding a biological marker of viability for oocytes and sperm has been a challenge for reproductive biologists. Such a marker could be used to choose gametes that have a higher chance of producing healthy descendents. This is especially important in humans and would benefit couples facing reproductive problems. The enormous amount of mitochondrial DNA (mtDNA) in oocytes and the fact the mtDNA is not replicated before implantation have led to speculation that the number of mtDNA copies in oocytes could be used as a marker of viability [1–4].

Mitochondrial DNA is present inside the mitochondrion and codes for proteins that are essential for cellular energy production [4]. Multiple copies of mtDNA in the same mitochondrion encode the proteins necessary for mitochondrial function, with the number of copies being directly proportional to the amount of ATP synthesized [4, 5]. Mitochondrial DNA from sperm does not contribute to the new embryo; all the mtDNA arises from the oocyte [4]. Hence, mtDNA copy number in oocytes could be a key determinant of development. Although some evidence supports this hypothesis (e.g., low mtDNA copy number in human oocytes has been linked to infertility [1–3]), little is known about how mtDNA copy number affects oocyte viability. In this issue, Wai et al. [6] report that the mtDNA content in mouse oocytes is involved in gamete viability, and a minimum number of copies is required by oocytes/embryos to support postimplantation development.

To study the importance of mtDNA copy number in oocytes and sperm, Wai et al. [6] partially depleted these cells of mtDNA using Cre recombinase-mediated excision of *Tfam*, which is an important component of nucleoids [4]. The heterozygous knockout *Tfam* mice obtained by expressing Cre recombinase in the germline reduced the mtDNA copy number by approximately 60% in oocytes and sperm. However, the investigators observed no effect on their reproductive performance when the heterozygous knockout *Tfam* mice were mated with wild-type animals. This suggests that mouse oocytes and sperm contain more mtDNA copies than are needed to support development to term. One might guess the large number of mtDNA copies in oocytes is necessary to provide ATP during fertilization and preimplantation [1–4], but the rate of mitochondrial respiration during early development is actually very low [5]. Moreover, embryonic development does not require mtDNA-encoded proteins up to the blastocyst stage in mice [7]. Replication of mtDNA does not appear to be essential for mouse development up to the egg cylinder stage [8], further confirming that mitochondrial function is not crucial for preimplantation in this species.

If, however, the mtDNA content in oocytes can be reduced by more than half with no effect on development, why do oocytes contain so many mtDNA copies? Previous reports have shown that the homozygous knockout of genes essential to the maintenance, replication, and expression of mtDNA in the mouse leads to developmental arrest between Embryonic Day (E) 8.5 and E10.5 [9–11]. Hence, re-establishment of mtDNA replication around E6 [8] must be essential to development. Otherwise, dilution of nonreplicating mtDNA molecules among daughter cells would result in mtDNA depletion and developmental arrest. Thus, one would expect to find a threshold mtDNA copy number in oocytes below which development after E8.5 to E10.5 is not possible.

Wai et al. [6] investigated this hypothesis using a gene promoter that allowed Cre recombinase expression to excise *Tfam* while mtDNA is intensively replicating during oocyte growth. As a result, the heterozygous females that were produced carried oocytes with mtDNA content depleted by approximately 90%; the majority of these oocytes were infertile. Although these oocytes were not able to develop to term, they developed into blastocysts with no obvious effect on rates of development. This confirms that mtDNA copy number in oocytes is critical for preimplantation in the mouse.

To further clarify whether female infertility was caused by the reduced levels of mtDNA in oocytes, blastocysts with low and with high mtDNA copy number were transferred into pseudopregnant females. Blastocysts were obtained by fertilization of *Tfam* heterozygous knockout oocytes (from females made heterozygous during early germline development) and from which a single cell had been biopsied at the 8-cell stage to determine the mtDNA content. Wai et al. [6] used 40 000–50 000 copies as a cutoff point, assuming that the lower limit of mtDNA copy number found in normal mouse oocytes defines the threshold number of copies necessary for development. In agreement with this, those authors found that development to either E10.5 or E12.5 failed when the number of mtDNA copies in 8-cell embryos was less than 50 000.

These findings [6] support the hypothesis that a minimum number of mtDNA copies in oocytes/embryos is necessary to allow the progression of postimplantation development (Fig.

[1]M.R.C. and F.V.M. were supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil.
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Received: 17 February 2010.
First decision: 23 February 2010.
Accepted: 24 February 2010.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

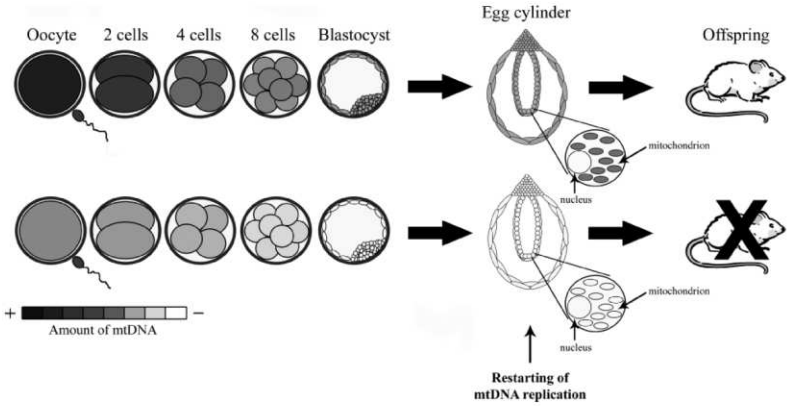

FIG. 1. Model of the effect of mtDNA copy number on developmental progress in mouse oocytes/embryos. This illustrates the postfertilization development of oocytes with mtDNA copy number above (**top**) and below (**bottom**) the threshold (40 000–50 000). After fertilization, embryo cleavage leads to mtDNA segregation among daughter cells, because mtDNA replication is downregulated up to E6 (egg cylinder stage). Thus, the number of mtDNA molecules per cell decreases after each cell cycle, leading to postimplantation developmental arrest if the starting copy number is below the threshold. Developmental arrest is thought to be caused by dilution of mtDNA copies below a minimum number necessary to support the cellular energy requirement. Therefore, mtDNA copy number can be used to select mouse oocytes/embryos with higher chances of development to term. Nevertheless, because the stage at which mtDNA replication starts again has been shown to differ among species, it remains to be proven whether mtDNA copy number can be used for the same purpose in other species.

1). Although mitochondrial function appears to be dispensable during preimplantation [5, 7], it is essential in subsequent stages of development [6, 9–11]. Thus, the oocyte needs enough mtDNA copies to provide a minimum number of molecules for each daughter cell before restarting of mtDNA replication around E6 [8]. These findings likely are of fundamental importance for human and livestock fertility, in which mtDNA copy number could be used as a viability marker to select oocytes/embryos with higher chances of development. Because preimplantation genetic diagnosis has been used in humans to select unaffected embryos for several disorders [12], quantification of mtDNA could be easily adapted to select embryos with higher levels of mtDNA. However, other selection criteria (e.g., embryo morphology) should be regarded as equally or even more important until studies have been carried out in species other than mice. In cattle, depletion of mtDNA content in zygotes can be replenished up to the blastocyst stage by a compensative replication [13], which minimizes the importance of mtDNA copy number in oocytes. The data reported by Wai et al. [6] also highlight the importance of mtDNA segregation among daughter cells, which could contribute to selection of wild-type mtDNAs to populate the germline. Dilution of mtDNA molecules to only a few copies per cell may enhance the effect of mtDNA mutations (normally mixed with wild-type molecules) on cell phenotype, and this might enhance selection of mtDNA molecules at the cellular level [14].

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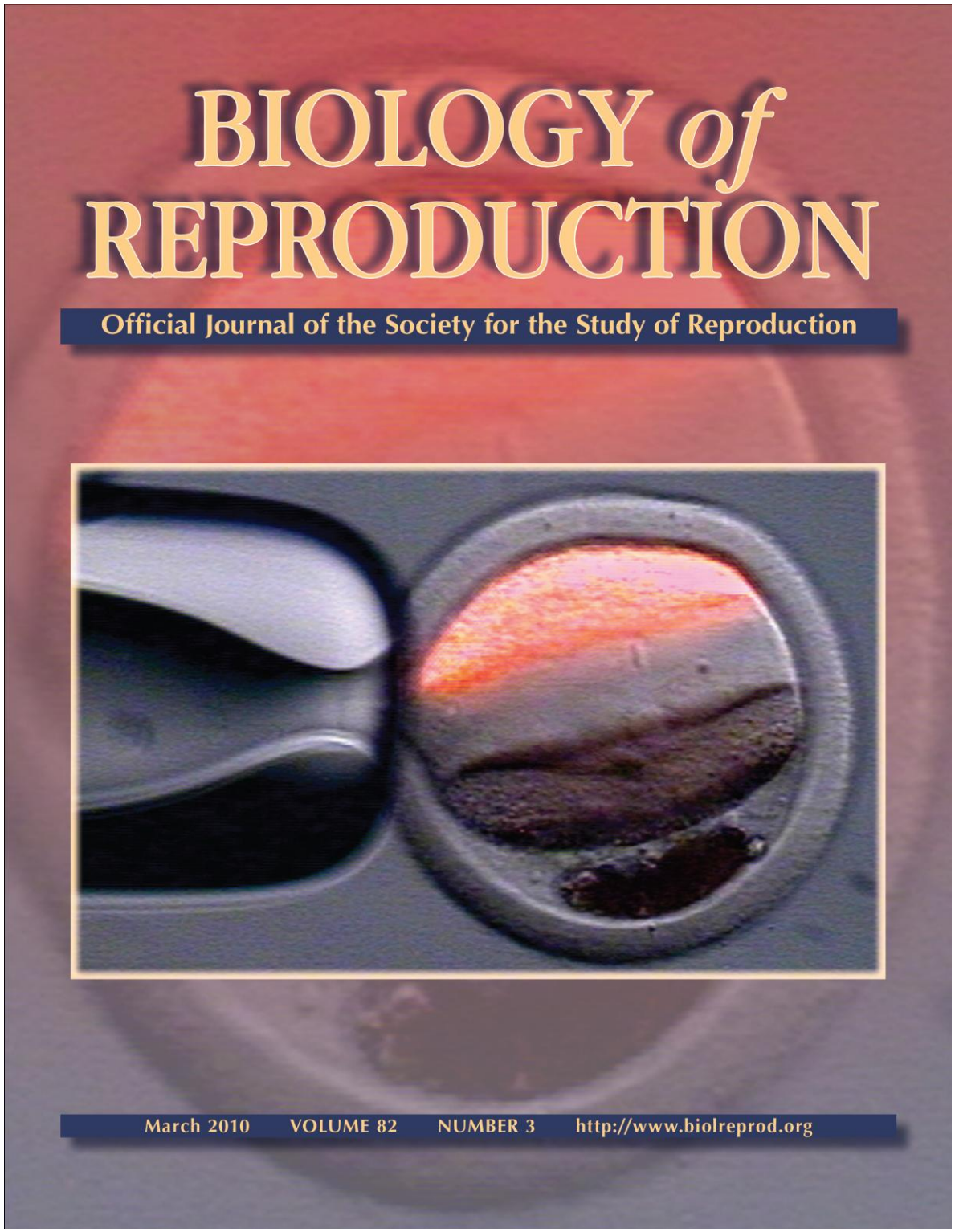
BIOLOGY of REPRODUCTION
Official Journal of the Society for the Study of Reproduction
March 2010 VOLUME 82 NUMBER 3 http://www.biolreprod.org

BIOLOGY OF REPRODUCTION **82,** 563–571 (2010)
Published online before print 2 December 2009.
DOI 10.1095/biolreprod.109.080564

Pronounced Segregation of Donor Mitochondria Introduced by Bovine Ooplasmic Transfer to the Female Germ-Line[1]

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ABSTRACT

Ooplasmic transfer (OT) has been used in basic mouse research for studying the segregation of mtDNA, as well as in human assisted reproduction for improving embryo development in cases of persistent developmental failure. Using cattle as a large-animal model, we demonstrate that the moderate amount of mitochondria introduced by OT is transmitted to the offspring's oocytes; e.g., modifies the germ line. The donor mtDNA was detectable in 25% and 65% of oocytes collected from two females. Its high variation in heteroplasmic oocytes, ranging from 1.1% to 33.5% and from 0.4% to 15.5%, can be explained by random genetic drift in the female germ line. Centrifugation-mediated enrichment of mitochondria in the pole zone of the recipient zygote's ooplasm and its substitution by donor ooplasm led to elevated proportions of donor mtDNA in reconstructed zygotes compared with zygotes produced by standard OT (23.6% ± 9.6% versus 12.1% ± 4.5%; $P < 0.0001$). We also characterized the proliferation of mitochondria from the OT parents—the recipient zygote (*Bos primigenius taurus* type) and the donor ooplasm (*B. primigenius indicus* type). Regression analysis performed for 57 tissue samples collected from the seven OT fetuses at different points during fetal development found a decreasing proportion of donor mtDNA ($r^2 = 0.78$). This indicates a preferred proliferation of recipient taurine mitochondria in the context of the nuclear genotype of the OT recipient expressing a *B. primigenius indicus* phenotype.

bovine, developmental biology, embryo, gamete biology, mitochondrial DNA, ooplasmic transfer

INTRODUCTION

Microinjection of ooplasm containing mitochondria into another oocyte is called ooplasmic transfer (OT) or, less precisely, cytoplasmic transfer. Ooplasmic transfer was initially developed to treat infertility in patients exhibiting persistent poor embryonic development and recurrent implantation failure after in vitro fertilization (IVF). The transfer of 5% to 15% of ooplasm from a presumptively young fertile donor into a putatively defective recipient oocyte resulted in heteroplasmic human offspring [1]. The inheritance of the donor- and recipient-derived mitochondrial genotypes (mitotypes) and possible physiological consequences of this deviation from the normal, uniparental maternal mtDNA inheritance have been addressed in animal studies [2].

In mouse, intrasubspecies OT [3] or karyoplast transplantation [4] performed to study the transmission and segregation of heteroplasmy produced offspring with moderate proportions of the donor-derived mitotype (7%–19%). Heteroplasmic mice have been used to study the persistence of heteroplasmy over generations [4], the mitochondrial bottleneck [5], and the identification of genes regulating mtDNA segregation [6].

Common *Mus musculus domesticus* mitotypes determine differences in respiration capacity per mtDNA molecule, mitochondrial oxidative phosphorylation performance, and the production of reactive oxygen species (ROS) [7]. Production of ROS was found to cause complex phenotypes associated with murine mitotypes [7]. The mitotypes NZB/BinJ and BALB/c, which belong to different phylogenetic subbranches [8] and differ in amino acid residues in the respiratory chain complexes I, III, and IV, are subject to different tissue-specific selection attributed to factor(s) involved with mtDNA maintenance [9]. Furthermore, they show significantly altered basic physiological functions when in a heteroplasmic state [10].

[1]Supported by Austrian Science Fund grant P14840 to R.S., the European Union grant MEDRAT-LSHG-CT-2005-518240 to M.M. and R.S., the "Clonet" MC Research Training Network grant MRTN-CT-2006-035468 to M.M. and R.S., and The State of São Paulo Research Foundation grants 02/05054-7 (to C.R.F.), 04/01841-0 (to J.M.G.), and 2006/59074-0 (to F.V.M.).
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Received: 3 August 2009.
First decision: 22 August 2009.
Accepted: 18 November 2009.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

Ooplasm donation between the subspecies *M. musculus musculus* and *Mus musculus domesticus* generated heteroplasmic offspring in which the former mitotype was selected regardless of nuclear background in most of the tissues [11].

Somatic cell nuclear transfer (SCNT) represents another way to produce mitochondrial heteroplasmy in mammals. The segregation of the mitotypes derived from the SCNT partners has been studied in cattle [12, 13], mouse [12], sheep [13], and pig clones [14]. The cytoplasm of a somatic cell contains only around 1% of the mtDNA amount of an oocyte. Thus, SCNT generates clones with no or low-level heteroplasmy if the parental mitotypes segregate neutrally [13, 15]. Rarely, cases exhibiting a marked elevation of the donor mtDNA proportion have also been observed in each of the four mammalian species studied in this respect [13, 15, 16]. The donor-derived mitotype was generally elevated in its proportion [13, 16, 17] or was preferentially selected only in a specific tissue [12, 14].

Here, we performed OT in cattle to provide a large-animal model with higher relevance to human physiology and longevity. Humans and cattle are much more similar to each other than humans and mice regarding mitochondrial ROS production and basal metabolic rate resulting from mitochondrial oxidative phosphorylation [18, 19]. Using recipient and donor mitotypes from two subspecific origins, *Bos primigenius taurus* and *Bos primigenius indicus*, we addressed the conceptual issue of a putative female germ-line transmission of the donor mtDNA. In addition, we tested two alternative OT strategies to further increase the donor mtDNA proportion transferred. One of the modifications of standard OT used centrifugation of the recipient zygote to generate a phase enriched in mitochondria that was subsequently substituted by a fraction of donor ooplasm. The other manipulation tested was an attempt to block the replication of recipient mtDNA with ethidium bromide (EB). The moderate levels of heteroplasmy produced by transferring 10% to 15% of ooplasm during OT allowed us to study the selection of parental mtDNAs during fetal development and in the offspring.

MATERIALS AND METHODS

Biological Material

Two nucleomitochondrial combinations of Nellore cattle (*B. primigenius indicus*) were used for OT. For the production of recipient zygotes, ovaries of Nellore cattle with a *B. primigenius taurus* mitotype generated by backcrossing *B. primigenius indicus* males to *B. primigenius taurus* females were obtained from a local slaughterhouse (Ferreira et al. [20] and the current study). Nellore cows possessing a *B. primigenius indicus* mitotype based on their Pure Imported Origin pedigrees [21] were used as ooplasm donors.

Cattle were maintained on pasture with free access to water and mineral supplements. The procedures for the use of animals for the investigations performed in this work were approved by The Institutional Animal Care and Use Committee at the Jaboticabal Campus of São Paulo State University (protocol no. 017256-06).

Recipient Zygote Production

Oocytes were obtained postmortem by follicular aspiration from the ovaries of unregistered Nellore cows. Follicles with diameters between 3 and 8 mm were aspirated using an 18-Gauge needle attached to a 20-ml syringe. In vitro maturation (IVM) of cumulus-oocyte complexes (COCs) was performed as described previously [20]. Oocytes and embryos (see below) were cultured in vitro at 38.5°C under a humidified atmosphere of 5% carbon dioxide and 95% air. Chemicals and culture media were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Oocytes were fertilized in Tyrode Albumin-Lactate-Pyruvate (TALP)-IVF medium [22] supplemented with 0.6% (w/v) bovine serum albumin (BSA), 30 μg/ml heparin, 18 μM penicillamine, 10 μM hypotaurine, and 1.8 μM epinephrine as described previously [23]. Oocytes and sperm were incubated for 10–12 h for IVF. Fertilized oocytes were washed in synthetic oviduct fluid medium (SOF) [24] supplemented with 10% fetal calf serum (FCS) and maintained in the incubator in this medium until used as recipient zygotes in OT.

Production of Donor Ooplasm

In vivo-derived oocytes were obtained by ovum pickup (OPU) [25] from Nellore cows with Pure Imported Origin pedigrees. Ovarian follicular waves were synchronized with dominant follicle ablation 96 h prior to OPU. At 24 h prior to OPU, animals received 50 units of follicle-stimulating hormone and 50 units of luteinizing hormone (Pluset; Laboratorios Calier S.A., Barcelona, Spain). The COCs recovered by OPU were transported in PBS at 25°C to 30°C to the laboratory and submitted to IVM for 21 h. The OPU-collected COCs were denuded of cumulus cells by gentle pipetting in 0.5% hyaluronidase solution and were selected by the presence of the first polar body (PB). Denuded oocytes were maintained in the incubator in IVM medium until enucleation and aspiration of donor ooplasm.

Microsurgery, Parthenogenetic Activation, and Standard OT

Microsurgery was performed using an inverted microscope (Olympus IX-70; Tokyo, Japan) equipped with a stage maintaining a temperature of 38.5°C, micromanipulators, and microinjectors (Narishige, Tokyo, Japan). Denuded oocytes having a first PB were incubated in SOF medium [24] supplemented with 10% FCS, 10 μg/ml Hoechst 33342, and 7.5 μg/ml cytochalasin B for 15 min. Removal of metaphase II-arrested chromosomes was performed in a 300-μl drop of SOF buffered with 20 mM HEPES (HSOF). HSOF was then supplemented with 10% FCS and 7.5 μg/ml cytochalasin B under mineral oil in a plastic Petri dish. Using a 25-mm (external diameter) glass pipette, metaphase II chromosomes and the first PB were removed and exposed to ultraviolet light to confirm enucleation. At 24 h after IVM, enucleated oocytes were then chemically activated by incubation in HSOF supplemented with 5 μM ionomycin for 5 min, followed by incubation for 4 h in SOF supplemented with 2 mM 6-dimethylaminopurine, 2.5% FCS, and 0.5% BSA. After activation, ooplasts were transferred to 100-μl drops of SOF supplemented with 2.5% FCS and 0.5% BSA for 10–12 h before OT. Immediately before OT, cumulus cells were removed from presumptive zygotes by gentle pipetting in 0.5% (w/v) hyaluronidase solution and were selected for the presence of the second PB. For microsurgery, selected zygotes and activated ooplasts were preincubated in SOF with 10% FCS and 7.5 μg/ml cytochalasin B for 30 min. In order to provide space for donor ooplasm, the ooplasm surrounding the second PB was removed from recipient zygotes using the same micromanipulation system described above. Subsequently, approximately 10%–15% of donor ooplasm was introduced into the perivitelline space of the recipient zygote. Each single, enucleated donor oocyte was used to reconstruct up to seven zygotes. The resulting couplet was placed in electrofusion solution (0.28 M mannitol, 0.1 mM $CaCl_2{\cdot}2H_2O$, 0.1 mM $MgSO_4{\cdot}7H_2O$, and 0.3% BSA) and exposed to two electrical pulses of 1.5 kV/cm for 30 μs (ECM-2001; BTX, San Diego, CA). Fused zygotes were selected before in vitro culture (IVC).

OT Modifications

Two OT modifications involving centrifugation or chemical treatment (Supplemental Fig. S1, all Supplemental Data are available online at www.biolreprod.org) were tested in comparison with the standard (control [CO]) protocol to increase the proportion of donor mtDNA in reconstructed embryos.

The physical approach used centrifugal force to generate a mitochondria-enriched ooplasm fraction. In detail, recipient zygotes extruding the second PB were placed for 30 min in SOF medium containing 10% FCS and 7.5 μg/ml cytochalasin B. Centrifugation for 15 min at 10 000 × *g* [26] concentrated the mitochondria at one of the zygote's poles. This part of the recipient zygote's ooplasm was removed by micromanipulation and discarded. Therefore, the resulting recipient zygotes were regarded as partially depleted (PD) of their mtDNA because they contained less mitochondria than their CO group counterparts. The integrity of mitochondria in the mitochondria-enriched zone was demonstrated by staining with the mitochondrion-selective red-fluorescent dye chloromethyl-X-rosamine (MitoTracker Red CMXRos; Invitrogen), which accumulates dependent upon membrane potential in mitochondria of live cells. For staining, the zygote was incubated for 30 min in SOF medium supplemented with 500 nM MitoTracker Red CMXRos and 7.5 μg/ml cytochalasin B. After centrifugation at 10 000 × *g* for 15 min, mitochondria enrichment and cellular subfractionation were visualized by epifluorescence microscopy using excitation and emission at 579 nm and 599 nm, respectively

(Fig. 1; a video illustrating the key points of the standard OT and PD treatment is provided as Supplemental Movie S1).

The chemical approach (EB group) used exposition of recipient oocytes to 7 μg/ml EB, a known inhibitor of mtDNA synthesis [27], during the last 4 h of IVM in the presence of 50 μg/ml uridine and 100 μg/ml pyruvate. The EB dose slightly exceeded the range of low concentrations (0.1–2 μg/ml [27, 28]), resulting in either partial or complete inhibition of mtDNA replication but having no effect on the replication of nuclear DNA (King and Attardi [27] and Hashiguchi and Zhang-Akiyama [28], and references therein). The EB concentration and the duration of administration were chosen based on a pilot experiment in which different doses were tested during IVM and evaluated with respect to their effect on oocyte maturation and embryonic development (data not shown). After 18–22 h of IVM, oocytes usually reached the metaphase II stage (expanded cumulus and first PB). To avoid hampering meiotic resumption and the migration of cortical granules, exposure of oocytes to EB was restricted to the last 4 h of IVM (Hours 20–24). At this time, most of the oocytes had completed maturation, and thus had reached their final mtDNA content.

After EB treatment, oocytes were washed thoroughly with TALP-IVF medium and submitted to IVF to be used later as recipient zygotes for OT.

Embryo Culture and Sampling

After OT, reconstructed zygotes were washed in HSOF and cocultured in vitro with a monolayer of granulosa cells in 100-μl drops of SOF supplemented with 2.5% FCS and 0.5% BSA, under mineral oil. For determination of the content and proportion of mtDNA, zygotes and blastocysts of each treatment group were collected after fusion or at Day 7 of IVC, respectively.

Embryos were placed individually in 0.2-ml PCR microtubes containing 5 μl of ultrapure water and were stored at −80°C until DNA extraction.

Embryo Transfer and Tissue Collection

A total of 43 blastocyst-stage embryos from all groups (CO: n = 12; PD: n = 12; and EB: n = 19) were transferred nonsurgically to the uterus of 32 recipient cows (CO: n = 9; PD: n = 9; and EB: n = 14) synchronized as reported previously [29]. Animals were diagnosed by ultrasound examination at Day 30 of pregnancy. A total of 6 of the 10 pregnancies obtained were interrupted for fetal collection (CO: n = 1; PD: n = 1; and EB: n = 4). The other four pregnancies were allowed to complete fetal development (CO: n = 1; PD: n = 1; and EB: n = 2).

Fetuses at different stages of gestation were recovered from pregnant uteri within 1 h after slaughter. Tissue samples were isolated and stored at −80°C until DNA extraction. Placenta and somatic tissue samples taken by needle biopsy (skin, muscle, and blood) or laparoscopy (liver) were recovered at birth. Somatic tissue biopsies of OT calves were also sampled at the ages of 6, 10, and 16 mo. Tissue samples were placed in 1.8-ml cryotubes and stored at −80°C until DNA extraction. The COCs recovered from OT heifers by OPU without hormonal stimulation were submitted to IVM. After removal of cumulus cells, single oocytes were placed in 0.2-ml microtubes containing 5 μl of ultrapure water and were stored at −80°C until DNA extraction.

A tissue sample list is given as Supplemental Table S1.

Quantification of the mtDNA Content and the Heteroplasmy Level by Real-Time PCR

Total DNA was isolated from oocytes, zygotes, blastocysts, and tissues using the NucleoSpin Tissue Kit (Macherey & Nagel, Dueren, Germany) according to the protocol provided by the manufacturer.

The mtDNA content of zygotes and blastocysts was measured by a quantitative real-time PCR (qPCR) assay targeting an *MT-RNR2* consensus sequence present in the recipient and donor mtDNAs of *B. primigenius taurus* (GenBank no.: AY526085) and *B. primigenius indicus* (AY126697).

The proportion of donor-derived mtDNA was determined by *B. primigenius indicus*-specific qPCR and normalized to changes in the input mtDNA amount measured by the latter consensus assay. A second set of qPCR assays targeting the mitochondrial control region and consisting again of a *B. primigenius indicus*-specific assay and a subspecies-consensus assay for normalization were used for confirmation of heteroplasmies in the tissues. Application of this assay was more demanding because it required sequence analysis of all samples because of the higher genetic variation in the control region compared with the highly conserved *MT-RNR2* gene.

Primers and TaqMan probes (Table 1) were designed using Primer Express version 1.5 (Applied Biosystems) and were synthesized at Invitrogen (Lofer, Austria) and Metabion (Martinsried, Germany), respectively. Duplicate qPCR reactions of 25-μl volume contained 80 mM Tris-HCl (pH 9); 20 mM $(NH_4)_2SO_4$; 0.02% w/v Tween-20; 300 nM each primer; 100 nM probe; 0.2 mM each dinucleotide triphosphate (MBI Fermentas, St. Leon-Rot, Germany); 4.5 or 3.5 mM $MgCl_2$ for the consensus and the donor mtDNA-specific assays, respectively; 1 unit HOT FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia); and 1.5 μl of target DNA or water in the case of the no-template control. DNA denaturation and enzyme activation were performed for 15 min at 95°C. DNA was amplified over 40 cycles consisting of 95°C for 20 sec and 63°C or 60°C for 1 min for the *MT-RNR2* assay and the control region assay, respectively. Amplification was performed on the ABI PRISM 7900HT Sequence Detection System (SDS) run under the SDS software version 2.3 (Applied Biosystems). The standard curve method [13], which yielded amplification efficiencies between 87% and 92% ($r^2 > 0.990$) for the four qPCR assays, was used for quantification.

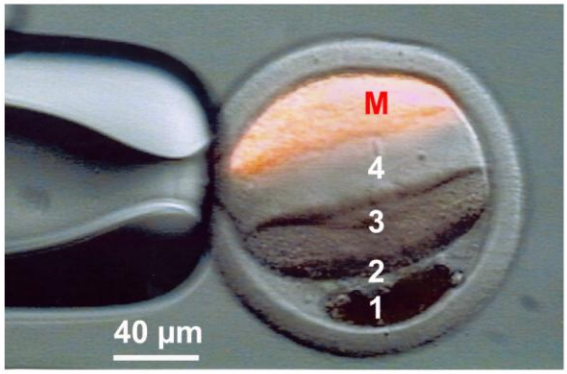

FIG. 1. Membrane potential-maintaining mitochondria were fractionated in a mature bovine zygote by centrifugation at 10 000 × *g*. For the purpose of demonstration only, mitochondria were visualized by staining with chloromethyl-X-rosamine (MitoTracker Red CMXRos; Invitrogen). This mitochondrion-selective red fluorescent dye stains mitochondria in live cells and accumulates dependent on membrane potential. Zones formed are: 1, lipid; 2, membrane-bound vesicles; 3, smooth endoplasmic reticulum; 4, organelle-free ooplasm; and M, mitochondria [26]. Full OT procedure is shown in Supplemental Movie S1. Bar = 40 μm.

The mtDNA content of zygotes and blastocysts was given as the mean calculated from three qPCR runs using the consensus *MT-RNR2* assay. Heteroplasmy levels of oocytes and tissue samples were determined by a single qPCR assay run targeting *MT-RNR2* and normalization by *MT-RNR2* consensus assay data.

In order to calculate relative mtDNA amounts, C_T values of the runs per sample were entered into the regression formula of the respective standard curve, giving rise to logarithmic DNA amounts in arbitrary units. Finally, the mtDNA content was expressed as mean ± SEM in relation to the CO zygote mean.

Sequence Analysis

Partial sequences for the *MT-RNR2* gene and the D loop were determined for the five Nellore cows used as ooplasm donors to exclude interferences from mutations in the primer and probe-binding regions. The primer pairs bMT2773-f (5′-GGTTTACGACCTCGATGTT)/bMT3174-r (5′-CCACTAACGTAAG GAATGCT) and D-f (AGTCTCACCATCAACCCCCA)/D-r (AGGATTTT CAGTGCCTTGC) were used to amplify PCR products for determination of partial *MT-RNR2* and D-loop sequences, respectively. BigDye terminators (Applied Biosystems, Foster City, CA) were used for cycle sequencing of PCR products. Sequences were submitted to the GenBank under the accession numbers GQ412276 to GQ412280 and GQ412281 to GQ412285 for *MT-RNR2* and D loop, respectively.

Statistical Evaluation and Data Presentation

Embryo data (percentage of donor mtDNA and total mtDNA amount) were analyzed as a factorial distribution considering OT group (CO, PD, and EB) and stage of development (zygote and blastocyst) as main factors. When necessary, means were compared by least square means. Heteroplasmy data determined for fetuses and calves were compared within the same group by ANOVA using the Statistical Analysis System version 8.3 (SAS Institute Inc.,

TABLE 1. Primers and TaqMan probes used for mtDNA quantification of *B.p. indicus* and *B.p. taurus* mitotypes.

Assay type[a]	Target site	Primer or probe	Sequence (5′ to 3′)[b]
ND	*MT-RNR2*	bMT3010-f	GCCCTAGAACAGGGCTTAGT
		bMT3096-r	GGAGAGGATTTGAATCTCTGG
		bMT3030-FAM	FAM-AAGGTGGCAGAGCCCGGTAATTGC-BHQ1
D	*MT-RNR2*	iMT2974-f	CCAATGACAGCATCTCAATCA
		iMT3095-r	GAGAGGATTTGAATCTCTGGG
		bMT3030-FAM	FAM-AAGGTGGCAGAGCCCGGTAATTGC-BHQ1
ND	Control region	bMT16162-f	TAATTACCATGCCGCGTGAAA
		bMT16287-r	GGCCCTGAAGAAAGAACCAGA
		bMT16204-FAM	FAM-TCCCTCTTCTCGCTCCGGGCC-BHQ1
D	Control region	iMT16185-f	CAACCCGCTAAGCAGAGG
		iMT16264-r	GCCTGGTAA AATTCATTAA
		bMT16204-FAM	FAM-TCCCTCTTCTCGCTCCGGGCC-BHQ1

[a] ND, nondiscriminative (consensus) assay for *B.p. taurus* and *B.p. indicus*; D, discriminative (*B.p. indicus*-specific) assay.
[b] Specific nucleotides are underlined.

Cary, NC). If necessary, the Tukey posthoc test was performed subsequently. Percentage data were transformed using arcsine. In all analyses, $P = 0.05$ was considered as the level for statistical significance. Exponential regression analysis of the donor mtDNA proportion throughout embryogenesis and generation of box whiskers plots were performed with the software package Microcal Origin version 6 (Microcal Software Inc., Northampton, MA).

RESULTS

Here, we used OT to generate heteroplasmic embryos. Two strategies based on physical or chemical treatment (Fig. 1 and Supplemental Fig. S1) were evaluated to increase the level of ooplasmic donor mitochondria. The physical treatment based on centrifugation-mediated enrichment of mitochondria in a fraction of the recipient ooplasm which was subsequently substituted with normal ooplasm was performed to generate recipient zygotes with partially depleted mitochondria (PD group). The chemical treatment was intended to impair the recipient mtDNA replication by exposition of recipient oocytes to EB (EB group). Standard OT (CO group) was performed for comparison.

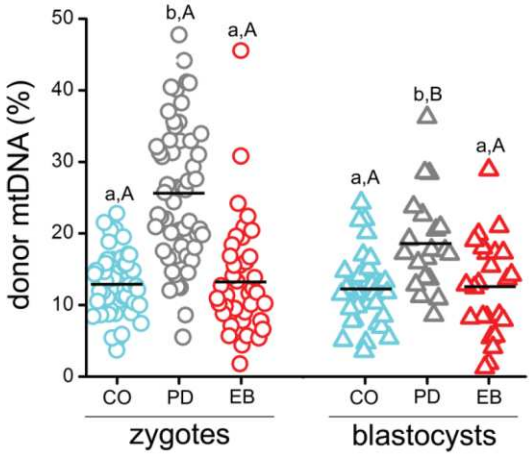

FIG. 2. Percentage of donor mtDNA in postfusion zygotes and blastocysts. Statistical significance ($P < 0.05$) is denoted by different superscript letters when difference was observed between groups of a development stage (a, b) or between developmental stages (A, B). The mean of individual proportions is depicted by a bar.

mtDNA Heteroplasmy in Early Embryos

A total of 225 embryos were collected for quantification of the donor:recipient mtDNA ratio (no. of zygotes/blastocysts in CO group: n = 49/36, PD group: n = 55/20, and EB group: n = 41/24, respectively). Overall, mean rates for embryo cleavage and blastocyst development of 69.7% and 31.7% were obtained, respectively. No difference in the two parameters was found among the groups ($P = 0.73$ and 0.42, respectively).

The percentage of donor mtDNA (e.g., the level of heteroplasmy) was analyzed at the zygote and the blastocyst stages (Fig. 2). The standard OT protocol, in which approximately 10% to 15% of the donor oocyte volume is transferred to a recipient zygote, yielded similar levels of heteroplasmy for zygotes and blastocysts, respectively (12.1% ± 4.5% and 11.2% ± 4.8%, respectively; $P = 0.52$). An increase in the level of donor mtDNA was achieved in the PD group. The removal of a mitochondria-enriched fraction of ooplasm from the recipient zygote before OT yielded zygotes

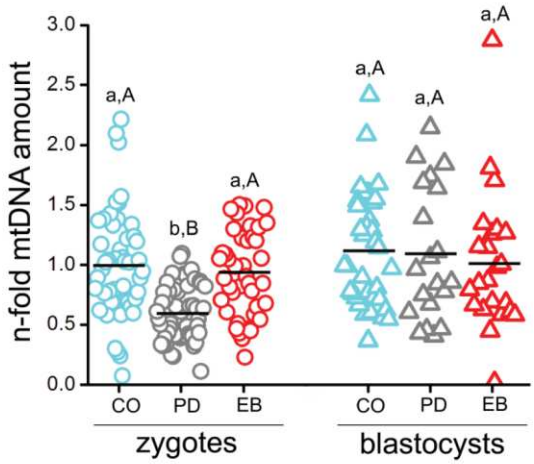

FIG. 3. Reconstructed zygotes with partially depleted mtDNA produced by OT into recipient zygotes being removed of a mitochondria-enriched fraction. The mtDNA content is expressed as mean ± SEM in relation to the CO zygotes' mean. Statistical significance ($P < 0.05$) is denoted by different superscript letters when difference was observed between groups of a development stage (a, b) or between developmental stages (A, B). The mean of individual data is depicted by a bar.

(mean: 2.5% ± 1.5%; Supplemental Table S2). See below for heteroplasmy levels found in the live twin (male 1).

In the EB group, 19 blastocysts were transferred to 14 recipient cows. Heteroplasmies quantified in fetal tissues of three Day 35 fetuses (22%, 36% ± 6.5%, and 19% ± 2.7%; Supplemental Table S2) included outliers with elevated heteroplasmy not covered by heteroplasmies found in the EB blastocysts analyzed (n = 24; mean: 13% ± 7%). Low heteroplasmy values were seen in a Day 117 fetus from this group (mean: 4.9% ± 2.6% across 14 tissues).

Regression analysis performed throughout fetal development using all heteroplasmy data determined for fetuses of all three OT protocols demonstrated a negative exponential relationship between the time of gestation and the proportion of donor mtDNA ($r^2 = 0.78$; Fig. 4 and Supplemental Table S2).

Heteroplasmy During Postterm Development

Next, we quantified heteroplasmies after term in three of the four live OT animals showing a donor-derived mtDNA proportion of above 1% in blood and skin at birth (e.g., in male 1 and females 1 and 3, but not female 2). The three heteroplasmic live animals exhibited completely normal development and had no obvious health problems until manuscript submission (4 yr). An additional analysis of blood parameters performed only for the female animals yielded values within the reference range (Supplemental Table S3).

First, the issue of a tissue-specific segregation of donor mtDNA was addressed in female 1 (CO group). The blood of this animal exhibited higher heteroplasmies at birth and at 6 and 16 mo (13%, 20%, and 17%, respectively) compared with skin (6% and 4%) and muscle (2% and 1%) at birth and 6 mo after term, respectively (paired *t*-test: $P < 0.05$).

We also asked whether the level of heteroplasmy changed in a given tissue of an individual over time. Samples collected at birth and during the first months of postnatal development indicated a trend for a decreasing proportion of donor mtDNA in muscle and skin but not blood (Supplemental Table S4 and data not shown). The limited sample size (n = 3) precluded rigorous statistical analysis.

Transmission of Heteroplasmy to the Female Germ-Line

The heteroplasmic OT animals female 1 and female 3 belonging to the CO and EB groups, respectively, were analyzed for a putative transmission of the mixed mtDNAs from mother to offspring (e.g., through the mitochondrial genetic bottleneck). The donor mtDNA was detectable only in 25% and 65% of the oocytes collected (n = 24 or 23, respectively; Supplemental Table S4). In cases of germ-line transmission, its proportion varied considerably (1.1% to 33.5% and 0.4% to 15.5%, respectively; Fig. 5).

DISCUSSION

Elevation of the Donor mtDNA Proportion Introduced by OT

Mitochondrial heteroplasmies of only a few percent are produced by SCNT if genotypes are transmitted neutrally [12, 13, 15, 36]. These low levels are sometimes lost before birth, preventing further segregation or (patho)physiological studies. The microinjection of 5%–15% ooplasm donates more mtDNA than a somatic cell, and thus generates animal models with higher levels of heteroplasmy (Jenuth et al. [3] and the current study). In humans, the transfer of small amounts of up to 15% of donor ooplasm to the recipient's oocyte [37] resulted in the transmission of donor mtDNA into human offspring [1, 36]. Here, we provided the first quantitative data for the mtDNA proportion transferred in a standard OT protocol (Fig. 2). The 10% to 15% of donor ooplasm introduced into a recipient zygote by standard OT resulted in zygotes and blastocysts exhibiting heteroplasmies of 12.1% ± 4.5% and 11.2% ± 4.8%, respectively. To further enhance the level of OT-generated heteroplasmy, we explored ways like EB treatment and partial mitochondria depletion of recipient zygotes.

Given that an mtDNA replication inhibitor (2′,3′-dideoxycytidine) applied during IVM successfully decreased the mtDNA amount of porcine oocytes [32], we attempted to reduce the proportion of recipient zygote-derived mtDNA by treating recipient oocytes with the DNA-intercalating dye EB during the last hours of IVM. The lack of effect in the blastocysts of the EB group could be due to a rapid reversion of the drug effect after its removal from culture medium [38], or species-specific limitation in causing mtDNA depletion [28]. Alternatively, this could be due to a rapid mitochondrial fusion process creating a connected compartment that facilitates content exchange and access to mtDNA products [39], thus distributing the drug between donor and recipient mitochondria and resulting in equal access to their replication machinery between the morula and blastocyst stages of bovine embryogenesis [30].

Elevation of the donor mtDNA proportion, however, was achieved by PD treatment. Using centrifugation of recipient zygotes, a recipient ooplasm fraction enriched in mitochondria is produced at the pole (Fig. 1) and is subsequently substituted by donor ooplasm having a normal density of mitochondria. Consequently, the ratios of donor:recipient mitotypes at the zygote and the blastocyst stages increased significantly (Fig. 2). The reestablishment of normal mtDNA levels in PD blastocysts (Fig. 3) is consistent with the maintained ratio of mtDNA copies per unit ooplasm volume reported for early embryogenesis up to the blastocyst stage [34]. The effect of elevated heteroplasmy was less pronounced in the resulting blastocysts (Fig. 2). This decrease can be explained by the turnover of mtDNA (e.g., decrease and neosynthesis of mtDNA before the blastocyst stage [30–33]) and selection against donor mtDNA (Fig. 4, and see below). It was not due to a difference in the blastocyst development rate, thus excluding a developmental selection of embryos with different mtDNA content as a cause.

Selection Against Donor Mitochondria

Here, we used OT to mix donor and recipient mitochondria from a similar developmental/differentiation stage. We demonstrated that throughout fetal development, the donor-derived *B. primigenius taurus* mitotype is selected over the *B. primigenius indicus* recipient mtDNA in the context of a phenotypically *B. primigenius indicus* genetic background derived by repeated backcrossing of *B. primigenius indicus* males to ''native'' Brazilian *B. primigenius taurus* cows (Fig. 4).

First, mtDNA transmission and segregation experiments of this kind have been performed in mice. The introduction of a considerable amount of donor ooplasm during OT performed between *Mus musculus domesticus* and *M. musculus molossinus* mice led to low heteroplasmy levels in most tissues in the offspring (references in Smith and Alcivar [40]). Authors regarded the reduced proportion of transmitted donor mtDNA compared with its input as indication that the introduced

mtDNA was lost at some stage between fetal development and adulthood by random drift or by selective replicative disadvantage of the transplanted mtDNA. A deviation from the original parental mtDNA ratio in the offspring is not surprising, considering that 390 amino acid residues are substituted between these *Mus musculus* subspecies [8]. This number exceeds the number of residue changes determined for the *B. primigenius taurus* and *B. primigenius indicus* parental mtDNAs of this work by more than 20-fold [41], and therefore could explain the presumed nonneutral transmission of parental mitotypes.

In mice, the *M. musculus musculus* mtDNA of the RR strain was selected over the *M. musculus domesticus* mitotype of C57BL/6 in the two nuclear genetic constellations analyzed [8, 11, 42]. Generally, the proliferation of mitotypes should be considered an interplay between the mitochondrial and nuclear genomes. In fact, there is genetic evidence that the nucleus controls mammalian mtDNA segregation [6].

In cattle, SCNT between donor and recipient cells possessing *B. primigenius taurus* mitotypes [16, 43] generated three clones with elevated levels of the donor-derived mitotype. This finding was attributed to a replicative advantage of the donor mtDNA over recipient mtDNA during the course of embryogenesis [16].

In humans, the surprisingly high proportions of 36%, 43%, and 70% donor mtDNA found in the blood of three OT infants, respectively [44], could have been caused by random genetic drift, but could also be indicative of a difference in the proliferative potential of mitotypes under the given nucleomitochondrial interactions.

In sheep, the high heteroplasmy of an SCNT clone indicated that the recipient oocyte-derived mitotype was negatively selected [13]. This putatively mildly deleterious mtDNA possessed three nonconservative amino acid substitutions, one of which was found at an evolutionary conserved site.

OT Donor Mitochondria Are Transferred to the Female Germ Line, Thereby Segregating Considerably

In mammals, mtDNA variants are observed to segregate rapidly between generations, despite the high mtDNA copy number in the oocyte (reviewed in Cree et al. [45]). Early in prenatal development, a restriction in the mtDNA content to about 200 copies per early primordial germ cell has been documented [5, 46]. At this time of development, in our bovine OT model, the levels of donor mtDNA were still moderate and began to diminish as a result of selection against it (see above). Here, we asked whether the OT donor mtDNA is transmitted to and segregated by the female germ line.

For a representative number of oocytes collected by OPU at the age of 14 or 20 mo from two females, we demonstrated donor mtDNA transmission in 25% and 65% of oocytes, and its pronounced segregation ranging from 0.3% to 33.6% in heteroplasmic oocytes. In higher mammals, the segregation of donor mtDNA through the germ-line bottleneck was assessed previously only indirectly, using tissues of a few SCNT offspring [47].

The finding of germ-line transmission of donor mtDNA and its pronounced segregation are consistent with published data reporting random genetic drift of heteroplasmy through the female germ line in mice generated by ooplasm donation [3]. Our data from a higher mammalian species formally answer the question of whether the moderate amount of mtDNA introduced in case of OT will be passed to the next generation, thereby solving the important conceptual issue of classification of OT as an assisted reproductive technology modifying the germ line [48].

Further Perspectives

As outlined above, we proved the expectation that the moderate amount of 10% to 15% of donor ooplasm transferred to the recipient zygote by standard OT produces (only) moderate mtDNA heteroplasmy levels of about 11% to 12% (see blastocyst-stage data in Supplemental Table S1). Even the PD pretreatment before transferring the donor ooplasm yielded only slightly higher values of 18% to 24%. However, to prevent the transmission of mtDNA disease to offspring from women suffering from mtDNA disorders, a larger amount of up to 50% of donor ooplasm is needed. It is questionable whether it is possible to introduce such an amount of ooplasm into the oocyte. The consequence is that the relative proportion of mutant to wild-type mtDNA is unlikely to change enough to prevent clinical disease (reviewed in Bredenoord et al. [48]). It remains to be seen whether future research in the fields of mtDNA transmission and segregation and nucleomitochondrial interaction will open a way to reach this threshold (e.g., by using a donor mtDNA of ''lagging'' proliferative potential). To fall short of this physiologically critical threshold of pathological mitotypes is currently only conceivable using spindle-chromosomal complex transfer developed in the nonhuman primate *Macaca mulatta*. This technique was reported to decrease the mtDNA proportion transplanted along with nuclear genetic material into an enucleated egg containing normal mtDNA below a threshold of 3% [49], thus reaching at least 97% of the normal mitotype in the reconstituted embryo.

In addition to the PD treatment studied in this work, we believe that centrifugation of the donor oocyte and transfer of aspirated donor ooplasm enriched in mitochondria to PD zygotes partially depleted in mitochondria could be a way to further elevate the proportion of donor mtDNA in OT embryos.

Future research in a large-animal system like cattle should also assess the potential of OT to affect paternal genome function and for more subtle phenotypic changes or transient effects [50–52].

Model Prospects

Because of the ethical issues involved with medical research involving human embryos, an appropriate animal model is needed for studying early embryogenesis, to demonstrate the safety of human infertility therapeutics related to OT [53], and to assess ways to prevent transmission of human mitochondrial disorders [48, 54]. The only large-animal models of relevance are the nonhuman primates *Callithrix jacchus* (common marmoset) and *Macaca mulatta* (rhesus monkey) [49, 55]. In the nonhuman primate, basic research is at the frontiers in the field of spindle replacement [49], but the success of other micromanipulation techniques is restricted to two offspring obtained by embryonic cell nuclear transfer [56]. All efforts to produce clone monkeys by SCNT for biomedical research have failed so far [53]. Because of this lack of SCNT success and numerous ethical and welfare issues regarding nonhuman primates compared with the economic and well-established breeding of cattle, basic research in the bovine model could assist and precede experiments in the nonhuman primate.

First, there is mounting evidence that the bovine model is better than the murine model for the study of human embryonic development with respect to the timing of genome activation, intermediate metabolism, and interaction with the culture medium [57]. Second, human and mouse differ in their

maximum lifespan potential. Evidence shows that long-lived vertebrates consistently have low mitochondrial free radical generation rates, a crucial factor determining their aging rate [58, 59]. Oxidative damage to mtDNA is also lower in long-lived vertebrates than in short-lived vertebrates [60]. Therefore, the longer-lived cow (30 yr) would be much more adequate concerning these issues than the mouse as a shorter-lived species (2–4 yr in mice, depending on the strain).

Third, for determining the efficacy of any therapeutic for human mtDNA disease, a higher mammalian model in addition to mouse models heteroplasmic for pathogenic mtDNA mutations would be beneficial. The bovine model represents an appropriate candidate in this regard. This large-animal model is an intensively studied model for mtDNA transmission and segregation [61], provides a large reservoir of different mitotypes [41, 62], would allow the use of a number of cost-efficient dwarf breeds [41, 63–66], and would respect ethical concerns regarding trapping and sampling of endangered species, especially apes.

In conclusion, here we documented germ-line transmission of the OT-generated heteroplasmy in a large-animal model for the first time. Our OT modification based on partial depletion of mitochondria in the recipient ooplasm to elevate levels of heteroplasmy extends the repertoire of strategies for introduction of mitotypes. It might be especially important in light of gene therapy of human mitochondrial disease requiring demonstration of efficacy in animal models before starting clinical trials.

Generally, OT can assist in broadening understanding of nucleomitochondrial interaction and identifying mitochondrial and nuclear genotypes which can influence proliferation of mitochondria and/or cells. In this regard, it can help to fine tune new therapeutic approaches that could prevent transmission of mtDNA mutations from mother to child, thus avoiding recurrence of mtDNA diseases.

ACKNOWLEDGMENTS

We thank Claus Vogl for valuable discussion, Alistair McGregor for comments on the manuscript, Roberta Vantini for IVF, and Gottfried Brem for support.

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9- ANEXOS

ANEXO 1

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Cidade Universitária "Zeferino Vaz",
19 de julho de 2006.

Prof. Dr. ANIBAL EUGÊNIO VERCESI
Marcos Roberto Chiaratti
Departamento de Prótese e Periodontia
FOP - Unicamp

Prezado Senhor:

A Comissão de Ética em Experimentação Animal da Unicamp (CEEA/IB/Unicamp) esclarece que não há necessidade de submissão do projeto intitulado **"Efeito da quantidade de mitocôndrias e de DNA mitocondrial sobre o desenvolvimento embrionário bovino: dois modelos originais"**, uma vez que o material biológico, células germinais obtidas de fêmeas bovinas após o abate, utilizado neste estudo será adquirido pronto no mercado.

A CEEA/IB/Unicamp emite apenas pareceres circunstanciados para projetos que envolvam manipulação animal.

Atenciosamente,

Profa. Dra. ANA MARIA APARECIDA GUARALDO
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