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ARTICLE

Ooplast-mediated developmental rescue of bovine oocytes exposed to ethidium bromide

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Abstract Ooplasm transfer has been used successfully to treat infertility in women with ooplasmic insufficiency and has culminated in the birth of healthy babies. To investigate whether mitochondrial dysfunction is a factor in ooplasmic insufficiency, bovine oocytes were exposed to ethidium bromide, an inhibitor of mitochondrial DNA replication and transcription, during in-vitro maturation (IVM). Exposure of immature oocytes to ethidium bromide for 24 h during IVM hampered meiotic resumption and the migration of cortical granules. However, a briefer treatment with ethidium bromide during the last 4 h of IVM led to partial arrest of preimplantation development without affecting oocyte maturation. Ooplasm transfer was then performed to rescue the oocytes with impaired development. In spite of this developmental hindrance, transfer of normal ooplasm into ethidium bromide-treated oocytes resulted in a complete rescue of embryonic development and the birth of heteroplasmic calves. Although this study unable to determine whether developmental rescue occurred exclusively through introduction of unaffected mitochondria into ethidium bromide-damaged oocytes, e.g. ethidium bromide may also affect other ooplasm components, these results clearly demonstrate

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that ooplasm transfer can completely rescue developmentally compromised oocytes, supporting the potential use of ooplasm transfer in therapeutic applications.

KEYWORDS: embryo, ethidium bromide, mitochondrial DNA, oocyte, ooplasm, transfer

Introduction

Ooplasm transfer has been used in women with recurrent implantation failure after IVF or intracytoplasmic sperm injection (ICSI) to improve the outcome of assisted reproduction (Cohen et al., 1997, 1998; Huang et al., 1999; Lanzendorf et al., 1999; Malter and Cohen, 2002). During ooplasm transfer, 5–15% of the ooplasm from a presumed fertile donated oocyte is microinjected along with a single spermatozoon into the compromised patient oocyte during ICSI. This procedure can rescue the capacity of the recipient oocyte to support normal embryonic development and has culminated in the birth of healthy babies (Cohen et al., 1997, 1998; Dale et al., 2001; Huang et al., 1999; Hwang et al., 2002). Thus, it has been suggested that one or more factors transferred into the recipient oocyte during this procedure are able to restore its developmental potential (Barritt et al., 2001; Levy et al., 2004; Malter and Cohen, 2002). However, the nature of these factors is not understood. Significant differences in ATP (reviewed by Dumollard et al., 2007) and mitochondrial DNA (mtDNA) content in human, mouse and porcine oocytes (May-Panloup et al., 2007; Wai et al., 2010) have been linked to the developmental competence of an embryo. Furthermore, age-related accumulation of mtDNA deletions (Chen et al., 1995) and mutations (Barritt et al., 1999; Brenner et al., 1998; Keefe et al., 1995) and decreased levels of mtDNA gene expression (Hsieh et al., 2004) have been reported in compromised human oocytes and embryos. Nonetheless, other factors, e.g. RNA and proteins, may also be able to rescue the development of compromised oocytes, which highlights the potential relevance of these factors for oocyte quality (Barritt et al., 2001; Levy et al., 2004; Malter and Cohen, 2002).

Despite the benefits of ooplasm transfer, some questions about the safety of the technique have been raised since it involves the mixing of ooplasmic factors of different origins (Barritt et al., 2001; Hawes et al., 2002; Malter and Cohen, 2002; St John et al., 2002; Levy et al., 2004). For instance, a mixture of mitochondria from both donor and recipient oocytes (heteroplasmy) can persist after birth and, if the donor oocyte harbours mtDNA with pathological mutations, might result in mitochondrial-encoded diseases in the infant (Barritt et al., 2001; Brenner et al., 2000). Notwithstanding possible mitochondria effects, other organelles and ooplasmic molecules such as RNA and proteins (e.g. cell cycle regulators) may also have genetic and epigenetic consequences (Barritt et al., 2001; Levy et al., 2004; Liang et al., 2009; Malter and Cohen, 2002).

Since ooplasm transfer began in humans without prior studies in animal models, further research is needed to identify potential benefits and possible consequences of the technique for human fertility. Thus, the aim of this work was to develop a biological model to study the consequences of ooplasm transfer based on the bovine species. In order to mimic failed embryonic development, ethidium bromide was utilized during in-vitro maturation (IVM) of bovine oocytes.

Materials and methods

Animals

The present study was approved by the Institutional Animal Care and Use Committee of the São Paulo State University (UNESP) at the Jaboticabal Campus (protocol no. 017256–06). Animals were maintained in a pasture, with free access to water and mineral supplements.

Since South American zebus have different mtDNA origins but the same nuclear background, they have been used in studies involving mitochondrial inheritance (Ferreira et al., 2007, 2010; Meirelles et al., 2001). In this study, crossbred cows harbouring *Bos taurus* mtDNA and *Bos indicus* nuclear DNA (Meirelles et al., 1999) (due to backcrossing zebu males with European cows) were used to obtain oocytes later used for recipient ooplasm. On the other hand, Nellore cows with pure imported origin pedigrees (Meirelles et al., 1999), which carry both *B. indicus* mitochondrial and nuclear DNAs, were used to obtain donor ooplast.

Chemicals and in-vitro culture conditions

Unless mentioned otherwise, chemicals and culture media were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). In-vitro experimental conditions were carried out in humidified incubators maintained at 38.5°C in air with 5% CO_2.

Oocyte collection and in-vitro maturation

Oocytes were obtained post mortem by follicular aspiration from the ovaries of crossbred cows slaughtered at a local slaughterhouse. Ovaries were transported in saline solution 0.9% at 25–30°C to the laboratory, and follicles with 3–8 mm diameter were aspirated using an 18-gauge needle attached to a 20 ml syringe. Cumulus–oocyte–complexes (COC) with at least three layers of cumulus cells and homogeneous ooplasm were washed in HEPES-buffered tissue culture medium-199 (TCM-199; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) that had been heat-inactivated at 55°C for 30 min, 0.20 mmol/l sodium pyruvate and 83.4 μg/ml amikacin sulphate. Groups of 20–25 COC were placed in 100 μl of bicarbonate-buffered TCM-199 supplemented with 10% FCS, 1 μg/ml FSH (Folltropin; Bioniche Animal Health, Belleville, Canada), 50 μg/ml human chorionic gonadotrophin (Profasi, Serono, São Paulo, Brazil), 1 μg/ml oestradiol, 0.20 mmol/l sodium pyruvate

and 83.4 μg/ml amikacin sulphate under mineral oil during 24 h for IVM.

Experiment I: effect of ethidium bromide on oocyte maturation and embryonic development

The objective of this experiment was to determine a protocol for the treatment of COC with ethidium bromide in order to compromise their development into normal blastocysts. Ethidium bromide is a dye that intercalates DNA and inhibits mtDNA replication and transcription (Nass, 1970, 1972; Zylber et al., 1969). The effects of a dose-dependent treatment on the nuclear and ooplasmic maturation of oocytes and on embryonic development after IVF were determined.

The optimal drug dose and incubation interval were chosen based on partial impairment of embryonic development without affecting oocyte maturation. Initially, COC were treated with increasing concentrations of ethidium bromide (0.0, 0.5, 1.5, 2.5, 3.5, 4.5, 5.5 and 7.0 μg/ml) during IVM following the protocol described by King and Attardi (1996). Moreover, the IVM medium was supplemented with 50 μg/ml uridine 5′-triphosphate (UTP; King and Attardi, 1996). Treatments were performed in a 24-well plate, and after 24 h of IVM, COC were denuded of cumulus cells by gentle pipetting in 0.5% hyaluronidase solution (Hyalozima, Aspen, São Paulo, Brazil). Denuded oocytes were stained for cortical granules and nuclear DNA using a previously described protocol (Cherr et al., 1988) with some modifications. Briefly, the zona pellucida was removed through rapid incubation in phosphate-buffered saline (PBS; pH 2.5). Oocytes were fixed in 3% formaldehyde in PBS (pH 7.4) for 30 min at room temperature. Next, oocytes were incubated overnight in 0.1% bovine serum albumin (BSA), 100 mmol/l glycine (Pharmacia Biotech, Uppsala, Sweden) and 0.2% sodium azide in PBS (blocking solution; pH 7.4) at 4°C. Oocytes were then permeabilized for 5 min in blocking solution (BS) containing 0.1% Triton X-100, incubated for 15 min in BS containing 10 μg/ml fluorescein isothiocyanate-conjugated *Lens culinaris*, washed in BS and stained for 10 min in BS containing 10 μg/ml Hoechst 33342. Finally, oocytes were washed in BS, mounted onto microscope slides with coverslips and analysed with an epifluorescence microscope (Axioplan; Carl Zeiss, Zeppelinstrassen, Germany) for cortical granule migration (fluorescein isothiocyanate; 488 nm excitation/518 nm emission) and meiotic resumption rates (Hoechst 33342; 350 nm excitation/450 nm emission). The software AxioVs40 version 4.6.1.0 (Carl Zeiss) was used for image processing.

As a follow-up to this experiment, oocytes were treated with 0.0, 2.5 and 7.0 μg/ml ethidium bromide. COC were treated with these doses of ethidium bromide as described above, washed serially (three times) in IVM medium without ethidium bromide and used for IVF. Oocytes were fertilized in TALP (Tyrode's albumin lactate pyruvate)-IVF medium supplemented with 0.6% BSA, 30 μg/ml heparin, 18 μmol/l penicillamine, 10 μmol/l hypotaurine and 1.8 μmol/l epinephrine. Thawed motile spermatozoa from a Nellore bull (*B. indicus* mitochondrial and nuclear DNA) were separated by centrifugation at 900 *g* for 30 min in a 45% and 90% Percoll density gradient. Final sperm concentration was 2.5×10^6 live spermatozoa/ml. For fertilization, oocytes and sperm were co-incubated for 24 h. Presumptive zygotes were then denuded of cumulus cells by gentle pipetting in 0.5% hyaluronidase solution and co-cultured *in vitro* on a monolayer of granulosa cells in 100 μl droplets of modified synthetic oviduct fluid (SOF; Vajta et al., 1999) supplemented with 2.5% FCS and 0.5% BSA (SOF+) under mineral oil. Cleavage and blastocyst rates were assessed at 48 h and 168 h post insemination, respectively. Finally, blastocysts were stained with 10 μg/ml Hoechst 33342 and the number of cells counted using an epifluorescence microscope.

Experiment II: ooplasm transfer

The objective of this experiment was to study the effect of transferring a small portion of ooplasm from untreated ooplasts into fertilized oocytes with ethidium bromide-compromised development. The effect of ooplasm transfer was assessed by analysis of developmental rates. Embryos were also transferred to recipient cows to verify pregnancy rates, to determine whether they could develop to term and to ascertain the offspring were heteroplasmic.

COC were treated with 7 μg/ml ethidium bromide during the last 4 h of IVM (ethidium bromide group) or left untreated (control). Afterwards, oocytes were fertilized and used as recipient zygotes (at the pronuclear stage) for ooplasm transfer or immediately cultured without manipulation for IVF, resulting in four groups: ethidium bromide + ooplasm transfer, control + ooplasm transfer, ethidium bromide + IVF and control + IVF.

For donor ooplasm production, in-vivo-derived COC were obtained by ultrasound-guided oocyte retrieval from five pure imported origin Nellore cows (Chaubal et al., 2006). Ovarian follicular waves were synchronized with dominant follicle ablation 96 h prior to oocyte retrieval. Twenty-four hours prior to oocyte retrieval, animals received 50 U FSH and 50 U LH i.m. (Pluset; Laboratorios Calier, Barcelona, Spain). The recovered COC were transported in PBS (pH 7.4) at 25–30°C to the laboratory, subjected to IVM for 21 h, denuded of cumulus cells by gentle pipetting in 0.5% hyaluronidase solution and selected by the presence of the first polar body (PB1).

Microsurgery was performed using an inverted microscope (IX-70; Olympus, Tokyo, Japan) equipped with a stage warmed at 38.5°C, micromanipulators and microinjectors (Narishige, Tokyo, Japan). At first, PB1-selected oocytes were incubated in SOF+ with 10% FCS, 10 μg/ml Hoechst 33342 and 7.5 μg/ml cytochalasin B for 15 min. Removal of metaphase II (MII)-arrested chromosomes was performed in a 300 μl droplet of HSOF (SOF containing 20 mmol/l of HEPES; Wells et al., 1999) supplemented with 10% FCS and 7.5 μg/ml cytochalasin B under mineral oil in a plastic Petri dish. Using a 20-μm (internal diameter) glass pipette, both MII chromosomes and the PB1 were removed and exposed to ultraviolet light to confirm oocyte enucleation. Enucleated oocytes (ooplasts) were chemically activated (at 24 h post IVM) by incubation in 5 μmol/l ionomycin in HSOF for 5 min, followed by incubation for 4 h in 2 mmol/l 6-dimethylaminopurine in SOF+. Ooplasts were then transferred to 100 μl droplets of SOF+ for 10–12 h prior to ooplasm transfer.

Immediately before ooplasm transfer, cumulus cells were removed from presumptive zygotes (control and ethidium bromide groups) by gentle pipetting in 0.5% hyaluronidase solution and selected by the presence of the second polar body (PB2). The rate of PB2 extrusion was determined in relation to the total number of oocytes used for IVM. For microsurgery, selected zygotes and ooplasts were pre-incubated in SOF supplemented with 10% FCS and 7.5 μg/ml cytochalasin B for 30 min. Then, to provide space for donor ooplasts, some ooplasm surrounding the PB2 was removed from recipient zygotes using the same micromanipulation system described above. Next, approximately 10–15% of the ooplasm from the ooplast was introduced into the perivitelline space of the recipient zygote. Approximately 70% of each ooplast was used to reconstruct up to seven zygotes. The resulting couplet was placed in electrofusion solution (0.28 mol/l mannitol, 0.1 mmol/l $CaCl_2{\cdot}2H_2O$, 0.1 mmol/l $MgSO_4{\cdot}7H_2O$ and 0.3% BSA) and exposed to two electrical pulses of 1.5 kV/cm for 30 μs each (ECM-2001; BTX, San Diego, CA, USA). Zygotes were selected for fusion before in-vitro culture.

Embryos from the four treatment groups had cleavage and blastocyst rates assessed at 48 and 168 h post insemination, respectively. For the control + oocyte transfer and ethidium bromide + oocyte transfer groups, sample embryos (10 per group and per stage) were collected post fusion (at the pronuclear stage) and at 168 h post insemination (at the blastocyst stage) for mtDNA analysis. These embryos were placed individually into 0.2-ml polystyrene microtubes containing 5 μl of ultrapure water and stored at −80°C. Some of the blastocysts (12 and 19 blastocysts, respectively) were also non-surgically transferred transcervically into the uterus (one or two blastocysts per cow) of previously synchronized recipient cows (9 and 12 cows, respectively) following a standard protocol (Nasser et al., 2004). These cows were evaluated for pregnancy by ultrasound visualization of fetal heart beat on day 30 of development. One pregnancy from the control + ooplasm transfer group and two pregnancies from the ethidium bromide + ooplasm transfer group were allowed to develop to term.

Quantitative real-time PCR

Prior to quantitative real-time PCR (qRT-PCR), each embryo was treated with 1 mg/ml proteinase K in water at 37°C for 1 h, after which the proteinase K was inactivated at 94°C for 10 min. qRT-PCR was performed as described in Ferreira et al. (2010), with few modifications. Briefly, the total mtDNA content was measured by a qRT-PCR assay targeting a *MT-RNR2* consensus sequence present in the recipient and donor mtDNA of *B. taurus* and *B. indicus* (GenBank accession numbers AY526085 and AY126697, respectively). This non-discriminating assay used the primers bMT3010-f (5′-GCCCTAGAACAGGGCTTAGT-3′) and bMT3096-r (5′-GGAG AGGATTTGAATCTCTGG-3′) in combination with the TaqMan probe bMT3030-Fam (5′-FAM-AAGGTGGCAGAGCCCGGTAA TTGC-BHQ1-3′). The proportion of donor-derived mtDNA was determined by species-specific qRT-PCR for *B. indicus* using the primers iMT2974-f (5′-CCAATGACAGCATCTCAA TCA-3′, discriminating nucleotides are underlined) and bMT3096-r. For signal detection, a TaqMan probe with the same sequence as in the consensus assay was used. Oligonucleotide sequences were designed using Primer Express version 1.5 (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes were purchased from Invitrogen (Carlsbad, CA, USA) and Metabion (Martinsried, Planegg, Germany), respectively.

Duplicate 25-μl qRT-PCR reactions contained 0.9 μmol/l of each primer, 0.25 μmol/l probe, 1× TaqMan Gene Expression Master Mix (Applied Biosystems) and 5 μl of template 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 s and 63°C for 1 min. Quantitative PCR was run on a ABI PRISM HT 7500 Sequence Detection System using the system's software version 2.3 (Applied Biosystems). The results obtained by qRT-PCR were quantified using the the standard curve method as described (Livak and Schmittgen, 2001).

Measurement of ATP content and mitochondrial membrane potential

Finally, this study investigated whether the effect caused by the ethidium bromide treatment on the oocyte was due to a mitochondrial dysfunction. To assess the effect of ethidium bromide treatment on mitochondrial function, COC were treated with 7.0 μg/ml ethidium bromide and 50 μg/ml UTP during the last 4 h of IVM (referred to as ethidium bromide group). Control groups were COC incubated with 50 ng/ml UTP and without. Treatments were performed in a 24-well plate and after 24 h of IVM, COC were denuded of cumulus cells by gentle pipetting in 0.5% hyaluronidase solution. Following, denuded oocytes were used either for measuring the ATP content or for assessing mitochondrial membrane potential ($\Delta\psi m$).

The ATP content was measured as reported by Tamassia et al. (2004) by using a commercial assay kit based on the luciferin–luciferase reaction (FLASC; Sigma-Aldrich Chemical). Briefly, oocytes were washed three times in PBS (pH 7.4) containing 0.1% polyvinyl alcohol (PBS + PVA), placed individually into 0.2-ml polystyrene microtubes with 5 μl PBS + PVA and stored in liquid nitrogen until use. The volume of each sample was adjusted to 50 μl by adding cold water, then 50 μl of somatic cell ATP releasing reagent was added and the whole volume was transferred to a 1.5 ml tube. Finally, 100 μl of ATP assay mix stock solution (25-fold diluted as suggested by the manufacturer) was added to the reaction tube and the fluorescence read in a luminometer (Modulus; Tuner Biosystems). For each run, a standard curve was generated using seven 2-fold serial-dilutions (20–0.313 pmol/l) of the ATP stock provided by the kit.

The $\Delta\psi m$ was assessed as described by Chiaratti et al. (2006), with few modifications. Oocytes were incubated in 500 nmol/l JC-1 (Molecular Probes, Eugene, OR, USA) diluted in PBS (pH 7.4) for 30 min at 38.5°C and mounted onto microscope slides with coverslips using anti-fade (Vecta Shield; Vector Labs, Yuongstown, OH, USA). Using a fluorescence microscope (Axioplan), JC-1 staining was visualized at 530-nm excitation/485-nm emission and 590-nm excitation/535-nm emission. Under these conditions, JC-1 produces green and red fluorescence, that are representative of the mitochondrial population (regardless of the $\Delta\psi m$) and

the $\Delta\psi m$, respectively. The relative $\Delta\psi m$ was calculated as the ratio between red and green fluorescence estimated using ImageJ version 1.36b (National Institute of Healthy, Bethesda, MD, USA).

Statistical analysis

In experiment I, the triplicate data for cortical granule migration and meiotic resumption rates were transformed using arcsine and analysed by a regression analysis. The regression coefficient (R^2) is presented. Development rates (five replicates) were subjected to ANOVA followed by Tukey's post-hoc analysis. In experiment II, two main factors were studied as a cross-classification (eight replicates): no treatment or treatment with ethidium bromide (control and ethidium bromide groups) and zygotic production with or without ooplasm transfer (ooplasm transfer and IVF groups). Data were analysed using the generalized linear model with binomial distribution and logistic link functions. The ATP concentrations and relative $\Delta\psi m$ were compared among groups by ANOVA. Differences with $P < 0.05$ were considered significant. Values are displayed mean ± standard error of the mean.

Results

Ethidium bromide treatment blocks early development

To determine the best protocol for ethidium bromide treatment, the effect of increasing concentrations of this drug on oocyte maturation was tested first (**Figure 1**). A strong correlation between ethidium bromide concentration and both cortical granule migration ($R^2 = 0.59$; **Figure 1**A) or meiotic resumption ($R^2 = 0.89$; **Figure 1**B) rates was found. The correlation between ethidium bromide concentrations and cortical granule migration rate persisted with up to 4—5 μg/ml ethidium bromide, at which point further increases in drug concentration did not lead to greater decreases in migration rate. The effect of ethidium bromide on cortical granule migration was determined to fit into one of three categories: absent (**Figure 1**C), partial (**Figure 1**D, E) and complete migration (**Figure 1**F). In contrast to untreated oocytes (0.0, 49.3 ± 7.7 and 51.0 ± 7.9%, respectively), treatment with 7 μg/ml ethidium bromide led to higher rates of both absent and partial cortical granule migration and a lower rate of complete migration (10.0 ± 3.2, 77.0 ± 0.7 and 13.0 ± 3.8%, respectively). In regards to meiotic resumption, the correlation between drug concentration and MII-establishment rate was found to persist with up to 7 μg/ml ethidium bromide. Compared with untreated oocytes (**Figure 1**G), ethidium bromide-treated oocytes showed an increase in patterns of dispersed-MII (**Figure 1**H), arrested-telophase I chromosomes (**Figure 1**I) as well as nuclear fragmentation (**Figure 1**J). In addition, the treatment had an unexpected effect on cumulus cell expansion (**Figure 1**K, L). Oocytes treated with 3.5—7.0 μg/ml ethidium bromide were denuded of cumulus cells more easily than those left untreated. Together, these results illustrate a dose-dependent effect of ethidium bromide on oocyte maturation.

To evaluate the developmental competence of ethidium bromide-treated (0.0, 2.5 and 7.0 μg/ml) oocytes, their cleavage and blastocyst rates were assessed after IVF and in-vitro culture (Table 1). Interestingly, cleavage rates were only affected at 7.0 μg/ml (2.6 ± 1.38%) when compared with the untreated group (82.4 ± 5.68%). On the other hand, blastocyst rates were considerably lower at both 2.5 (6.6 ± 4.43%) and 7.0 μg/ml (0.3 ± 0.46%) ethidium bromide compared with the untreated group (25.4 ± 5.27%). Moreover, morphological observations made under light microscopy showed that blastocysts produced after treatment with ethidium bromide were of poor quality characterized by a smaller blastocoel and a poorly defined inner cell mass. The number of cells in blastocysts treated with 2.5 μg/ml ethidium bromide (114 ± 22.5) was significantly lower than in blastocysts resulting from untreated oocytes (149 ± 14.3). Since the blastocysts produced from oocytes treated with 7 μg/ml ethidium bromide were of very poor quality they were not taken into account for cell number analysis. Therefore, the treatment with ethidium bromide during IVM for 24 h compromises the ability of an oocyte to develop into a normal blastocyst.

Ooplasm transfer can rescue the development of ethidium bromide-treated oocytes

This experiment sought to evaluate the developmental rates of partially compromised oocytes that received ooplasm from normal donor ooplasts. However, as discussed above, the treatment of oocytes with ethidium bromide during IVM for 24 h impairs oocyte maturation and, therefore, cannot be used as recipients for ooplasm transfer at the pronuclear stage. Considering that most oocytes have completed their ooplasmic and nuclear maturation at 20 h post IVM (Ferreira et al., 2009), oocytes were treated during the last 4 h of IVM only (20—24 h). The highest concentration of ethidium bromide (7.0 μg/ml) was chosen to account for the shorter treatment period. Oocyte maturation and fertilization were not compromised by such treatment, as shown by the similar PB2-extrusion rates (control + ooplasm transfer = 52.4 ± 0.4% and ethidium bromide + ooplasm transfer = 54.5 ± 0.2%). In spite of this, decreases in the ethidium bromide + IVF group compared with the control + IVF group were observed for both cleavage rates (49.3 ± 2.51% and 64.5 ± 2.12%) and blastocyst (8.3 ± 1.38% and 25.6 ± 1.93%) rates ($P < 0.05$; Table 2), respectively.

To test the potential of ooplasm transfer in rescuing development of treated oocytes, zygotes from the ethidium bromide + ooplasm transfer and control + ooplasm transfer groups received ooplasm donated by untreated donor ooplasts and their developmental rates were assessed (Table 2). Both cleavage (68.7 ± 3.79% and 67.7 ± 3.62%) and blastocyst (33.3 ± 3.85% and 34.1 ± 3.67%) rates were similar in the ethidium bromide + ooplasm transfer and control + ooplasm transfer groups, respectively. Moreover, both cleavage (68.7 ± 3.79% and 49.3 ± 2.51%) and blastocyst (33.3 ± 3.85% and 8.3 ± 1.38%) rates were increased in the ethidium bromide + ooplasm transfer as compared with the ethidium bromide + IVF group (Table 2), providing evidence of a developmental rescue mediated by ooplasm transfer. It is interesting to mention that oocytes in the

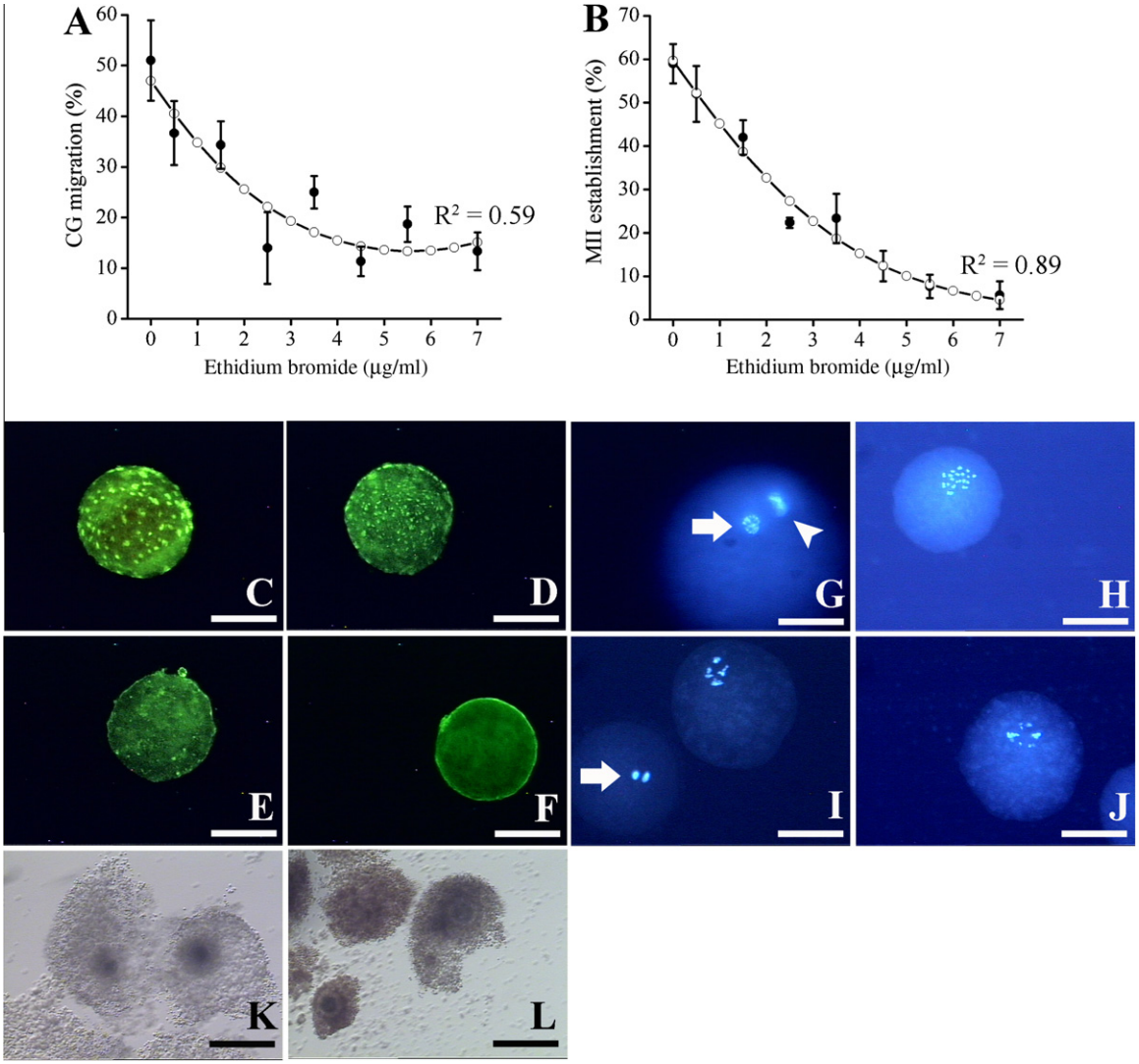

Figure 1 Oocyte maturation is compromised by treatment with ethidium bromide. (**A**,**B**) Dose-dependent effect of ethidium bromide on (**A**) complete cortical granules (CG) migration and (**B**) metaphase II (MII) establishment rates. Filled circles with bars are observed mean values ± standard error of the mean. Empty circles are estimated values determined by a regression analysis ($P < 0.05$), which allowed adjusting a second degree polynomial curve for both panels. (**C**—**F**) Characteristic patterns of cortical granules that are (**C**) absent, (**D**,**E**) partially migrated and (**F**) completely migrated. (**G**) Complete nuclear maturation (arrow = arrested MII chromosomes; arrow head = first polar body). (**H**) Pattern of dispersed metaphase I. (**I**) Incomplete nuclear maturation (arrow = arrested telophase-I chromosomes). (**J**) Nuclear fragmentation. (**K**) Expanded cumulus—oocyte—complexes untreated with ethidium bromide. (**L**) Partially expanded cumulus—oocyte—complexes after treatment with 7 µg/ml ethidium bromide). Bars = 50 µm (**C**—**J**) and 500 µm (**K**,**L**).

Table 1 Effect of ethidium bromide exposure during oocyte maturation on embryonic development.

Ethidium bromide (µg/ml)	*Developmental rates (%)*			*Cell numbers (n)*	
	No. embryos used	*Cleavage*	*Blastocyst*	*No. embryos used*	*Cells*
0.0	149	82.4 ± 5.68[a]	25.4 ± 5.27[a]	20	149 ± 14.3[a]
2.5	162	52.1 ± 9.42[a]	6.6 ± 4.43[b]	13	114 ± 22.5[b]
7.0	182	2.6 ± 1.38[b]	0.3 ± 0.46[b]	0	NA

Values are mean ± standard error of the mean.
NA = not analysed.
[a,b]Different letters within columns denote significant difference ($P < 0.05$).

Table 2 Effect of ooplasm transfer on developmental rates of ethidium bromide-treated oocytes after IVF.

Protocol	*Cleavage (%)*		*Blastocyst (%)*	
	Control	*Ethidium bromide*	*Control*	*Ethidium bromide*
IVF	64.5 ± 2.12[c]	49.3 ± 2.51[a,d]	25.6 ± 1.93[a,e]	8.3 ± 1.38[a,f]
Ooplasm transfer	67.7 ± 3.62	68.7 ± 3.79[b]	34.1 ± 3.67[b]	33.3 ± 3.85[b]

Values are mean ± standard error of the mean.
Total number of presumptive zygotes cultured for each group: Control + IVF = 512, ethidium bromide + IVF = 398, control + transfer = 167 and ethidium bromide + transfer = 150.
[a,b]Different letters within columns denote significant difference ($P < 0.05$).
[c,d,e,f]Different letters within rows denote a significant difference ($P < 0.05$).

control + ooplasm transfer group had a higher rate of blastocyst formation (34.1 ± 3.67%) compared with oocytes in the control + IVF group (25.6 ± 1.93%). This result likely reflects the fact that oocytes not subjected to ooplasm transfer were not selected for the presence of the PB2. Nonetheless, similar PB2-extrusion rates between oocytes treated or untreated with ethidium bromide provide evidence the developmental rescue was mediated by introduction of normal ooplasm, not by a side-effect of the micromanipulation procedure.

To further confirm the rescue of development by ooplasm transfer in the control + ooplasm transfer and ethidium bromide + ooplasm transfer groups, 12 and 19 blastocysts, respectively, were transferred to recipient cows. Gestation rates between the control and the ethidium bromide-treated groups were similar (17 and 32%, respectively). Moreover, one gestation resulting from a control embryo and two gestations resulting from treated embryos were allowed to develop to term, resulting in the birth of three calves (**Figure 2**). Together, these results indicate that the ooplasm transfer procedure utilized was effective in rescuing both pre- and post-implantation development, allowing the birth of normal offspring.

Mitochondrial DNA content and heteroplasmy remain unchanged during early development

The ooplasm transfer of donor mitochondria was studied by assessing the levels of donor and total (donor + recipient) mtDNA in zygotes shortly after ooplast fusion (pronuclear stage) and in embryos at the blastocyst stage. The percentage of donor mtDNA observed in zygotes at the pronuclear stage did not differ between control and ethidium bromide-treated groups (15 ± 3.2% and 13 ± 2.8%, respectively). Moreover, the relative content of mtDNA per zygote after ooplasm transfer was not significantly different between the two groups (1.0 ± 0.13 and 1.6 ± 0.25). At the blastocyst stage, the percentage of donor mtDNA (12 ± 1.9% and 11 ± 1.8%, respectively) and the content of mtDNA (1.4 ± 0.25 and 1.9 ± 0.41, respectively) were similar and remained the same as that found at the pronuclear stage. Furthermore, the calves born after ooplasm transfer inherited the mtDNA introduced by the procedure (Ferreira et al.,2010). These results show that the mtDNA content during preimplantation was not affected by the treatment with ethidium bromide. Moreover, embryos and the offspring produced by ooplasm transfer were heteroplasmic.

Mitochondrial function is unaffected soon after ethidium bromide treatment

In order to determine whether the effect of ethidium bromide on oocyte competence was specific to mitochondria leading to impairment of their function, the ATP content was measured immediately after IVM (**Figure 3**A). Surprisingly, the ATP content of oocytes treated with ethidium bromide was not different to that of control oocytes whether incubated with UTP or not. The average ATP content was 3.66 ± 0.149 pmol/oocyte for the control group, 3.42 ± 0.129 pmol/oocyte for the control with UTP group and 3.87 ± 0.133 pmol/oocyte for the ethidium bromide group. This finding was further confirmed by assessing the $\Delta\ \psi m$ (**Figure 3**B and C). The $\Delta\ \psi m$ did not differ among the control (1.00 ± 0.076), control with UTP (0.97 ± 0.062)

Figure 2 Ooplasm transfer rescues the development of ethidium bromide-treated oocytes allowing the birth of healthy offspring. Calves produced after ooplasm transfer using recipient zygotes either (**A**) untreated or (**B**,**C**) treated with ethidium bromide.

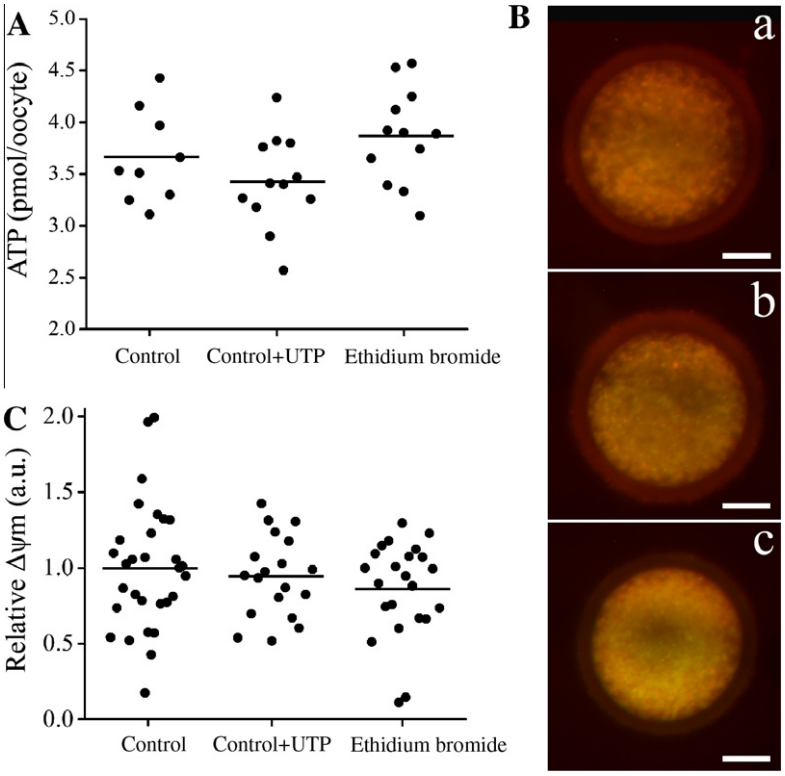

Figure 3 Mitochondrial function is not affected by ethidium bromide treatment. (**A**) Effect of 7.0 μg/ml ethidium bromide plus 50 μg/ml uridine 5′-triphosphate (UTP) during the last 4 h of in-vitro maturation on oocyte ATP content in comparison to oocytes matured either in the absence of both ethidium bromide and uridine (control) or in the presence of only uridine (control + UTP). (**B**,**C**) The mitochondrial membrane potential (Δ ψm) was also assessed using (**B**) JC-1 in (a) control, (b) control + UTP and (c) ethidium bromide groups and (**C**) relative Δ ψm, estimated as the ratio between Δ ψm and mitochondrial population. a.u. = arbitrary units. Bars = mean (**A**,**C**) and 30 μm (**B**).

and ethidium bromide (0.93 ± 0.065) groups. Therefore, these results indicate that the effect of ethidium bromide on oocyte competence was not caused by mitochondrial dysfunction immediately after ethidium bromide treatment. Since analysis of mitochondrial function at later periods after ethidium bromide treatment would be biased by developmental effect, these results cannot exclude the possibility that ethidium bromide acts on mitochondria at a later time.

Discussion

Ooplasm transfer has been adopted by a few clinics worldwide since the report of babies being born following the transfer of a small amount of ooplasm to the oocytes of women with a history of poor embryonic development and recurrent implantation failure (Cohen et al., 1997, 1998; Huang et al., 1999; Levy et al., 2004). However, it is widely accepted that further research based on animal models is needed to better understand the genetic and epigenetic consequences of transferring ooplasm from donor to recipient oocytes (Barritt et al., 2001; Levy et al., 2004; Liang et al., 2009; Malter and Cohen, 2002). This research is needed in order to evaluate the benefits to the fertility of patients and the potential risks to the resultant babies and their descendants (Barritt et al., 2001; Hawes et al., 2002; Malter and Cohen, 2002; St John et al., 2002; Levy et al., 2004; Sills et al., 2004). Here in the present study, a new animal model was examined, in which ethidium bromide was used to partially impair preimplantation development of bovine in-vitro fertilized oocytes. Evidence is given to show that oocyte development can be rescued in cases in which infertility is caused by a defect in the ooplasm supporting the use of this experimental model for the study of ooplasm transfer.

Ethidium bromide was chosen for this experimental model with the aim of impairing mitochondrial function. Although widely used in somatic cells (Chiaratti and Meirelles, 2006; King and Attardi, 1996; Piechota et al., 2006; von Wurmb-Schwark et al., 2006), reports on the effects of the drug on mammalian oocytes and embryos are less common (Pikó and Chase, 1973). Thus, dosages and incubation intervals used in the present study were based on previous reports using somatic cells and invertebrate embryos (Geuskens, 1971; King and Attardi, 1996; Chiaratti et al., 2006; Piechota et al., 2006; von Wurmb-Schwark et al., 2006). During oocyte maturation, the cortical granules are reorganized in the ooplasm while meiotic division is resumed to the MII stage. Both events are critical steps for the gamete to acquire developmental competence (reviewed by Kane, 2003). In the present study, a clear dose-dependent toxic effect of ethidium bromide on oocyte maturation was seen for both cortical granule migration and

meiotic resumption. Moreover, an effect on the expansion of cumulus cells was also detected. Like most events in oocyte maturation, these events require energy (Eichenlaub-Ritter et al., 2004; Van Blerkom, 1991; Van Blerkom and Runner, 1984) that is provided mainly by the mitochondrion (Dumollard et al., 2007). Thus, a mitochondrial dysfunction leading to lower levels of ATP production could compromise cortical granule migration and meiotic resumption. Earlier reports describe a correlation between mitochondria or ATP content and oocyte maturation (Connors et al., 1998; Takeuchi et al., 2005). Thus, it is reasonable to consider that oocyte maturation was compromised by a mitochondrial dysfunction caused by ethidium bromide treatment. However, the present study cannot discard a possible side-effect of ethidium bromide on other cellular components that are unrelated to mitochondria but could lead to the same outcome.

A series of events occurring during oocyte maturation in both nucleus and ooplasm are responsible for preparing the cell to be fertilized and to support the development of the embryo (reviewed by Kane, 2003). Therefore, embryonic development was expected to be compromised after treatment of oocytes with ethidium bromide during the entirety of IVM. It is possible that blastocyst rate, but not cleavage rate, was affected by the lower ethidium bromide dosage because the oocyte quality is much more put to test to reach the blastocyst stage than to reach the 2-cell stage (cleavage). At the blastocyst stage the embryo needs a higher amount of energy than at the 2-cell stage to support protein synthesis and blastocoel formation (Dumollard et al., 2007; May-Panloup et al., 2007; Thompson et al., 1996). Moreover, even when the treatment was restricted to the last 4 h of IVM, cleavage and blastocyst rates remained low compared with untreated oocytes. After 20 h of IVM, most of the events related to oocyte maturation, including cortical granule migration and meiotic resumption, are complete (Ferreira et al., 2009). The present study confirmed this by analysis of the PB2-extrusion rate, which was not affected by ethidium bromide exposure at the end of IVM. Thus, it is possible that in spite of the short period of treatment, mitochondrial function was compromised and resulted in the observed developmental arrest. This hypothesis is also supported by reports showing a negative effect on embryonic development when mitochondria were damaged after oocyte maturation (Thouas et al., 2004, 2006). The number of mitochondria and the levels of ATP in the oocyte have been correlated to its fertilization and developmental potential (Dumollard et al., 2007; May-Panloup et al., 2007; Wai et al., 2010), although this is controversial in bovine (Chiaratti et al., 2010). But again, the present results do not exclude a possible side-effect of ethidium bromide on other cellular processes that might lead to similar results.

To test the potential of ooplasm transfer to rescue preimplantation development, ooplasm donated by untreated ooplasts was transferred into zygotes previously treated with ethidium bromide during the last 4 h of IVM. Ooplasm transfer was first performed in mice during the early 1980s to overcome in-vitro-induced cleavage arrest at the 2-cell stage (Muggleton-Harris et al., 1982). During the late 1990s, this procedure was applied in humans to rescue oocytes of patients with a history of poor embryonic development and recurrent implantation failure (Cohen et al., 1997, 1998; Huang et al., 1999; Lanzerdorf et al., 1999; Dale et al., 2001; Hwang et al., 2002). The present study postulated that ooplasm transfer would enhance the developmental rates of oocytes exposured to ethidium bromide. This is confirmed by data showing that, after ooplasm transfer, ethidium bromide-treated oocytes develop to the blastocyst stage similarly to control embryos. Furthermore, transfer of blastocysts to recipient cows resulted in the same gestation rates for both groups. The birth of calves from ethidium bromide-treated oocytes proves not only that preimplantation developmental rates are restored, but also that these embryos are able to develop to term. Similarly, Takeuchi et al. (2005) completely rescued the preimplantation development of oocytes with damaged mitochondria after germinal vesicle transfer. The factors responsible for the beneficial effects of ooplasm transfer are not clearly understood, but it has been suggested that mitochondria and products of mitochondrial metabolism (e.g. ATP) are responsible for such effects (Barritt et al., 2001; Levy et al., 2004; Malter and Cohen, 2002). For instance, Hua et al. (2007) rescued the development of bovine oocytes by injecting mitochondria isolated from cumulus cells. As shown by Van Blerkom et al. (1998), injection of ooplasts enriched with mitochondria resulted in increased ATP production in recipient oocytes. Thus, it is tempting to propose that the developmental rescue observed in the present experiment was mediated by mitochondria or products of their metabolism (e.g. ATP) that were transferred into the zygotes.

One of the main concerns with ooplasm transfer in humans is the generation of mtDNA heteroplasmy, a condition that is not commonly found in normal individuals but is observed in patients with mitochondrial-encoded diseases (reviewed by Wallace, 2010). Thus, the present study assessed the quantity of donor mtDNA introduced during ooplasm transfer and that remaining at the blastocyst stage. The introduced mtDNA does not segregate until the blastocyst stage as observed by others when mitochondria in the same differentiation stage are mixed (Ferreira et al., 2007). Furthermore, the introduced mtDNA was inherited by the offspring, confirming earlier reports in babies born after ooplasm transfer (Barritt et al., 2001; Brenner et al., 2000; St John, 2002).

Finally, to confirm that the detrimental effect of ethidium bromide on development was caused by disruption of mitochondrial function in oocytes, the mtDNA content during preimplantation development was assessed. It was expected that ethidium bromide would affect mitochondrial function by means of inhibiting mtDNA replication and transcription (King and Attardi, 1996; Pikó and Chase, 1973; Chiaratti et al., 2006). Nonetheless, the present study showed no effect on mtDNA content either at the pronuclear or at the blastocyst stage. If ethidium bromide had disrupted mitochondria by interfering with mtDNA replication, higher levels of heteroplasmy in embryos should be found, but instead there were similar levels between embryos treated and untreated with ethidium bromide. Even the total mtDNA content of pronuclear and blastocyst stages was unchanged by the treatment. These results indicate mtDNA replication was not compromised by the ethidium bromide treatment at the end of IVM. Then, this study

investigated the hypothesis that mitochondrial function is affected by an effect of ethidium bromide on mtDNA transcription by assessing the ATP content and the relative $\Delta\ \psi m$ in oocytes. Surprisingly, none of these parameters were disrupted in oocytes treated with ethidium bromide. Although part of the cellular ATP is synthesized in the cytoplasm by glycolysis, a mitochondrial-independent mechanism, most of the ATP is produced in mitochondria. Thus, lower endogenous ATP amounts were expected if the ethidium bromide treatment had impaired mitochondrial activity. The absence of an effect of ethidium bromide on mitochondrial function was further confirmed by unaltered levels of relative $\Delta\ \psi m$ in treated and untreated oocytes. These results suggest the detrimental effect of ethidium bromide on oocytes, which is reversed by ooplasm transfer, is not mediated by a mitochondrial dysfunction. In this context, a side-effect of ethidium bromide on nuclear and cytoplasmic systems (e.g. transcription, translation) might have accounted for the observed result as reported previously (Geuskens, 1971; Nass, 1972; Pikó and Chase 1973; Zylber and Penman, 1969). According to these authors this side-effect is dependent on the ethidium bromide dosage used resulting in embryonic developmental arrest due to impairment of total cellular RNA synthesis (Pikó and Chase, 1973). However, a side-effect of ethidium bromide on RNA synthesis in the present work does not seem reasonable since at 20 h of IVM the nuclear DNA is highly condensed in MII and, therefore, RNA synthesis is inactive. An alternative explanation is that mitochondrial dysfunction was not observed in oocytes treated with ethidium bromide due to the techniques employed. For instance, a compensatory response of mitochondria to ethidium bromide might have prevented a decrease in the levels of mtDNA copy number, ATP content or relative $\Delta\ \psi m$. This compensatory mechanism is typical of many cell types in response to an oxidative phosphorylation deficiency which reflects an attempt of the cell to survive (Chiaratti et al., 2006; Miranda et al., 1999; Miranda et al., 2009; Piechota et al., 2006; Wai et al., 2010). It might be mediated by an up-regulation of mtDNA replication or transcription or increase of glycolysis-derived ATP synthesis, for example, thus preventing a proper evaluation of ethidium bromide effect on mitochondria.

Therefore, it remains to be conclusively demonstrated that ooplasm transfer can rescue the development of human oocytes with defective mitochondria. Moreover, although there are studies showing an effect of mtDNA copy number on embryonic development (Almeida Santos et al., 2006; May-Panloup et al., 2005a; Reynier et al., 2001; Wai et al., 2010), this effect may vary among species depending on the time mtDNA replication recommences (Aiken et al., 2008; Chiaratti and Meirelles, 2010). For instance, restarting of mtDNA replication at the blastocyst stage in bovine was shown to reverse mtDNA depletion preventing an effect of mtDNA copy number on development (Chiaratti et al., 2010). It is unclear when mtDNA replication recommences in humans, but if it is anticipated, as reported for cattle (May-Panloup et al., 2005b), mtDNA copy number might exert a lower effect on development than that reported in murine (Chiaratti and Meirelles, 2010; Wai et al., 2010). Although human ooplasm transfer was suggested to rescue development due to the introduction of healthy mitochondria (Cohen et al., 1997, 1998; Huang et al., 1999; Lanzendorf et al., 1999), this has not been confirmed until now (Barritt et al., 2001; Levy et al., 2004; Malter and Cohen, 2002). Other factors than mitochondria are introduced into oocytes during ooplasm transfer which prevent exclusion of the hypothesis that these factors (e.g. proteins, RNAs, lipids) are responsible for the developmental rescue seen in humans. During oogenesis and/or folliculogenesis, the oocyte stores mitochondria and other factors that are necessary to support early embryonic development and therefore affect oocyte competence. If any of these factors limit embryonic development, ooplasm transfer might restore oocyte competence allowing development to progress.

In conclusion, ethidium bromide can be used to impair both oocyte maturation and embryonic development which is reversed by ooplasm transfer. This rescue apparently occurs without negative consequences to the offspring in spite of generating mitochondrial heteroplasmy. As cytoplasmic factors (e.g. proteins, RNAs, lipids) other than mitochondria may be involved in the developmental arrest reported in humans, further studies using appropriate animal models are needed to address the cytoplasmic factors capable of affecting oocyte competence and the benefits of ooplasm transfer to restore competence in these cases.

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