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Increase in mitochondrial DNA quantity and impairment of oxidative phosphorylation in bovine fibroblast cells treated with ethidium bromide for 15 passages in culture

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ABSTRACT. Bovine fetal fibroblast cells were treated with ethidium bromide at a low concentration for 15 passages in culture to determine its effect on mitochondrial DNA copy number and on cell metabolism. Mitochondrial membrane potential and lactate production were estimated in order to characterize cell metabolism. In addition, mitochondrial DNA ND5 in proportion to a nuclear gene (luteinizing hormone receptor) was determined at the 1st, 2nd, 3rd, 10th, and 15th passages using semi-quantitative PCR amplification. Treated cells showed a lower mitochondrial membrane potential and higher levels of lactate production compared with control cells. However, the mitochondrial DNA/nuclear DNA ratio was higher in treated cells compared with control cells at the 10th and 15th passages. This ratio changed between the 3rd and 10th passages. Despite a clear impairment in mitochondrial function, ethidium bromide treatment did not lead to mitochondrial DNA depletion. It is possible that in response to a lower synthesis of ATP, due to an impair-

ment in oxidative phosphorylation, treated cells develop a mechanism to resist the ethidium bromide effect on mtDNA replication, resulting in an increase in mitochondrial DNA copy number.

Key words: Bovine, EtBr, Fibroblast, mtDNA, OXPHOS, ρ^0

INTRODUCTION

According to the endosymbiosis theory, the evolution of eukaryotic pluricellular organisms is closely related to the development of an organelle specialized in efficient oxidative energetic metabolism, the oxidative phosphorylation system (OXPHOS) (Schwartz and Dayhoff, 1978; Gray et al., 1999). The mitochondrion, the organelle responsible for OXPHOS, can be characterized as the main energy source for the cell. Five enzyme complexes located in the mitochondrial membrane are involved in the OXPHOS. The mitochondrial DNA (mtDNA) encodes only 13 polypeptides among the nearly 70 involved in the OXPHOS, as well as 22 transfer RNAs and two ribosomal RNAs. The rest of the OXPHOS subunits, as well as the factors responsible for control of the replication process, transcription and part of the translation machinery of the mtDNA, are encoded in the nuclear DNA (Hermann and Neupert, 2000). Mitochondrial biogenesis and function depend on a complex interaction between nuclear and mitochondrial genomes (Attardi and Schatz, 1988; Clayton, 1998; Garesse and Vallejo, 2001). A specific cell type may adapt mitochondrial function in response to metabolic needs or environmental signals (Williams, 1986).

The mammalian mtDNA is a closed circular molecule of approximately 16 kb. It is present in cells in a very high ploidy, ranging from 400 copies in some cells to over 100,000 copies in oocytes (Bogenhagen and Clayton, 1978; Pikó and Taylor, 1987; Robin and Wong, 1988). On average, a fibroblast cell has 4000 copies of mtDNA, segregated in 400 organelles. The number of copies can change according to cell metabolism (Williams, 1986); the number of mtDNA copies increases in direct proportion to the level of OXPHOS. Severe disorders in human tissues with greater ATP needs originate from mutations in mtDNA, ranging from simple deletions/mutations to large deletions (Wallace, 1999).

Several researchers have achieved partial or total depletion of mtDNA. King and Attardi (1996) found that the number of mtDNA copies decreases by a factor of $1/2^n$ (n corresponds to the number of cell doublings) when human cell lines are treated with low concentrations of ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide, EtBr, a non-specific inhibitor of mtDNA replication). After 3-4 weeks of growth in the presence of this drug, it was possible to isolate cells lacking mtDNA (ρ^0 derivates). However, these cells depend on supplementation with uridine (Morais and Giguere, 1979) and pyruvate to grow (King and Attardi, 1989). The EtBr mechanism causing mtDNA depletion involves intercalation into mtDNA inhibiting mtDNA replication. The ρ^0 cells survive under specific fermentation conditions (still poorly characterized). We treated bovine fibroblast cells in culture with an inhibitor of mtDNA replication to determine its effect on mtDNA quantity and on cell metabolism.

MATERIAL AND METHODS

Cell culture and treatment

A Nelore (*Bos taurus indicus*) breed bovine male fetus was collected at a 55-day gestation from an abattoir, and skin fragments were used to establish a primary cell culture in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 20% fetal calf serum (FCS). After the second passage in culture, the cells were cryopreserved and stored for further studies.

One vial of fibroblast cells was thawed and cultured on a 60-mm Petri dish in DMEM supplemented with 10% FCS until confluence. After trypsin treatment, 1 x 10^5 cells were transferred to two Petri dishes containing 3 mL α-minimum essential medium (Life Technologies) supplemented with uridine (50 µg/mL; Morais and Giguere, 1979), pyruvate (100 µg/mL; King and Attardi, 1989) and 10% FCS. When confluence was reached, the cells were treated with trypsin and plated on new dishes for 15 passages. The medium was changed frequently to assure the requirements of the cells. In the treatment group, the fibroblast cells were exposed to 50 ng/mL EtBr for 15 passages.

Mitochondrial membrane potential (ΔΨm)

The ΔΨm was estimated using the JC-1 probe (Molecular Probes). This probe accumulates in the mitochondria due to membrane potentials. Organelles with low ΔΨm accumulate a low number of JC-1 molecules, which produce a green fluorescence (530 excitation/485 emission). At high concentrations (high ΔΨm), the probe aggregates and exhibits a red-shifted fluorescence (590 excitation/535 emission). Loss of membrane potential is followed by a red to green shift.

Before confluence, at the 10th passage, the fibroblasts were incubated in the presence of 2 µM JC-1 for 50 min at 37°C, protected from light and examined with an epifluorescence microscope (Axioplan, Carl Zeiss). The cells were then trypsinized and transferred to 96-well ELISA plates, and the ΔΨm was estimated by the red/green fluorescence ratio, measured with the appropriate filters in a Fuji Fla 3000G Laser Scanner (Fuji Film Co., Japan).

Lactate production

Lactate production was measured based on its oxidation to pyruvate in the presence of NAD^+ and L-lactic acid dehydrogenase (LDH; Engel and Jones, 1978). To achieve complete oxidation of L-(+)-lactate, hydrazine and an alkaline reaction medium were used to drive the reaction to the NADH side.

$$\text{L-(+)-lactate} + NAD^+ + \text{hydrazine} \xrightleftharpoons{\text{LDH pH 9.0}} \text{pyruvate hydrazone} + NADH + H_3O^+$$

The 10th passage cells were cultured and collected at 0, 11, 19, and 26 h after seeding. NADH was then measured with a recording spectrophotometer at 340 nm (Beckman, DV 640) applying a previously proposed formula (Engel and Jones, 1978). The amount of lactate determined at 0 h was subtracted from that obtained at 11, 19 and 26 h.

Amplification of mtDNA ND5 and luteinizing hormone receptor gene (nuclear gene)

Fibroblast cells collected from both treated and control cultures at the 1st, 2nd, 3rd, 10th, and 15th passages were centrifuged and total DNA was extracted from the cell pellet according to standard procedures (Sambrook and Russel, 2001). Part of the mtDNA ND5 gene was amplified using previously described primers (Meirelles et al., 1999) 5'-CCCAACGAGGA AAATATACC-'3 (BosmtF1) and 5'-AACCGCAAACAACCTCTTCC-3' (BosmtR1) with 3.5 mM $MgCl_2$ for 12, 15, 18, 21, and 24 cycles of 30 s at 94°C, 45 s at 58°C and 45 s at 72°C. Part of the LH receptor gene (LHr) was amplified using the primers described by Lussier et al. (1995): (LHRBOV-F) 5'-GCCGTGTTGCCTCTTGTGGG-3' and (LHRBOV-R) 5'-GGTCCAT GACCATGGCCCGTC-3'. The amplification reaction for LHr was carried out with 1.5 mM $MgCl_2$ for 30, 32, 34, and 36 cycles of 1 min at 95°C, 30 s at 58°C and 2 min at 72°C. The PCR products for mtDNA and LHr were then submitted to electrophoresis on 1.5% agarose gel and stained with EtBr, and their quantities were determined with the appropriate filters in a Fuji Fla 3000G Laser Scanner (Fuji Film Co.) using Image Gauge V3.12 software. Arbitrary density was plotted against PCR cycle for the two amplification products (mtDNA and LHr; Figure 2C1 and C2). One cycle in each curve was selected for amplification and quantification. The selected points were 21 and 33 cycles for mtDNA and LHr, respectively. At these cycles, amplification was found to be exponential. mtDNA analysis at each passage was expressed as an mtDNA/LHr ratio to allow comparisons between the two groups. This ratio was determined by mtDNA ND5/nDNA LHr gene densitometry applying the Image Gauge V3.12 software (Fuji Film Co.).

Statistical analysis

Statistical analysis was performed using the SAS system (Version 8.0) with three individual replicates for each analysis (mtDNA, $\Delta\Psi m$ and lactate production). mtDNA and lactate production data were analyzed by a mixed procedure (PROCMIX), while $\Delta\Psi m$ data were evaluated by analysis of variance (ANOVA). Statistical significance was set at $P \leq 0.05$.

RESULTS

Cell culture and mitochondrial functions

Fibroblast cultures exhibited a classical appearance in both treated and control cultures, suggesting that the treatment did not affect cell growth rate or that the uridine plus pyruvate supplementation was capable of supporting normal cell growth. Cell staining with JC-1 dye clearly suggests a reduction in red fluorescence in the EtBr-treated group compared with the control (Figure 1A and B). This observation is in agreement with $\Delta\Psi m$ estimates at the 10th passage, which also showed a significantly lower ratio of red/green fluorescence density in the treated group (Figure 1C). Lactate concentration in the medium was higher in the EtBr-treated group at the 10th cell passage, which was evident after 10 h of culture (Figure 1D). In agreement with this result, the culture medium was yellowish in the treated group, indicating more acidification compared with the control group (see details on the corner of Figure 1A and B, respectively).

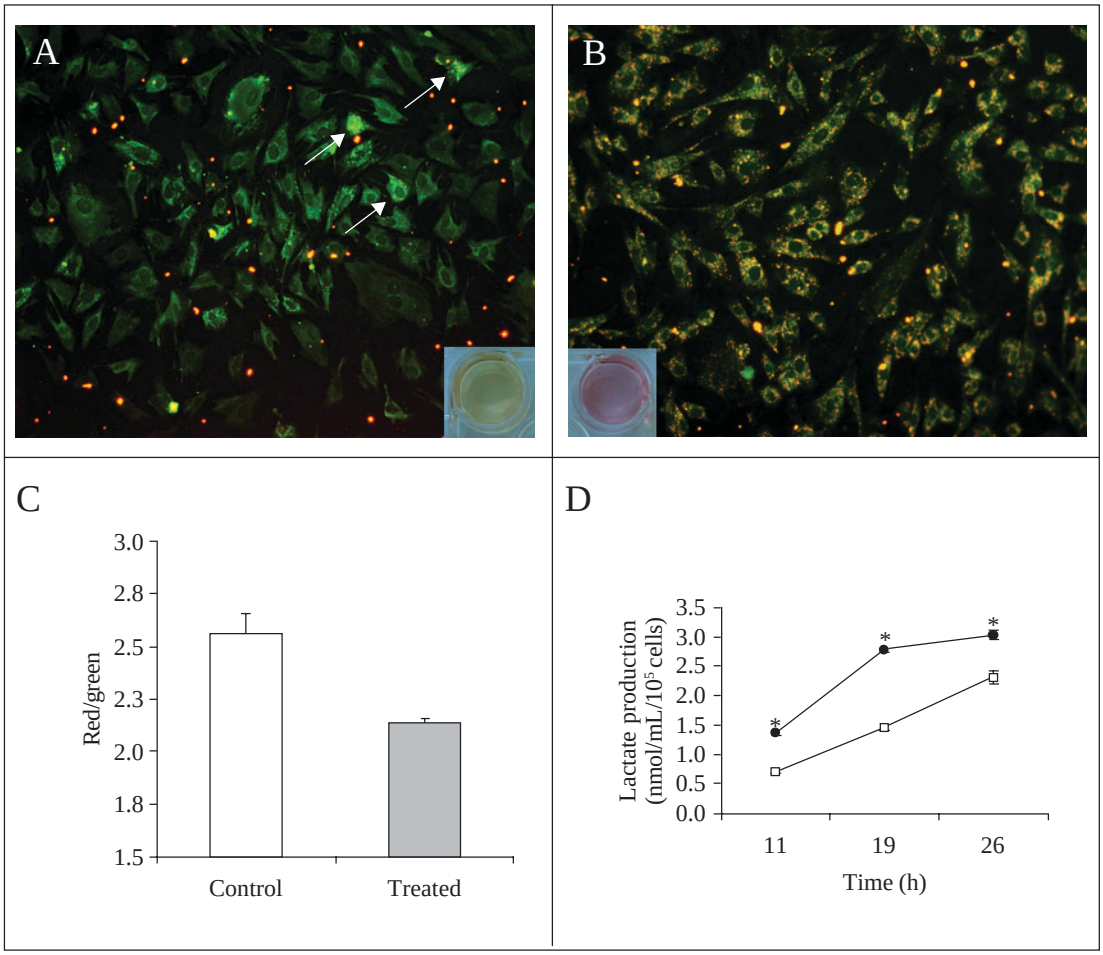

Figure 1. Estimation of the mitochondrial membrane potential (ΔΨm) using JC-1 probe and of lactate production. Epifluorescence photomicrography of nearly confluent fibroblast cells at 10th passage, ethidium bromide (EtBr)-treated (A) and control (B), stained with 2 µM JC-1 probe. In detail, the higher acid content in medium of treated cells compared to control (the presence of phenol red in the culture medium illustrates the lower pH where acidification leads to yellow color). Most of the cells in the control group show red fluorescence, while most cells in the treated group show a green fluorescence (only few treated cells are able to maintain some ΔΨm, as indicated by arrows). Red and green fluorescence was emitted in both groups after laser excitation, but the control group (mean and SD, 2.556 ± 0.096) showed a higher ΔΨm compared with the treated group (2.138 ± 0.019; C, statistically significant; $P \leq 0.05$). As determined by lactate production (D), EtBr-treated cells (circles, 1.375 ± 0.043, 2.769 ± 0.033 and 3.022 ± 0.082 to 11, 19 and 26 h, respectively) show a significant increase in fermentation compared with control (squares, 0.705 ± 0.034, 1.449 ± 0.047 and 2.310 ± 0.113; items marked with asterisk are different compared with the control group in the same passage; $P \leq 0.05$).

Mitochondrial DNA analysis

The amount of mtDNA in the control group did not differ from the 1st to the 15th passage (Figure 2, $P < 0.05$). In the treated group, the amount of mtDNA did not vary between the 1st, 2nd and 3rd passages ($P < 0.05$). However, at the 10th passage, there was a significantly higher concentration of mtDNA ($P = 0.010$), which was maintained until the 15th passage ($P = 0.011$). The amount of mtDNA at the 10th and 15th passages was higher ($P = 0.004$ and 0.054, respectively), when compared to the control.

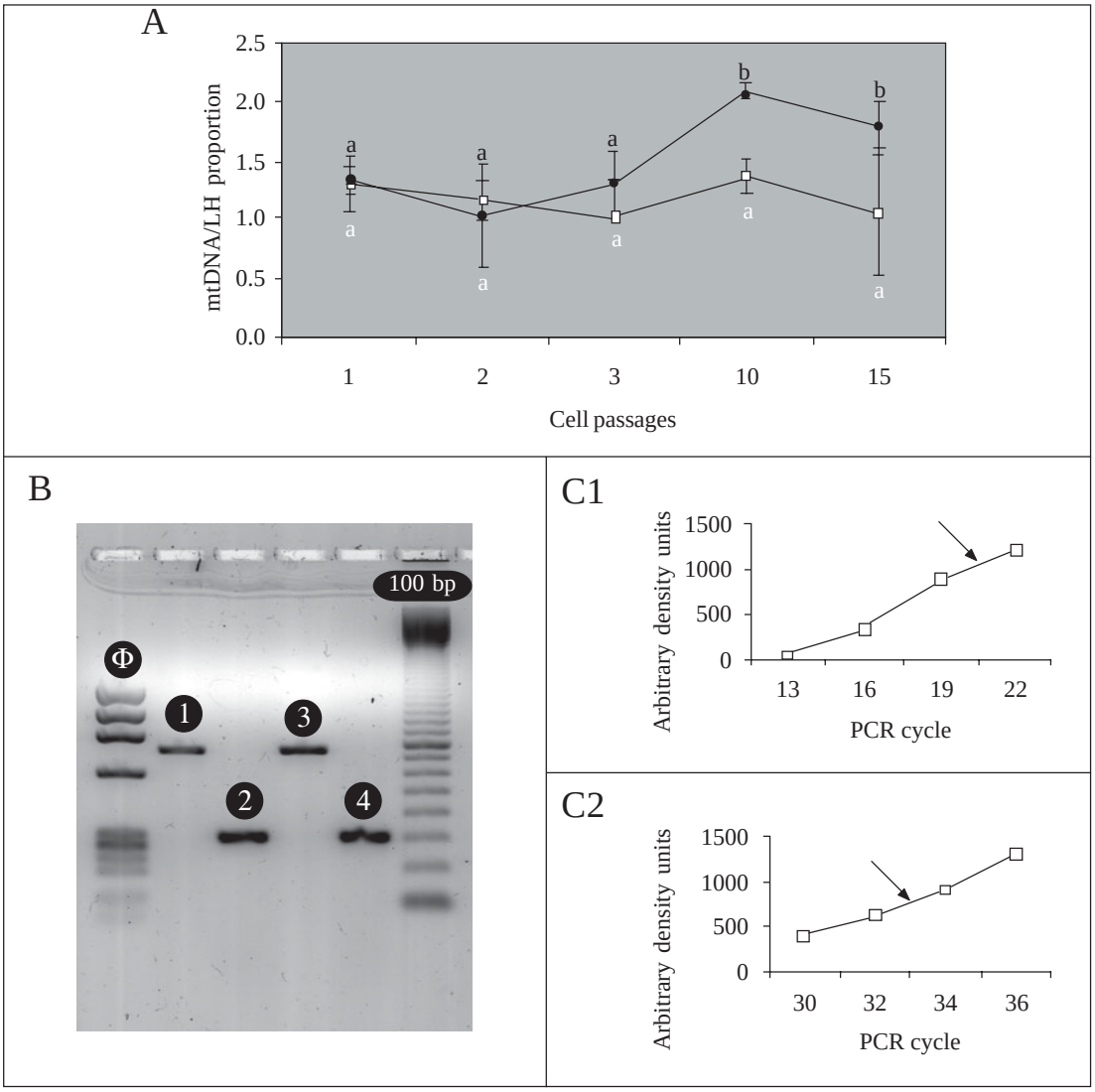

Figure 2. Effect of ethidium bromide (EtBr) on mtDNA quantities. The graph shows the ratio of mtDNA ND5 gene to nDNA LHr gene in EtBr-treated (circles, 1.34 ± 0.110, 1.02 ± 0.431, 1.30 ± 0.284, 2.09 ± 0.060 and 1.78 ± 0.225 to the 1st, 2nd, 3rd, 10th, and 15th passages, respectively, mean and SD) and control (squares, 1.29 ± 0.239, 1.16 ± 0.166, 1.01 ± 0.025, 1.36 ± 0.157, and 1.05 ± 0.544) cells at different passages in culture (A; items with different letters differ statistically; $P \leq 0.05$). EtBr-stained 1.5% agarose gel of ND5 mtDNA fragment and LHr gene amplification products in treated and control groups at the 10th passage (B; Φ-symbol depicts the Φx *HAE*III molecular marker, 1 and 2 are LHr and mtDNA ND5 amplification products in treated cells, respectively, and 3 and 4 are LHr and mtDNA ND5 amplification products in control cells, respectively. A 100-bp molecular marker is included. C1 and C2 show the PCR amplification curve determined for mtDNA and nDNA (arrows indicate the PCR cycle selected for analysis). mtDNA = mitochondrial DNA; LHr = luteinizing hormone receptor.

DISCUSSION

We detected a decrease in some parameters related to mitochondrial function in cells treated with EtBr. The decrease in $\Delta\Psi m$, together with the increase in lactate formation, sug-

gest OXPHOS impairment. These findings are in agreement with other reports (Dey and Moraes, 2000), suggesting a decline in mtDNA copy number or in organelle transcription rate. Although there was clearly a lower red/green fluorescence ratio in the treated group (Figure 1C), some cells failed to show a lower $\Delta\Psi m$ after EtBr treatment (Figure 1A, arrows). Furthermore, as described by Marchetti et al. (1996) and Buchet and Godinot (1998), some cells with ρ^0 status are able to maintain a normal $\Delta\Psi m$ independent of impaired OXPHOS.

Despite this apparent impairment in the OXPHOS in the treated group, the amount of mtDNA per cell did not decrease with time of EtBr treatment (Figure 2A). According to King and Attardi (1996), it is impossible to isolate ρ^0 derivates of all human cell lines using EtBr; they suggest that some cell lines are resistant to this treatment. These cells initially show a decrease in mtDNA amount when treated with EtBr, but they subsequently regain normal levels of mtDNA. We found that the mtDNA amount increases in treated cells compared with controls, and there was a typical appearance of fermentation, characterized by lower $\Delta\Psi m$ and higher levels of lactate production. It is possible that both conditions result from acquiring DNA polymerase γ resistance to the inhibitory effects of EtBr on mtDNA replication; however, RNA polymerase does not become resistant. Therefore, RNA polymerase fails to transcribe mtDNA, leading to a decline in $\Delta\Psi m$. Moreover, the finding of higher levels of lactate production corresponds to a cellular alternative for the generation of ATP by the glycolytic process. Poyton and McEwen (1996) demonstrated a retrograde communication from mitochondria to the nucleus in response to modifications in mitochondrial activity. In ρ^0 yeast and mouse myocytes with low mtDNA content and/or decreased $\Delta\Psi m$, transcription factors are translocated to the nucleus, allowing the expression of important proteins for the control of cellular homeostasis (Biswas et al., 1999); the disruption of the $\Delta\Psi m$ and the decrease in ATP synthesis lead to an increase in cytosolic Ca^{2+} concentration, which is likely to be responsible for the cellular signaling. Using electrical stimulation in skeletal muscle, Williams (1986) demonstrated that mtDNA, mtRNA and oxidative capacity increased, suggesting that mtDNA amount is somehow mediated by the expression of RNA transcripts of mitochondrial genes. However, the nuclear cytoplasm interaction may explain the increase in mtDNA content in treated groups, as nuclei may export a higher number of copies of DNA polymerase γ in response to lower $\Delta\Psi m$. Brown and Clayton (2002) used a nucleoside analogue, 2',3' dideoxycytidine, to treat mouse LA9 cells to reduce the number of mtDNA copies. They found a certain “relaxing” of the control of the mtDNA replication in response to this treatment, which supports the hypothesis of nuclear compensation.

Overall, we found that EtBr treatment affected bovine fibroblast mtDNA function. However, we were not able to establish a cell line without mtDNA. We suggest that the increased amount of mtDNA found in the treated group resulted from a feedback between mitochondria and nuclei as a possible response to a lower synthesis of ATP.

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