Placental Mitochondrial Toxicity, Oxidative Stress, Apoptosis, and Adverse Perinatal Outcomes in HIV Pregnancies Under Antiretroviral Treatment Containing Zidovudine

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Objective: To determine whether mitochondrial, oxidative, and apoptotic abnormalities in placenta derived from HIV and combined antiretroviral therapy (cART) containing zidovudine (AZT) could be associated with adverse perinatal outcome.

Design: Cross-sectional, controlled, observational study.

Methods: We studied obstetric results and mitochondrial, oxidative, and apoptotic state in placenta of 24 treated HIV-infected and 32 -uninfected pregnant women. We measured mitochondrial DNA (mtDNA) content by quantitative reverse transcriptase–polymerase chain reaction (mtND2/n18SrRNA), oxidative stress by the spectrophotometric quantification of lipid peroxidation and apoptosis by Western blot analysis of active caspase-3 respect to β-actin content and analysis of the terminal deoxynucleotidyl transferase dUTP nick end labeling.

Results: Global adverse perinatal outcome (defined as preterm delivery or/and small newborns for gestational age) was significantly increased in HIV pregnancies [or 6.7 (1.3–33.2); $P < 0.05$]. mtDNA content in HIV-infected women was significantly depleted (39.20% ± 2.78%) with respect to controls (0.59 ± 0.03 vs. 0.97 ± 0.07; $P < 0.001$). A significant 29.50% ± 9.14% increase in oxidative stress was found in placentas of HIV-infected women (23.23 ± 1.64 vs. 17.94 ± 1.03; $P < 0.01$). A trend toward 41.18% ± 29.41% increased apoptosis active caspase-3/β-actin was found in HIV patients (0.48 ± 0.10 vs. 0.34 ± 0.05; P = not significant), confirmed by transferase dUTP nick end labeling assay. Adverse perinatal outcome did not correlate mitochondrial, oxidative, or apoptotic findings.

Conclusions: Placentas of HIV-infected pregnant women under AZT cART showed evidence of mtDNA depletion, increased oxidative stress levels, and apoptosis suggestive of secondary mitochondrial failure, potential base of associated adverse perinatal outcome. Despite the fact that further demonstration of causality would need new approaches and bigger sample sizes, AZT-sparing cART should be considered in the context of pregnancy.

Key Words: apoptosis, cART, HIV, mitochondria, mitochondrial toxicity, mitochondrial function/dysfunction, perinatal outcome, placenta, pregnancy

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INTRODUCTION

In maternal HIV infection, the use of combined antiretroviral therapy (cART) has dramatically decreased perinatal transmission.[1–4] Nucleoside reverse transcriptase inhibitors (NRTIs) are the main components of cART and promote their therapeutic activity by competing with the natural nucleosides, inhibiting the reverse transcriptase of the virus but, as a secondary effect, blocking the mitochondrial DNA (mtDNA) replication that may lead to mitochondrial dysfunction[5,6] and adverse clinical manifestations including hyperlactatemia, lipodystrophy, or myopathy.[7–11] It is remarkably that not all the NRTI drugs have the same intensity or capacity to produce mitochondrial toxicity, as they present different affinities for DNA-polymerase-γ, the enzyme responsible of replicating and repairing mtDNA, as extensively described.[5,12] Indeed, a ranking of toxicities has been in vitro established as follows (from the most to the less harmful): 2′-3′-dideoxycytidine (ddC)>didanosine (ddI)> stavudine (d4T)>>Zidovudine (AZT)>lamivudine (3TC)>

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abacavir = tenofovir (TDF), being AZT among the most commonly prescribed NRTIs to prevent mother-to-child-transmission (MTCT) of HIV.

As mtDNA encodes for respiratory chain enzyme subunits, a defect in oxidative phosphorylation (OXPHOS) may result. There is now considerable evidence demonstrating that mitochondrial function is affected by HIV, cytokines, and/or cART, through a diverse array of mechanisms, including increases in reactive oxygen species (ROS) and apoptosis.[13–15]

Experimental studies have demonstrated that mitochondrial toxicity, evidenced by depletion in mtDNA and OXPHOS enzyme abnormalities, was manifested in placenta of AZT exposed monkey fetuses[16,17] or by decreased OXPHOS in heart and skeletal muscle from monkey's offspring.[18] Such mtDNA depletion, defects in OXPHOS, increased ROS, and consequent apoptosis have been also demonstrated in peripheral blood mononuclear cells of NRTI-treated HIV-infected adults,[19–21] infants,[22] and in HIV-pregnant women under cART including NRTIs or their in utero NRTI-exposed healthy newborns especially in case of classical antiretroviral as AZT.[23–25]

The placenta regulates fetal growth and development by transport of nutrients and gases and synthesis and secretion of steroid and peptide hormones. It is derived from the fetal genome and has a significant mitochondrial mass, which increases with gestational age. The placenta grows with extreme velocity to accommodate the increasing nutritional and oxygen needs of the fetus. Several authors have hypothesized a causal relationship between placental mitochondrial abnormalities and disorders of placental function, as occurs with intrauterine growth retardation (IUGR) and preeclampsia (PE); however, this remains unclear.[26–29] Growing epidemiological evidence supports that IUGR and PE may occur more frequently in HIV-infected women on cART.[23,30,31] However, the relationship between maternal HIV infection and disorders of placental or mitochondrial function has not been yet established.

NRTIs readily cross the placenta and their administration during pregnancy may also be associated with an increased fetal mitochondrial toxicity. In vitro studies have demonstrated ultrastructural degenerative changes in placental the villi of HIV-1–infected pregnant women under AZT zidovudine treatment[30] which may disturb placental function. These changes indicate that the cytopathic effect spreads out from peripheral syncytium to stromal zone suggesting that the damaged placental barrier does not have the best conditions for the transmission of gases, nutrients, and metabolites toward fetal circulation, being the potential cause of adverse obstetric outcomes. In addition, in vitro exposure of placenta to AZT zidovudine has been demonstrated to promote ROS, mitochondrial toxicity, and caspase-dependent cell death.[32] It is therefore conceivable that placenta, which is an accessible tissue, might be a potential indicator of NRTI-mediated mitochondrial toxicity in human pregnancies.

Some ex vivo studies have evaluated mtDNA content, mitochondrial function, or ROS in human placenta.[24,29,33,34] They showed controversial data regarding measured outcomes (including length or type of cART exposure), hampering comparison. Some of them showed evidence of mtDNA depletion and enzyme dysfunction in placenta of HIV-1–infected women exposed to AZT.[34] However, some other studies found similar mitochondrial content and oxidative stress in placentas of HIV-infected pregnancies with respect to controls.[24]

The objective of this study was to evaluate mtDNA content, oxidative stress, and apoptosis in placenta of HIV/cART exposed pregnant women compared with uninfected controls to evaluate the deleterious effect of current therapies (mainly based on AZT administration), as well as the aetiology, potential biomarkers, and putative therapeutic targets of adverse perinatal outcome associated to HIV pregnancies.

MATERIALS AND METHODS

We performed a single-site, cross-sectional, controlled, and observational study without intervention. Fifty-six pregnant women were prospectively and consecutively included in this study during their routine prenatal care at first trimester of gestation, in the Materno-Fetal Medicine Department of the Hospital Clinic of Barcelona in the period between June 2006 and December 2007; 24 asymptomatic HIV-1–infected pregnant women and 32 uninfected pregnant controls.

Controls and cases were matched by age and the inclusion criteria were as follows: age $\geq$18 years, single pregnancy, delivery after at least 22 weeks of gestation, and previous diagnosis of HIV infection before pregnancy (for cases) or absence of HIV infection (for controls). In all control patients, a negative HIV test during pregnancy (at first and third trimester) was documented. Exclusion criteria were familial history of mitochondrial disease.

All individuals were informed and signed written consent approved by the Ethical Committee of our hospital, following Declaration of Helsinki guidelines.

cART was administered to all pregnant HIV-infected women following the international guidelines recommendations. Therapeutic care of HIV women stratified them into different categories according to the use of antiretrovirals during pregnancy, being AZT the common backbone in all schedules.

Maternal epidemiological and obstetric parameters included information on maternal age, race, parity, and illicit substance abuse during pregnancy (obtained by self-report and confirmed by urine drug screen testing along pregnancy).

Immunovirological parameters for HIV-infected women throughout pregnancy consisted of registering potential hepatitis C virus coinfection, time from diagnosis of HIV infection to delivery, and measuring $CD4^+$ T-cell count (by flow cytometry) and plasmatic viral load (by HIV RNA copy quantification through Amplicor HIV Monitor; Roche Diagnostic Systems, Branchburg, NJ) at 32–36 weeks of gestation.

Information regarding obstetric and perinatal outcomes included the following: PE (new onset of hypertension of >140 mm Hg systolic or >90 mm Hg diastolic pressure and >300 mg proteins/24 h of urine), fetal death (>22 weeks of pregnancy), gestational age at delivery, mode of delivery, preterm delivery (PTD) (<37 weeks),

birth weight, newborn small for gestational age (SGA) (<10th percentile), 5-minute Apgar score <7, neonatal admission to intensive care unit, and global adverse perinatal outcome (PTD and/or SGA).

Mitochondrial Genetic, Oxidative, and Apoptotic Studies in Placenta

At the time of delivery, a placenta cotyledon was collected and frozen at −80°C. Mitochondrial genetic studies were performed from a piece of placental tissue and oxidative stress and apoptotic analysis in placental homogenates (5% wt/vol in mannitol buffer),[35] previous quantification of protein content by Bradford's assay.[36]

To evaluate mtDNA content, total DNA was obtained by standard phenol–chloroform extraction procedure. A fragment of the mitochondrial-encoded ND2 gene and the nuclear-encoded 18S rRNA gene were amplified in triplicate and separately by quantitative rtPCR using the Roche Lightcycler thermocycler.[10] The relative mtDNA content was expressed as the ratio between conserved mitochondrial ND2 gene with respect to the amount of the nuclear 18S rRNA (expressed as mtND2/n18SrRNA ratio).

Lipid peroxidation was measured as an indicative biomarker of ROS attack into lipid cell compounds by the spectrophotometric quantification of malondialdehyde (MDA) and 4-hydroxyalkenals (HAEs) normalized by protein content (μM MDA + HAE/mg prot).[37]

Apoptosis was measured by Western blot analysis of 20 μg of total cell protein by 7/13% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and posterior immune staining of active caspase-3 pro-apoptotic protein expression (cell signaling; 17–19 KDa) normalized by β-actin content (SIGMA; 47 KDa), further reacted with secondary antibodies against rabbit and mouse, respectively, and expressed in relative units as active caspase-3/β-actin.[38] The histological terminal deoxynucleotidyl transferase dUTP nick end labeling assay was used to detect nuclear DNA fragmentation[37] in frozen placentas cut in a slicing microtome at 7–8 μm and immunoreacted with the "In Situ Cell Death detection" (Roche 11 684 817 910) and expressed as the ratio of positive nuclei (red) per total 1000 nuclei (red and blue).[39]

Statistical Analysis

Clinical and epidemiological parameters were expressed as mean values and range interval and experimental results as mean values and standard error of the mean or as a percentage of increase/decrease with respect to healthy controls.

Differences between cases and controls and correlations among quantitative parameters were assessed using nonparametric tests: χ^2 test to calculate odds ratio (OR) values [OR; 95% confidence interval: significance], Mann–Whitney analysis to search for independent sample differences, and the Spearman rank coefficient for parameter correlation (R^2 and significance). SPSS Statistics 20 (IBM, Armonk, NY) was used for the statistical analysis. The level of significance was set at 0.05 for all the statistical tests.

RESULTS

The epidemiological, immunovirological, and therapeutic outcomes are summarized in Table 1. Most pregnant women were of white origin (87.5% of cases and 100% of controls), with an age ranging from 25 to 42 years.

Nonsignificant differences were observed in maternal epidemiological data between HIV-positive and HIV-negative women except for smoking status and primiparity. There were no cases of illicit maternal drug use during pregnancy. HIV/hepatitis C virus coinfected women (n = 3) had a history of drug use.

cART was given to all patients at the time of delivery to avoid MTCT and consisted, except for one case of AZT monotherapy, in a 2 NRTIs schedule plus either 1 protease inhibitor (PI), 1 non-NRTI, or a third NRTI (Table 1 for percentages of treatment assignment), always containing AZT. Most HIV women were under treatment before pregnancy (84%) and only 4 cases (16%) were naive for cART and started therapy at the second trimester of gestation. At delivery, all women had received at least 6 months of NRTI treatment. In addition, all infants were HIV uninfected and received 6-week AZT chemoprophylaxis to reduce perinatal transmission.

The obstetric and neonatal outcomes are summarized in Table 2. Results showed a trend toward increased PTD in HIV pregnancies with respect to controls [25% vs. 3%; OR 8 (0.9–70.9); P = not significant (NS)], lower birth weight children (2970 g vs. 3265 g; P = NS), and SGA [25% vs. 3%; OR 8 (0.9–70.9); P = NS]. Gestational age at delivery and neonatal intensive care unit admission trend to be increased among HIV pregnancies, although nonsignificantly. Global adverse perinatal outcome (including PTD or/and SGA), occurred significantly more frequently among HIV pregnancies with respect to controls [41.7% vs. 6.3%; OR 6.7 (1.3–33.2); $P < 0.05$].

Mitochondrial, Oxidative, and Apoptotic Analysis

Placental mtDNA content (Fig. 1) was significantly depleted (39.20% ± 2.78%; $P < 0.001$) in HIV-infected patients with respect to uninfected controls (0.59 ± 0.03 vs. 0.97 ± 0.07; $P < 0.001$).

We found a significant increase of 29.50% ± 9.14% ($P < 0.01$) in the levels of placental oxidative stress (Fig. 1) in HIV-infected pregnant women compared with noninfected controls (23.23 ± 1.64 vs. 17.94 ± 1.03; $P < 0.01$).

Placentas of HIV-infected pregnant women presented a marked trend toward increased apoptosis of 41.18% ± 29.41% (P = NS) through activation of caspase-3 compared with uninfected controls (0.48 ± 0.10 vs. 0.34 ± 0.05; P = NS) (Fig. 1). Transferase dUTP nick end labeling assay staining showed similar results.

Association Among Clinical and Experimental Parameters

A significant negative correlation was found between mtDNA levels and ROS. In addition, in HIV pregnancies,

TABLE 1. Clinical, Epidemiological, Immunovirological Characteristics and Therapeutic Schedules of HIV-Infected and -Uninfected Pregnant Women

	HIV Positive (n = 24)	HIV Negative (n = 32)	*P*	OR (95% CI)
Maternal age at delivery, yr*	35.0 (27–42)	33.6 (25–41)	NS	—
Race white	21 (87.5)	32 (100)	NS	—
Black-African	3 (12.5%)	0		
Primiparity, N (%)	8 (33.3)	21 (65.6)	<0.05	—
HCV infection, N (%)	3 (11.1)	1 (3.1)	NS	—
Tobacco use, N (%)	11 (45.8)	3 (9.4)	<0.05	—
Illicit drug use during pregnancy, N (%)	0	0	—	—
Alcohol use during pregnancy, N (%)	0	0	—	—
Time from diagnosis of HIV infection to delivery, mo*	104 (4–228)	NA	—	—
Viral load at delivery (RNA copies per mL)*	67.4 (49–250)	NA	—	—
$CD4^+$ lymphocyte count at delivery (cell count per mL at delivery)*	580.1 (211–1242)	NA	—	—
NRTI before pregnancy (mo)*	55.2 (0–106)	NA	—	—
Antiretroviral therapy		NA	—	—
Triple NRTI (n) (ABC + AZT + 3 TC)	1			
2 NRTIs + 1 PI (n) (AZT + 3 TC + NFV)	7			
2 NRTIs + 2 PI (n) (AZT + 3 TC + RTV + SQV)	7			
2 NRTIs + 1 non-NRTI (n) (AZT + 3 TC + NVP)	8			
AZT monotherapy, n	1			

*Data are presented as means and range.
3 TC, lamivudine; 95% CI, 95% confidence of the mean; ABC, abacavir; AZT; zidovudine; HCV, hepatitis C virus; N, number; NA, not available; NVF, nelfinavir; NVP, nevirapine; PI, protease inhibitor; RTV, ritonavir; SQV, saquinavir; tNRTI, nucleotide-analog reverse transcriptase inhibitor; ZDV, zidovidine.

ROS significantly and positively correlated with apoptosis (Fig. 2).

No correlations were found between mitochondrial, oxidative, and apoptotic placental experimental parameters and immunological status or cART during pregnancy.

Nonsignificant differences were observed in maternal epidemiological data between HIV-positive and HIV-negative women, according to global adverse perinatal outcome.

Similarly, no correlation was observed between experimental measures and adverse perinatal outcome in any cohort. However, global adverse perinatal outcome (PTD or/and SGA) significantly increased in HIV pregnancies, and these patients showed significantly decreased placental mtDNA content and increased oxidative stress, with strong trends toward increased rate of apoptosis development.

DISCUSSION

Not all antiretrovirals have an equal risk of mitochondrial toxicity. NRTIs have substantially decreased the risk of MTCT of HIV. However, concern has been raised about its perinatal safety because of evidence of mitochondrial toxicity demonstrated in vitro, in animal models, in newborns in utero exposed to NRTI, or in HIV-infected infants and adults on cART therapy,[16–26] especially classical NRTIs as AZT. Non-NRTI have not been associated with mtDNA alterations, although they have been controversially related to mitochondrial apoptosis, leading to mild mitochondrial secondary toxic effects. The secondary effects derived from the administration of PI are due to the alterations in glucose and lipid metabolism, which often lead to an energetic failure that can derive in apoptosis.[40,41] HIV infection, by its own, has also been documented to cause mitochondrial damage by

TABLE 2. Obstetric and Neonatal Outcomes of the Study Cohorts

	HIV Positive (n = 24)	HIV Negative (n = 32)	*P*	OR (95% CI)
Gestational age at delivery, wk*	38 (31–41.2)	39.4 (34.4–42)	NS	—
Preterm birth (<37 wk of gestation), N (%)	6 (25)	1 (3)	—	8 (0.9 to 70.9)
Birth weight, g*	2970 (1250–4040)	3265 (2380–4600)	NS	—
SGA (<10th percentile), N (%)	6 (25)	1 (3)	—	8 (0.9 to 70.9)
5-minute Apgar score <7, N (%)	1 (2.4)	0	—	4.1 (0.16 to 106)
Neonatal admission to intensive care unit, N (%)	3 (12.5)	1 (3.1)	—	4.42 (0.43 to 45)
Global adverse perinatal outcome (PTD and/or SGA newborn), N (%)	10 (41.7)	2 (6.3)	—	6.7 (1.3 to 33.2)

*Data are presented as means and range interval.
95% CI, 95% confidence interval of the mean; No, number; NS, not significant.

promoting apoptosis.[4] Fortunately, severe cases of mitochondrial toxicity during pregnancy are very rare, but milder forms and long-term effects in newborns are more frequent.

Despite some studies report preserved obstetric outcome in HIV pregnancies,[42,43] PTD and low birth weight have been described as major contributors to neonatal morbidity and mortality in HIV-pregnant women. Many studies support an association between cART and prematurity,[44–49] especially because of PIs,[50–57] while others do not.[42,44,58–60] In this study, 29% of the cohort of HIV patients were taking PIs and showed significantly increased risk of PTD and SGA. Interestingly, all HIV cohorts were under AZT administration, commonly associated with obstetric complications and one of the most widely administrated NRTIs with well-known mitochondrial toxicity. The present findings confirm the increased frequency of adverse obstetric events reported for this population and validate the source of sample.

To our knowledge, this is the first study reporting simultaneously mtDNA depletion, ROS, and apoptotic disarrangements in placenta of HIV-infected women receiving NRTIs during pregnancy, showing poor obstetric outcome. In terms of mtDNA depletion, our study confirms an effect in mtDNA content in placenta from the HIV cohort, as described by the significant decrease of the mitochondrial genome copies. In addition, the present findings show cell damage beyond mitochondria leading placental cells toward significantly enhanced ROS and trends toward increased apoptosis.

Independent of HIV or cART, thus, in physiologic conditions, pregnancy is associated with placental oxidative stress arising from increased mitochondrial activity and well-orchestrated process of tissue remodeling involving apoptosis. The placenta also produces other ROS including nitric oxide, carbon monoxide, and peroxynitrite which have pronounced effects on placental function including trophoblast proliferation and differentiation and vascular reactivity. Excessive production of ROS in association with increased trophoblast apoptosis may occur at certain windows in placental development and in pathologic pregnancies, such as those complicated by PE and/or IUGR, overpowering antioxidant defenses with deleterious effects.[26,29,61,62] It is therefore conceivable that the placental mtDNA depletion, ROS, and apoptosis herein demonstrated in HIV pregnancies under NRTIs may be the etiological base of the detrimental obstetric events observed in these pregnancies.[21,28,29]

In addition, the observed trend toward negative correlation found between mtDNA levels and ROS, accompanied by a significant positive correlation between ROS and apoptosis, suggests a potential mechanism of mitochondrial

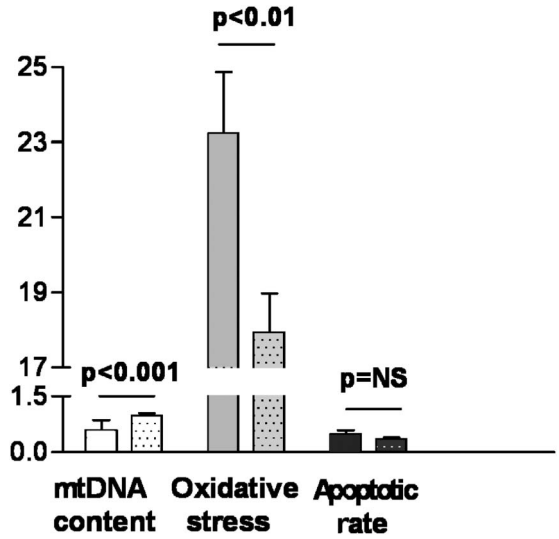

FIGURE 1. mtDNA content (mtDNA; white bars; mtND2/n18SrRNA units), oxidative stress (gray bars; μM MDA + HAE/mg prot units), and apoptotic rate (black bars; active caspase-3/β-actin units) in placenta of HIV-infected and cART-treated pregnant women (solid bars) compared with uninfected controls (dotted bars).

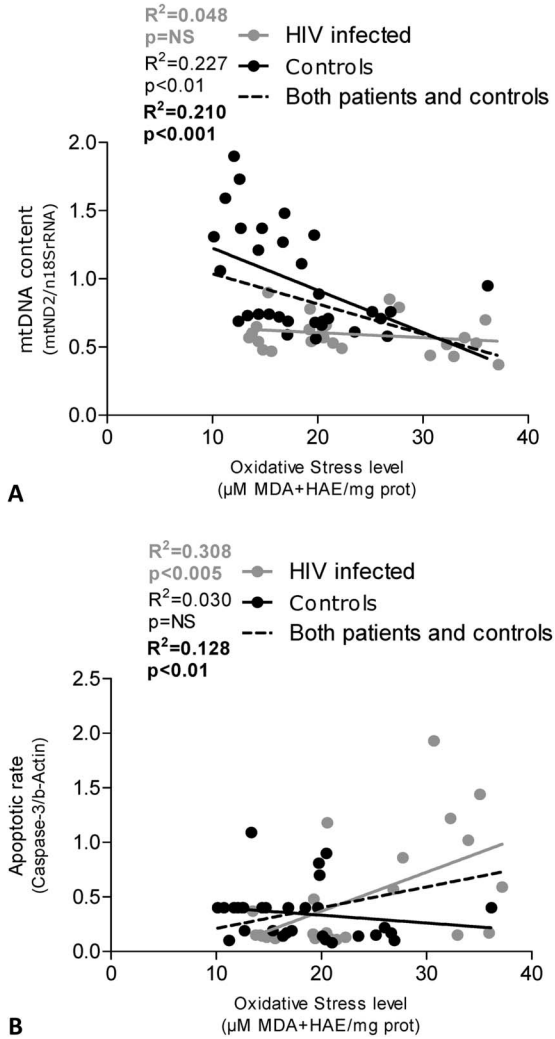

FIGURE 2. Association between levels of placental oxidative stress and mtDNA content (A) or apoptotic rate; (B) parameters are shown in the graphs for HIV patients (gray line and significance), uninfected controls (black line and significance) or for both patients and controls (discontinuous line and bold significance).

toxicity in HIV pregnancies that may be first triggered by HIV- and NRTI-mediated mtDNA depletion and could lead ultimately to increased ROS and mitochondrial failure as the potential cause of apoptosis development. Further demonstration of such causality would need novel studies and future approaches.

Whether these mitochondrial, oxidative, and apoptotic disarrangements rely on the basis of poor obstetric outcome in these pregnancies is still a matter of doubt. Our limited sample size and the intrinsic heterogeneity of each patient in this population studies hampered the statistical finding of a causal effect between molecular damage and obstetric complications. The small sample size of the studies makes it difficult to link mitochondrial toxicity or the development of apoptosis with adverse pregnancy outcomes because of the reduced statistical power when classifying the HIV cohort according to successful pregnancy results.

These findings have implications for the safe administration of NRTIs in pregnancy regarding the maintenance of integrity of the maternal–fetal barrier, at least when administering classical NRTIs as AZT. NRTIs cross the placenta leaving damaging effects behind and, consequently, placental examination may be useful to screen for neonatal mitochondrial, oxidative, and apoptotic toxicity. Further studies should consider investigating the effect of alternative AZT-sparing cART in the context of pregnancy as, despite its confirmed toxic effects, it is still the cornerstone in the cART backbone to prevent MTCT.

Some limitations have to be acknowledged in this study such as the potentially heterogeneous baseline characteristics in the study populations. The rate of smoking habit was significantly higher in the HIV cohort, and the rate of primiparity was significantly higher in the uninfected-pregnant women. However, no differences in experimental parameters were observed in the smoking or primiparous pregnant women when they were stratified by cohorts. In addition, the study design does not allow distinguishing whether molecular alterations may be attributed to maternal HIV-1 infection or to cART because all the cohorts of HIV-infected women were on cART to prevent fetal infection. Epidemiologic, clinical, immunovirological, and therapeutic characteristics at baseline in HIV women were similar, regardless further development of adverse perinatal outcome. However, mitochondrial measures in placental tissue were not performed until delivery and, thus, at baseline, any potential mitochondrial imbalance in both cohorts (or in controls) cannot be discarded. Finally, potential bias of socioeconomics or environmental events can neither be discarded, as they were not measured.

Further studies in larger cohorts and at long-term follow-up are needed to better understand morbidity associated with mitochondrial toxicity, oxidative damage, or apoptosis. The health care provider must be aware of these potentially serious events associated with NRTIs administration. Information about potential synergistic or additive toxicity in NRTIs combinations is scarce. More studies are required to find an individual pattern of treatment for each patient, by maintaining the efficacy to prevent vertical infection and minimizing the side effects of cART, especially when considering antiretrovirals as AZT, with demonstrated toxicity, that should be minimized in the context of human pregnancies.

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