Mitochondrial Evolution in HIV-Infected Children Receiving First- or Second-Generation Nucleoside Analogues

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Background: Highly active antiretroviral therapy (HAART) and HIV-related mitochondrial toxicity lead to several adverse effects and have become a major issue, especially in children. The main goal in the treatment of HIV-infected children is to maximize cost-effectiveness while minimizing toxicity. We aimed to study the evolution of mitochondrial parameters over time in children receiving different types antiretroviral regimens.

Methods: We followed-up 28 HIV-infected children receiving HAART including either first-generation nucleoside reverse transcriptase inhibitors (1gNRTIs; didanosine, zidovudine, or stavudine; n = 15) or second-generation NRTIs (2gNRTIs; the remaining drugs; n = 13) for a period of 2 years for their immunovirological and mitochondrial status, and compared these subjects with a group of untreated HIV-infected patients (n = 10) and uninfected controls (n = 27). We measured T-lymphocyte CD4+ content (flow cytometry), viral load (real-time polymerase chain reaction), and lactate levels (spectrophotometry); we assessed mtDNA content (real-time polymerase chain reaction), mitochondrial protein levels (Western blot), oxidative stress, mitochondrial mass, and electron transport chain function (spectrophotometry) in peripheral blood mononuclear cells.

Results: At the second time point, lactate levels were significantly higher in children on 1gNRTIs compared with those receiving 2gNRTIs (1.28 ± 0.08 vs. 1.00 ± 0.07 mmol/L, respectively; P = 0.022). MtDNA content was similar among all HIV-infected groups and significantly lower than in healthy controls at baseline. Oxidative stress tended to increase over time in all the groups, with no differences among them. However, a significant decrease in cytochrome c oxidase activity was found over time in HIV-infected patients; this decline was greater in the 1gNRTI group.

Conclusions: HIV infection and the use of 1gNRTIs caused greater mitochondrial damage than 2gNRTIs over time. The higher lactate levels and the significant decrease observed in cytochrome c oxidase activity argue against the use of 1gNRTIs in HIV-infected children when an alternative is available, in accordance with international recommendations.

Key Words: children, first- and second-generation antiretrovirals, HIV, mitochondrial toxicity, nucleoside reverse transcriptase inhibitors, therapeutic strategies

(INT J Acquir Immune Defic Syndr 2012;60:111–116)

INTRODUCTION

Highly active antiretroviral (ARV) therapy (HAART) reduces the mortality and morbidity of HIV infection and AIDS in both adults and children. Nonetheless, HAART may lead to adverse events, which have become a major issue, especially in HIV-infected children. It has been proposed that many of these adverse events have a mitochondrial basis. Currently, the main goal in the treatment of HIV infection is to reduce the risk of virological failure while maximizing cost-effectiveness and minimizing toxicity. Although a wide range of new drugs is available, few data have been reported on mitochondrial toxicity in children, and thus, further investigations are needed.

It has been previously reported that HIV is responsible for mtDNA depletion in adults and subsequently, it was shown that this effect was reflected in mitochondrial dysfunction.

Many studies have looked at how nucleoside reverse transcriptase inhibitors (NRTIs) trigger mitochondrial impairment through the inhibition of the gamma-polymerase enzyme, causing mtDNA depletion, which, in turn, may lead to mitochondrial failure. There has been less examination of the effects in perinatally HIV-infected pediatric patients, for whom this issue has special relevance because they constitute the first generation that will receive ARV treatment throughout their lives.

As in adult patients, HAART-related mitochondrial effects in children have been reported by our group. Rosso et al studied, for the first time, the mitochondrial effects observed after switching from mitochondrial-toxic drugs to less toxic compounds in children over a period of 18 months, finding no significant changes in mtDNA content. In fact, the mitochondrial toxicity of NRTIs may be different depending on the specific drug; accordingly, a ranking of toxicities in vitro has been described in the literature, from the most toxic to the least toxic.
didanosine (ddI) slightly modifies lamivudine (3TC)/emtricitabine (FTC) to abacavir (ABC) = tenofovir (TDF).\(^9\)\(^{10}\) The use of zalcitabine (no longer administered) and ddI has been related to pancreatitis.\(^11\)\(^{12}\) d4T has been associated with lipoatrophy.\(^9\)\(^{13}\)\(^{14}\) hyperlactatemia, and lactic acidosis;\(^15\) and ZDV has been linked to myopathy.\(^16\) In addition, 3TC/FTC and ABC present a lower affinity for gamma-polymerase,\(^5\)\(^7\) and are thus considered to be the least mitochondrial-toxic compounds, together with TDF.

We aimed to study the evolution of mitochondrial parameters along time in a series of pediatric patients receiving different types of ARV therapy, and we hypothesized that children receiving a HAART regimen based on highly mitochondrial-toxic NRTIs [first-generation NRTIs (1gNRTIs): ddI, ZDV, and d4T] develop more mitochondrial toxicity than those on an ARV combination that does not include these, but rather second-generation NRTIs (2gNRTIs): ABC, 3TC/FTC, and TDF. The results in these patients were compared with 2 control groups of children, one consisting of HIV-infected but untreated patients, in whom HIV is the only detrimental agent for mitochondria, and one group of uninfected healthy controls providing reference values of normality.

The main objective of this study was to provide more information related to the evolution of mitochondrial markers in HIV-infected pediatric patients undergoing different ARV regimens and to elucidate whether a given ARV therapy is safer than others from the mitochondrial point of view while also remaining effective.

**METHODS**

A longitudinal study was conducted over 2 years. The immunovirological and mitochondrial status of 28 vertically HIV-infected children (64% girls, median age ± SEM at baseline, 11.1 ± 0.7 years) on HAART that either included 1gNRTIs (n = 15) or 2gNRTIs (n = 13) was assessed and compared with the status of an untreated group of HIV-infected children (no ARV group; n = 10) and with the values of normality in a group of healthy uninfected children (n = 27). The HAART regimens of the 1gNRTIs and 2gNRTIs groups were maintained during the study period. Informed consent to participate in the study was obtained from parents or legal guardians, and approval from the local ethics committee was given.

T-lymphocyte CD4+ content was assessed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA). HIV RNA viral load was quantified by quantitative real-time polymerase chain reaction (CA HIV Monitor; Roche, Basel, Switzerland; limit <50 copies/mL) and plasma lactate levels (a surrogate biomarker of mitochondrial lesion, millimoles per liter) were measured with a spectrophotometric procedure (Cobas Fara II Analyzer; Roche).

Peripheral blood mononuclear cells were isolated from 5–10 mL of venous blood with a Ficoll gradient (Histopaque 1077; Sigma Diagnostics, St Louis, MO)\(^18\) for mitochondrial studies.

Mitochondrial mass was estimated by the measurement of citrate synthase (CS) enzymatic activity with spectrophotometry at 412 nm (Hitachi 2900; Hitachi Instruments Inc, San Jose, CA), as previously reported.\(^19\) Results were expressed as nanomoles of reduced substrate per minute and per milligram of cell protein (nanomoles per minute per milligram protein).

We assessed mtDNA content by quantitative real-time polymerase chain reaction (LightCycler FastStart DNA Master SYBR Green I; Roche Molecular Biochemical, Mannheim, Germany) through the amplification of a fragment of the highly conserved mitochondrial gene ND2 and a sequence within the housekeeping 18SrRNA nuclear gene, as reported elsewhere.\(^19\)\(^{21}\) The results of mtDNA content were expressed with respect to nDNA content as the ratio of ND2/18SrRNA and normalized by CS.

Mitochondrial protein levels of mtDNA-encoded subunit II (COXII) and nDNA-encoded subunit IV (COXIV) of cytochrome c oxidase or complex IV (CIV) were quantified by Western blot, using the porin voltage-dependent anion carrier protein as a marker of mitochondrial protein loading and β-actin as a marker of overall cell protein loading, as previously reported.\(^22\)

Oxidative stress was assessed by lipid peroxidation analysis with the measurement of malondialdehyde and hydroxyalkenals content at 586 nm,\(^23\)\(^24\) and the results were normalized per total protein content (micromolar malondialdehyde and hydroxyalkenals per milligram protein).

Measurement of the enzymatic activity (also in nanomoles per minute per milligram of protein) of the mitochondrial respiratory chain (MRC) complexes was performed by spectrophotometry (Hitachi 2900; Hitachi Instruments Inc), and the results were referred to overall cell protein and normalized by mitochondrial mass, estimated by CS activity. Because isolated complex I and V activities cannot be measured in whole cells due to the absence of activation of the former with decylubiquinone and due to an oligomycin-insensitive ATPase activity of the latter,\(^25\) we determined the following: CIV activity at 550 nm according to Rustin et al,\(^24\) slightly modified for minute amounts of biological samples\(^19\)\(^{25}\), complex II–III at 550 nm; glycerol-3-phosphate dehydrogenase–complex III (G3PDH–CIII) at 550 nm; complex II at 600 nm; and isolated G3PDH at 600 nm.

Statistical analysis was carried out with the SPSS 18.0 program. The results were expressed as mean ± SEM. Normality of values was confirmed with the Kolmogorov–Smirnov test. For cross-sectional analysis, the Mann–Whitney test was carried out, and for longitudinal analysis, the Wilcoxon test was used.

**RESULTS**

The clinical, immunovirological, and mitochondrial characteristics of the patients and controls at baseline are shown in Table 1. All patients on HAART showed significantly lower plasma viral loads (P < 0.001) at both time points when compared with children not receiving therapy; CD4+ T-cell percentages remained within normal limits in all HIV-infected patients but were higher in treated patients. The evolution of immunovirological parameters (with respect to baseline) of the patients included in the study is shown in Figure 1.

Lactate levels, which constitute a plasma biomarker of mitochondrial dysfunction, were similar in all groups at baseline (Table 1) but were higher in the 1gNRTIs group with respect to HIV-infected untreated controls (1.28 ± 0.08 vs. 0.85 ± 0.08 mmol/L; P < 0.001) and also with respect to the
TABLE 1. Clinical, Immunovirological, and Mitochondrial Data of Patients and Controls at Baseline

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>1gNRTIs</th>
<th>2gNRTIs</th>
<th>Untreated</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. subjects (n)</td>
<td>15</td>
<td>13</td>
<td>10</td>
<td>27</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>9.53 ± 1.39</td>
<td>13.13 ± 0.91</td>
<td>13.20 ± 1.44</td>
<td>9.96 ± 0.82*</td>
</tr>
<tr>
<td>Sex (girls, %)</td>
<td>53</td>
<td>77</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>Time on HAART (yr)</td>
<td>9.53 ± 1.39</td>
<td>13.13 ± 0.91</td>
<td>13.20 ± 1.44</td>
<td>—</td>
</tr>
</tbody>
</table>

Immunovirological data

CD4+ T-cell percentages | 32.76 ± 2.51 | 33.33 ± 2.73 | 26.00 ± 1.78† | — |
Viral load (log HIV RNA, copies/mL) | 0.84 ± 0.39 | 0.81 ± 0.8 | 4.46 ± 0.24‡ | — |

Mitochondrial data

Lactate levels (mmol/L) | 1.05 ± 0.13 | 1.09 ± 0.13 | 0.93 ± 0.1 | — |
mtDNA/nDNA (ND2/18SrRNA) | 4.25 ± 0.61 | 3.49 ± 0.61 | 3.26 ± 0.90 | 5.82 ± 0.48§ |
mtdNA/CS (ND2/18SrRNA) | 0.04 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.05 ± 0.01§ |
COXII/β-actin | 0.038 ± 0.006 | 0.065 ± 0.018 | 0.07 ± 0.029 | 2.90 ± 0.80| |
COXIV/β-actin | 0.14 ± 0.03 | 0.26 ± 0.08 | 0.34 ± 0.16 | 2.29 ± 0.533|| |
COXII/CS | 0.0003 ± 0.00008 | 0.0006 ± 0.00018 | 0.0006 ± 0.00025 | 0.03 ± 0.012|| |
COXIV/CS | 0.0013 ± 0.00038 | 0.0026 ± 0.00084 | 0.003 ± 0.0014 | 0.0234 ± 0.006|| |
COXII/COXIV subunits | 0.36 ± 0.06 | 0.39 ± 0.08 | 0.23 ± 0.03 | 1.08 ± 0.08|| |
MDA and HAE (µM/mg protein) | 0.57 ± 0.14 | 0.57 ± 0.05 | 0.43 ± 0.07 | 0.59 ± 0.09 |
CS (nmol/min/mg protein) | 127.87 ± 8.43 | 118.77 ± 8.64 | 107.33 ± 11.47 | 118.20 ± 6.10 |
CII/CS | 0.15 ± 0.02 | 0.15 ± 0.02 | 0.25 ± 0.07 | 0.21 ± 0.02 |
CII–III/CS | 0.13 ± 0.02 | 0.15 ± 0.02 | 0.15 ± 0.03 | 0.15 ± 0.02 |
G3PDH/CS | 0.33 ± 0.04 | 0.32 ± 0.04 | 0.35 ± 0.46 | 0.43 ± 0.03 |
G3PDH–CII/CS | 0.14 ± 0.01 | 0.16 ± 0.02 | 0.14 ± 0.019 | 0.15 ± 0.01 |
CIV/CS | 0.51 ± 0.04 | 0.51 ± 0.05 | 0.51 ± 0.06 | 0.45 ± 0.03 |

Data are mean ± SEM, except when stated otherwise.
*Significant differences in age of the uninfected controls with respect to the untreated group (P < 0.05).
†Significant differences in CD4+ T-cell percentages in untreated group compared with the 2gNRTIs group (26.00 ± 1.78 and 33.33 ± 2.73, respectively; P < 0.05).
‡Viral load (HIV RNA copies per milliliter) in the untreated group compared with the 2gNRTIs and 1gNRTIs groups (4.46 ± 0.24, 0.81 ± 0.38, and 0.84 ± 0.39, respectively; P < 0.001 for both).
§Significantly depleted in all the groups of HIV-infected children (2gNRTIs, 1gNRTIs, and untreated groups) with respect to the uninfected controls (P < 0.005 for all).
||Significant decrease in mitochondrial protein levels of COXII and COXIV compared with the overall cell protein (β-actin) or mitochondrial mass (CS) and in the ratio of COXII/COXIV in all groups of HIV-infected patients (2gNRTIs, 1gNRTIs, and untreated groups) with respect to the uninfected controls (P < 0.001 in all cases).

Mitochondrial protein levels of subunits COXII and COXIV were decreased in all HIV-infected patients (2gNRTIs, 1gNRTIs, and untreated groups) with respect to the uninfected controls (P < 0.001). Furthermore, the ratio of COXII and COXIV remained unaltered along time (Fig. 2B). An example of an immunoblot is provided in Figure 3.

The oxidative stress, estimated by lipid peroxidation measurement, tended to increase in all groups of HIV-infected children over time, although this change was not statistically significant (Fig. 2C). Mitochondrial mass was similar at baseline and after 2 years in all groups (Fig. 4).

Enzymatic activities of MRC were similar referred to overall cell protein and to mitochondrial mass. At baseline, MRC enzymatic activities (nanomoles per minute per milligram protein) relativized by CS (nanomoles per minute per milligram protein) were similar for all HIV-infected groups. However, at the second time point, after 2 years, G3PDH–CII/CS activity was significantly lower in the untreated group with respect to the 2gNRTIs and 1gNRTIs groups (0.064 ± 0.01, 0.13 ± 0.02, and 0.14 ± 0.02, respectively; P = 0.012 and P = 0.015; Fig. 4).

Mitochondrial function was preserved over time except for CIV/CS activity, which significantly decreased in the untreated patients and in the 1gNRTIs group (0.256 ± 0.023 and 0.292 ± 0.036, respectively) with respect to baseline (0.519 ± 0.06 and 0.512 ± 0.036; P = 0.015 and P = 0.006, respectively; Fig. 4). Absolute CIV enzymatic activity per overall cell
protein was significantly decreased in all HIV-infected groups with respect to baseline and with respect to the uninfected controls. At baseline, results on absolute CIV enzymatic activity were as follows: 52.20 ± 5.62 for the untreated patient group, 57.58 ± 5.64 for the 2gNRTIs group, and 65.06 ± 5.84 for the 1gNRTIs group. At the second time point, CIV enzymatic activity was significantly decreased by 26.58% in the untreated patient group (38.33 ± 13.29), by 33.54% in the 2gNRTIs group (38.27 ± 4.93), and by 48.31% in the 1gNRTIs group (33.60 ± 4.48; P < 0.05 for all).

**DISCUSSION**

Although a wide range of new drugs is available for the treatment of HIV infection and AIDS, there are scarce data on mitochondrial toxicity in children. We studied the evolution of mitochondrial parameters in a series of pediatric patients undergoing different ARV schedules to provide more information about these mitochondrial markers over time in HIV-infected children and to elucidate whether a given ARV therapy is safer than others from the mitochondrial point of view, while also remaining effective. The fact that after 2 years of treatment, lactate levels significantly increased in the 1gNRTIs group with respect to the untreated group and to the 2gNRTIs group is an indicator of a mitochondrial alteration in the former.

MtDNA content was decreased in all HIV-infected groups with respect to the controls at baseline. Thus, HIV-induced and HAART-induced mtDNA depletion was present in the untreated and treated groups, respectively. There was a slight, albeit nonsignificant, increase in this parameter over time, and after 2 years, mtDNA content remained significantly lower with respect to the control reference values in the untreated group. Nevertheless, in patients treated with either 1gNRTIs or 2gNRTIs, no significant mtDNA depletion was detected with respect to control values as described by Rosso et al. Indeed, HIV infection-related phenomena, regardless of treatment, can cause mtDNA depletion. At the second time point in our series, we found that HIV was more harmful than HAART regarding mtDNA depletion. Many proteins encoded by HIV genome are apoptogenic, such as Env, gp120, gp41, Vpr, Nef, or Tat. Most of these induce mitochondrial apoptosis through the depolarization of the mitochondrial membrane.

**FIGURE 1.** Immunovirological parameters. Differences between groups of HIV-infected patients in CD4+ T-cell percentages and HIV plasma viral load (HIV RNA copies per milliliter). The x axis represents the baseline of the 3 HIV-infected groups and the columns represent the increase or decrease of the parameters with respect to baseline. Bars represent mean ± SEM. *Viral load (log HIV RNA copies per milliliter) significantly decreased in the 2gNRTIs group compared with baseline (from 0.81 ± 0.38 to 0.33 ± 0.22; P < 0.05). †Despite a decrease of almost 1 log at the second time point, the viral load (log HIV RNA copies per milliliter) was still higher in the untreated group when compared with the 2gNRTIs and 1gNRTIs groups (3.81 ± 0.51, 0.33 ± 0.22, and 0.94 ± 0.45, respectively; P < 0.05 for both). ‡At the second time point, CD4+ T cells (%) were significantly lower in the untreated group compared with the 2gNRTIs and 1gNRTIs groups (25.70 ± 1.70, 35.53 ± 2.35, and 33.53 ± 3.13, respectively; P < 0.05 for both).

**FIGURE 2.** A, Lactate levels (millimoles per liter). The x axis represents the baseline of the 3 HIV-infected groups and the columns represent the increase or decrease of the parameters with respect to baseline. Bars represent mean ± SEM. *After 2 years, at the second time point, lactate levels were significantly higher in the 1gNRTIs group with respect to the untreated group and the 2gNRTIs group (1.28 ± 0.083, 0.85 ± 0.081, and 1.00 ± 0.071, respectively; P < 0.05 for both). B, MtDNA content and mitochondrial protein levels over time in the 3 groups of HIV-infected children. C, Evolution of oxidative stress measurements over time in the 3 groups of HIV-infected children. HAE, hydroxyalkenals; MDA, malondialdehyde.
The apoptotic process, derived from HIV infection, is mainly associated with mitochondrial abnormalities, such as mtDNA depletion, mitochondrial dysfunction, and increase of oxidative stress. Of note, there has recently been shown to be an increase in plasma mtDNA released from damaged or dead cells, which, in turn, may explain an inflammatory response in the organism.

As expected, after the mtDNA depletion observed in untreated HIV-infected children at the second time point, HIV infection irrespective of ARV drugs also damaged G3PDH–CIII/CS enzymatic activity, which was significantly lower in the untreated group compared with both treated groups. Along this line, in the literature it has been described how HIV triggers mitochondrial impairment, not only at a genetic level but also at a functional level in adults. In contrast, this enzymatic activity was not compromised in the groups treated with NRTIs, suggesting that these ARVs do not alter MRC function at this point.

All the enzymatic activities were preserved over time, except for cytochrome c oxidase activity. CIV/CS activity significantly dropped in the untreated and 1gNRTIs groups with respect to baseline, suggesting that HIV infection and 1gNRTIs, respectively, triggered mitochondrial dysfunction of cytochrome c oxidase activity. It is remarkable that this alteration was not found in the group receiving 2gNRTIs in which almost normal CIV/CS activity (75%) was preserved in comparison with healthy controls. Absolute CIV enzymatic activity dropped in all HIV-infected groups over time; this decline was greater (a half percent) in the 1gNRTIs group. These results support the idea that a therapy including 2gNRTIs, other than ddI, ZDV, or d4T, could preserve the mitochondria from significant alterations in the functionality of MRC over time.

Some limitations of our study are the sample size and the lack of a longitudinal assessment of the healthy controls, due in both cases to the complexity of the recruitment of such samples in the pediatric age. Furthermore, there is a lack of a direct clinical repercussion, although it is possible that clinical manifestations might arise in the future.

In conclusion, our findings support the contention that HIV infection and the use of 1gNRTIs cause higher mitochondrial damage than the use of 2gNRTIs over time in perinatally HIV-infected children. Current recommendations strongly encourage the early start of HAART in these children in the first year of life, regardless of their clinical or immunologic status. Likewise, HAART changes are often required in pediatric patients, usually because of toxicity or resistance. According to our results, the use of 1gNRTIs should only be considered in the HIV-infected child when 2gNRTIs are no longer an option for the patient.
ACKNOWLEDGMENT
The authors are indebted to Mireia Nicolás for her technical laboratory support.

REFERENCES