Mitochondrial dysfunction and human immunodeficiency virus infection

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Abstract
Human immunodeficiency virus (HIV) infection and the pharmacological treatment thereof have both been shown to affect mitochondrial function in a number of tissues, and each may cause specific organ pathology through specific mitochondrial pathways. HIV has been shown to kill various tissue cells by activation of mitochondrial apoptosis. Nucleoside analogues, used extensively to treat HIV infection, are known to influence a number of steps affecting mitochondrial DNA integrity. This review describes the basic physiology, pharmacology and pathophysiology of HIV infection and the nucleoside analogues regarding mitochondrial function and discusses the progress made in this field with respect to the measurement of these effects and the prediction of potential drug toxicity.

Introduction
The success of combination highly active antiretroviral therapy (HAART) for advanced human immunodeficiency virus (HIV) infection, introduced in 1996, marked the beginning of an era that would result in HAART becoming the principal regimen used to treat millions of HIV-infected adults and children worldwide. Unfortunately, the agents used in these multi-drug regimens are not without side-effects, and nucleoside reverse transcriptase inhibitors (NRTIs), included in most regimens, are responsible for significant morbidity and mortality mostly associated with lipatrophy and severe lactic acidosis in patients on HAART. NRTIs were known by the early 1990s to be mitochondrial toxins causing mitochondrial deoxyribonucleic acid (mtDNA) depletion. Since then, toxic effects have been well characterised, less toxic NRTIs have been developed and the risk factors associated with mitochondrial toxicity have been identified. However, what has remained elusive is the ability to identify preclinical mitochondrial toxicity and in so doing prevent NRTI-associated morbidity.

More recently, HIV infection itself has emerged as a cause of mitochondrial dysfunction, further complicating the diagnosis and management of NRTI-associated mitochondrial toxicity. These factors, combined with recent data suggesting that NRTIs in addition may undermine mitochondrial DNA integrity, have renewed concern over the long-term side-effects of these agents, particularly in growing children who could very well be exposed to them for life.

Objectives
The objectives of this review can be summarised as follows:
• To describe the basic physiology of mitochondrial function and how this relates to the pathophysiology of NRTIs and HIV infection regarding mitochondrial function.
• To describe the history of research in the field of HIV-associated mitochondrial toxicity, citing important studies that have contributed significantly towards our current understanding of the problem and discussing the major strengths and weaknesses of these studies.
• To identify areas requiring further research.

Background
Mitochondria are found in all human cells, with the exception of mature red blood cells, and host a number of essential biochemical reactions largely concerned with fuel oxidation and adenosine triphosphate production. In addition, mitochondria are intimately involved in a number of cellular processes, including cell differentiation, signalling and division, and are recognised as being critically involved in programmed cell death, or apoptosis, and ageing. Mitochondria also contain their own unique circular DNA, an evolutionary remnant that, at 16.5 kilobases, contains 37 genes that code for 13 respiratory chain peptides, together with two ribosomal ribonucleic acids (rRNAs) and 22 transfer ribonucleic acids (tRNAs) involved in mitochondrial
protein synthesis.\textsuperscript{13,14} Most mitochondria contain at least two copies of mtDNA, and the number of mitochondria in cells range from a couple of hundred to hundreds of thousands in tissues with high energy demands, such as muscle, renal tubular cells and neurons.\textsuperscript{14,15}

Mitochondria and their DNA are maternally inherited, derived directly from thousands of maternal oocytic mitochondria. A number of maternally inherited mitochondrial cytopathies with specific mutations in the mitochondrial DNA are well described.\textsuperscript{14} Two principles govern the manifestation of inherited mitochondrial disease. Firstly, the “threshold effect” describes the fact that dysfunctional mitochondria need to reach a critical threshold before a tissue manifests with mitochondrial failure, as a result of the high reserve of oxidative capacity inherent in most tissues. Secondly, mitochondria are randomly distributed in the growing zygote in a process known as “heteroplasmy”.\textsuperscript{15} This means that two patients with identical mtDNA mutations may manifest with different tissue symptoms due to differences in the diseased mitochondrial load in each tissue.\textsuperscript{14} Mitochondrial DNA is replicated by the enzyme DNA polymerase \(\gamma\), an enzyme that performs both polymerase and exonuclease activities and differs from the nuclear DNA (nDNA) polymerases in that it is more susceptible to interference by a number of nucleotide analogues designed to inhibit HIV reverse transcriptase.\textsuperscript{16}

### Nucleoside reverse transcriptase inhibitors and mitochondrial DNA integrity

Phosphorylated nucleoside analogues interfere with viral reverse transcriptases by actively competing with endogenous nucleotides for incorporation into the growing nucleic acid chain. After incorporation, they prevent chain elongation as they do not contain a 3’-hydroxyl group for addition to the next nucleotide.\textsuperscript{17-19} Examples are shown in Figure 1.

In this manner, NRTIs have been shown to interfere with mtDNA polymerase \(\gamma\) \textit{in vitro},\textsuperscript{17} causing depletion of mtDNA that is assumed to result in mitochondrial dysfunction. Lipoatrophy is a common manifestation of mitochondrial dysfunction, and less frequent complications include potentially fatal lactic acidosis, skeletal myopathy, cardiomyopathy, neuropathy, pancreatitis and nephro-toxicity that usually manifests as tubulopathy.\textsuperscript{2,20-27} Although all NRTIs potentially affect mtDNA polymerase \(\gamma\), didanosine (ddI) and stavudine (d4T) have repeatedly been implicated in the most severe cases of mitochondrial toxicity with fatal lactic acidosis.\textsuperscript{28-30}

Apart from their direct effect on mtDNA polymerase \(\gamma\), nucleoside analogues may also compete with endogenous nucleosides for phosphorylation by mitochondrial thymidine kinase 2 and also for transport into the mitochondria.\textsuperscript{31,32} Evidence is now emerging that NRTIs may increase the rate of accumulation of mtDNA mutations, and increased mutations have been demonstrated longitudinally in peripheral leukocytes in

![Figure 1: Selected endogenous nucleosides and their corresponding antiviral drug analogues](image-url)
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patients on HAART. In addition, Maagaard et al found increased mtDNA deletions in the skeletal muscle of patients exposed to NRTIs, and reduced mitochondrial gene expression has been demonstrated in adipocytes exposed to NRTIs. Taken together, this evidence suggests that NRTIs as a group may have far-reaching effects on mitochondrial function in the short as well as long term. Regarding predisposing risk factors for NRTI mitochondrial toxicity, obese adult women are known to be at increased risk. Furthermore, genetic predisposition linked to the mitochondrial DNA sequence also appears to play a role. Individuals belonging to mitochondrial haplogroup T, which is best characterised as a European mitochondrial lineage, have a higher incidence of NRTI-associated neuropathy, and more recently a known T9098C polymorphism in the mitochondrial gene for adenosine 5'-triphosphate synthase subunit 6 has been associated with severe mitochondrial toxicity in a child after in utero exposure to zidovudine (AZT) and lamivudine (3TC).

HIV and mitochondrial function

HIV causes apoptosis and cellular death in many tissues, most notably in immune cells such as CD4 lymphocytes. It is not surprising therefore that given the importance of mitochondria in regulating cellular apoptosis, HIV should exploit mitochondrial apoptotic pathways to initiate destruction of immune cells involved in the immune response against infection. Apoptosis is triggered by two major pathways: the first through exogenous factors that bind cell membrane receptors such as Fas ligand (FasL; CD178) and ultimately give rise to cytoplasmic caspase 8 and 10 activation through a cytoplasmic signaling pathway, leading to apoptosis; and the second through intrinsic factors within the cell that trigger cytochrome C and other proapoptotic factors to be released from the mitochondrial intermembrane space, which in turn result in caspase 9 activation and apoptosis. HIV is implicated in both of these pathways.

A number of HIV proteins have been shown to activate apoptosis directly and initiate cell death, the most important of these being Env, Nef, Tat and viral protein R (Vpr). Env, also known as gp-120, together with Nef and Tat, has been shown to activate the extrinsic apoptotic pathway through the Fas/FasL receptor, and Env, Nef, Tat and Vpr have all been shown to trigger cytochrome C release from the mitochondrial intermembrane space with subsequent caspase activation through the internal pathway. This form of HIV-induced apoptosis has been demonstrated in CD4 cells, haematopoietic cells, cardiac myocytes and neurons. In addition to the direct effect of HIV-1 viral proteins, the massive inflammatory response and immune activation associated with HIV and the associated cytokines, including tissue necrosis factor , interleukin 2 and interferon , are also capable of inducing apoptosis.

It is clear from the above mechanisms that both NRTIs and HIV infection itself directly influence mitochondrial function in a number of tissues. HIV infection by activating mitochondrial apoptotic pathways to trigger cell death and NRTIs by directly or indirectly interfering with mtDNA integrity. It is therefore not surprising that the clinical manifestations of HIV organ pathology and NRTI mitochondrial toxicity should overlap in a number of organs with high dependence on mitochondrial function. Examples include neuropathy, renal tubulopathy, lipodystrophy and myopathy that may be associated with either agent.

Research history of nucleoside reverse transcriptase inhibitor toxicity

The mechanism of nucleoside analogue interference with mitochondrial DNA was first described in 1990 in early cases of AZT-associated myopathy. In 1995 Lewis and Dalakas put forward their “polymerase gamma hypothesis” based on the principle that tissues dependent on high levels of oxidative phosphorylation, where NRTIs were incorporated and phosphorylated, were most likely to manifest with mitochondrial dysfunction. With the widespread use of dual NRTIs in HAART regimens and the associated increase in cases of fatal lactic acidosis, researchers looked for markers that could predict mitochondrial toxicity. Blood lactate was the obvious choice and lactate has been extensively measured and monitored in large cohorts of patients on antiretroviral treatment (ART) combination regimens. Many of these studies confirmed the increased relative risk of lactataemia with d4T- and ddI- vs. AZT-based regimens. However, lactate measurement as a predictive marker of decompensated mitochondrial toxicity was limited by the fact that many patients on full-time NRTI therapy and neonatal recipients of short courses of NRTIs have asymptomatic hyperlactataemia that does not progress to decompensated lactataemia. Also, lactate lacks specificity and is influenced by technical and physiological variability.

Another approach was to quantify mtDNA against nDNA using real-time polymerase chain reaction (PCR) techniques in peripheral blood leukocytes (PBLs), mainly to avoid invasive tissue biopsies. Most techniques employ separation of a leukocyte buffy coat from platelets because platelets contain mitochondria but not nDNA, which could theoretically confound results.
Quantitation is usually performed by simultaneous real-time PCR amplification with fluorometric detection of a mitochondrial and a nuclear gene, and the results are either expressed as mtDNA:nDNA ratios or as absolute mtDNA copies per cell if separate commercial mtDNA and nDNA standards are used. This approach yielded promising data in 2002 when Coté et al followed up eight HIV-infected hyperlactataemic patients on NRTIs longitudinally. The authors found significant depletion of PBL mtDNA in these patients, that improved after cessation of therapy to levels comparable to 47 ART-naive HIV-infected patients. In addition, the authors reported lower levels of PBL mtDNA in the ART-naive HIV-infected patients vs. 24 uninfected controls. The strength of this study was the fact that it was the first to demonstrate the clinical application of PBL mtDNA quantitation as a marker of NRTI mitochondrial toxicity and could be used as a laboratory marker for this purpose, but its weakness lay in the fact that the patients selected for longitudinal study all had overt symptomatic hyperlactataemia on NRTI regimens of d4T or d4T/ddI, drugs already known to be associated with a high risk for lactataemia. The study was therefore unable to demonstrate whether PBL mtDNA could be used to detect presymptomatic mitochondrial toxicity before the onset of clinical lactataemia or improve on the specificity or sensitivity of lactate as a marker of mitochondrial toxicity.

Subsequent to this work, a number of studies were conducted to evaluate PBL mtDNA in HIV-infected cohorts to answer these questions. Five adult studies confirmed the findings of Coté et al, namely that PBL mtDNA levels in ART-naive HIV-infected patients were depleted vs. HIV-negative control groups but not significantly different from ART-exposed patients, and a single cross-sectional study in South Africa demonstrated the same findings in a paediatric population. However, conflicting findings occurred in the small (n=10) study by Henry et al that demonstrated no difference between HAART-exposed patients and healthy controls, and in the longitudinal study of Miura et al that demonstrated complete amelioration of PBL mtDNA depletion to normal levels with AZT/3TC-based HAART, suggesting that HIV was the major cause of depleted PBL mtDNA. These findings have more recently been supported by Aldrovandi et al who measured PBL mtDNA in babies exposed to NRTIs in utero and as neonates. The authors found that PBL mtDNA was decreased in HIV-exposed neonates compared to controls, but was higher in ART/HIV-exposed neonates than HIV-exposed neonates who were not exposed to ART. These data support the notion that HIV itself, and not NRTIs, is the major contributor towards PBL mtDNA depletion. Regarding ART-exposed patients in larger studies, Chiappini et al and Coté et al all found lower levels of PBL mtDNA associated with d4T, DDI and particularly d4T/DDI combination regimens. In the largest of these, a cross-sectional study by Coté et al of 214 ART-treated individuals exposed to ART for more than four months, clearly demonstrated progressive PBL mtDNA depletion with d4T/ddI, ddi, d4T and AZT combinations, in this order. Today, neither d4T nor ddi are recommended for first-line HAART regimens and have been replaced by safer NRTI combination regimens, owing to their association with mitochondrial toxicity. Stavudine is currently only used as a first-line drug in resource-constrained settings because of its lower cost.

As a result of the conflicting findings with PBL mtDNA measurement, researchers turned their attention to tissue mtDNA quantitation. Mitochondrial DNA measured in renal biopsies of patients without HIV, with HIV on tenofovir (TDF) treatment and with HIV without TDF, demonstrated reduced mtDNA levels in both HIV-infected groups vs. uninfected biopsies, but no difference between the two HIV-infected groups, suggesting that HIV itself also contributed toward mtDNA depletion in renal tissue. Adipose tissue mtDNA measured in a large cohort of HIV-infected individuals with lipodystrophy on HAART demonstrated depleted mtDNA levels vs. controls and a significant association between d4T, ddi and mtDNA depletion. However, this work was limited by the fact that only two HIV-infected ART-naive patients were studied, and so the contribution of HIV infection to adipose tissue mtDNA depletion was not assessed. More recently, Garrabou et al found significant mtDNA depletion and reduced mitochondrial oxidative function in adipocytes of HIV-infected ART-naive individuals compared to uninfected controls. The value of tissue mtDNA measurement has been questioned by Kim et al, who confirmed findings of depleted mtDNA in adipose tissue of lipodysplatic HAART-treated patients, but found no decrease in mtDNA-dependent mitochondrial function and an actual compensatory increase in nuclear-driven mitochondrial biogenesis, suggesting that mtDNA depletion was not a good marker for mitochondrial function. In another cross-sectional study by Magaad et al, mitochondrial DNA was lower in muscle biopsies from 24 patients with HIV on HAART than 10 ART-naive HIV patients, but both groups demonstrated decreased PBL mtDNA compared to 11 healthy controls. The authors concluded that PBL mtDNA does not correlate with organ mtDNA depletion. Taken together, the measurement of mtDNA in end organs appears to deliver similar information to PBL measurements, namely that NRTIs, particularly d4T and ddi, cause tissue mtDNA depletion. HIV infection...
may cause depletion independently, but this is likely to be a tissue-dependent phenomenon, and mtDNA levels in tissue do not correlate with PBL mtDNA levels or with actual mitochondrial function, as a result of the initiation of compensatory mechanisms to preserve essential mitochondrial functions. Currently most authors have concluded that measurement of mtDNA in PBLs and tissue contributes little toward predicting NRTI-induced functional mitochondrial toxicity, and mtDNA measurements are not used in routine practice, though there may still be some value in performing serial measurements of PBL mtDNA in at-risk individuals.

Other methods used for direct assessment of mitochondrial function without invasive tissue biopsy include measurement of lymphocyte mitochondrial transmembrane potential by flow cytometry and 13C methionine breath testing.76-78 Flow cytometric techniques employ a cationic lipophilic fluorochrome with an affinity for mitochondria with high membrane potential. This method has the added advantage that circulating leukocyte subsets are easily separated and compared. Using flow cytometry, Polo et al found mitochondrial dysfunction independently associated with HIV infection and the use of d4T, in concordance with the findings of others.79 The 13C methionine breath test assesses hepatic mitochondrial function by quantification of exhaled 13CO2 after oral methionine loading and is based on the fact that intact mitochondrial metabolism is required for methionine catabolism. Using this method, Milazzo et al found reduced 13CO2 excretion in HIV-infected patients on AZT- or d4T-based regimens when compared to ART-naïve controls, with further reductions in lactataemic patients. No differences were apparent between healthy controls and infected ART-naïve patients. The authors concluded that hepatic mitochondrial dysfunction is drug related as opposed to HIV related and that the 13C methionine breath test is superior to lactic acidosis measurement for detection of subclinical toxicity.77

Paediatric HIV presents additional scenarios where children who do not contract HIV are exposed to ART, either in utero during maternal treatment, or perinatally during prevention of mother to child transmission (PMTCT) of HIV. In addition, most HIV-infected children are not ART naive prior to initiation of therapy, and clinical mitochondrial dysfunction has been described in over 30% of perinatally infected children followed up longitudinally.80 Mitochondrial toxicity associated with PMTCT was highlighted in 1999 when eight HIV-uninfected children in a French cohort exposed to AZT and/or 3TC presented with severe mitochondrial dysfunction, and two died.81 Subsequent prospective cohort studies have been conflicting, with some reporting evidence for an increased incidence of mitochondrial dysfunction up to 18 months post-PMTCT,82 and others not.83,84 Despite this, transient hyperlactatemia that rarely progresses to fatal lactic acidosis in ART-exposed neonates is well known.57,58 Based on the outcomes of the South African arm of the Children with HIV Early Antiretroviral Therapy (CHER) study,85 treatment guidelines for paediatric HIV have been changed and now include ART for all HIV-infected children under 12 months of age, regardless of clinical condition.86 In addition, a move to reduce the use of d4T in resource-constrained settings is being advocated, with substitution of d4T with TDF in adults and abacavir in children as one of the first-line NRTIs.86

Further research

No study to date has managed to answer a number of pressing questions regarding the combination effect of HIV infection and NRTIs on mitochondrial function. A number of these questions may only be answered in longitudinal studies and all are particularly applicable to children. Research questions that need to be answered are the following:

What are the long-term complications of NRTIs, specifically regarding mitochondrial DNA integrity and its effect on growth, ageing and end-organ function, and to what extent are tissues able to compensate for NRTI-induced mtDNA depletion?

Are the mitochondrial effects of HIV infection and NRTIs synergistic? In other words, are patients with severe HIV disease at higher risk of mitochondrial toxicity from NRTIs than patients with less severe HIV disease, and if so, which tissues are most likely to be affected? This question is especially relevant in resource-constrained settings where access to ART is limited and many patients have more severe disease prior to initiation of treatment with regimens often containing d4T.

While safer, cost-effective ART is developed, is there another laboratory marker or tissue-specific marker that can be used to identify patients at high risk of NRTI toxicity pre-emptively so that preventative management can be employed in time?

Conclusion

The availability of HAART has made an enormous contribution worldwide to the treatment of people living with HIV infection, and the focus has now shifted to prevention of the long-term side-effects of the drugs used in the treatment of this disease. While lipodystrophy and its associated metabolic changes are associated with protease inhibitors, the major toxic effect of NRTIs is fatal decompensated lactic acidosis due to mitochondrial toxicity. There is also growing concern about the cumulative long-term effects of
a class of drugs that influence mtDNA synthesis and repair. Although the availability of safer ART regimens has reduced the incidence of severe toxicity, new drugs are often not available in resource-constrained settings where the majority of patients with HIV are found, and though measures to limit NRTI toxicity, such as regimen switching, are available, there is a pressing need to implement protocols that promote the use of safer first-line NRTIs and restrict the use of d4T. It is also crucial to continue further research into the understanding and prevention of ART-related toxicity, particularly as it relates to children with HIV.

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