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ORIGINAL PAPERS

METHODS FOR MONITORING MITOCHONDRIAL TOXICITY OF NUCLEOSIDE-ANALOGUE REVERSE-TRANSCRIPTASE INHIBITORS IN HIV PATIENTS DURING ANTIRETROVIRAL THERAPY

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Abstract. Various side effects have been associated to HIV antiretroviral therapy (ART) containing nucleoside reverse transcriptase inhibitors (NRTIs) as a result of mitochondrial toxicity. Mitochondrial DNA (mtDNA) depletion has important clinical consequences: lipodystrophy, hepatic toxicity, lactic acidosis, myopathy and polyneuropathy. Several methods have been developed in recent years in order to prevent the clinical and biological side effects during NRTIs treatment. These methods aim to predict the metabolic risk using non invasive tools. The present study aims to evaluate the current laboratory methods for mitochondrial toxicity and to assess their utility for monitoring ART toxicity.
Keywords: HIV, Mitochondrial DNA, Nucleoside reverse transcriptase inhibitors, ART

Background

Mitochondrial dysfunction is the main pathogenic mechanism for more than 75 diseases. Mitochondrial toxicity frequently targets the cardiovascular and central nervous system, peripheral sensory and motor neurons [1].

Each human mitochondrion contains approximately 2 to 10 mitochondrial DNA (mtDNA) molecules. The mtDNA molecules are circular and have 37 genes. One cell may contain hundreds to thousands of mitochondria [1].

Antiretroviral therapy (ART) introduction in 1996 has modified the evolution of HIV infection and led to a decrease in opportunistic infections and an increase in life expectancy. Several years after ART introduction, reports of HIV-infected individuals experiencing changes in body metabolism began to appear [2]. The term "lipodystrophy" was initially used to summarize these metabolic changes. The most important metabolic abnormalities observed in lipodystrophy include: hypercholesterolemia hypertriglyceridemia, low levels of HDL-cholesterol, insulin resistance, type 2 diabetes mellitus, lactic acidemia and elevated hepatic transaminases [3].

Lipodystrophy also associates clinical changes: subcutaneous fat wasting at Bichat's fat pad, of temporal fat, or at the buttocks and extremities, abnormal fat accumulation intra-abdominally and in the dorsocervical region [1].

These clinical and metabolic abnormalities may appear as a result of the different effects of the various ART combination drugs. MtDNA depletion is the main pathogenetic mechanism through which ART precipitates metabolic changes and organ toxicities [4]. MtDNA depletion is known to be the result of nucleoside reverse transcriptase inhibitors (NRTIs). The reduction of the mtDNA copy number causes cellular respiratory chain impairment and may have many clinical consequences: lipodystrophy, peripheral neuropathy, cardiomyopathy, hepatic steatosis and hepatotoxicity, hyperlactatemia and lactic acidosis, and type 2 diabetes or insulin resistance [1]. Mitochondrial liver toxicity may cause hepatomegaly, steatosis and elevated serum liver transaminases.

Mitochondrial neuropathy appears weeks or months after ART initiation. The axonal toxicity may manifest predominantly as a sensitive, distal, symmetrical polyneuropathy of the extremities [1].

Objectives

The study aims to evaluate the current methods of laboratory diagnosis of mitochondrial toxicity and to assess their utility for monitoring ART toxicity.

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Materials and methods

Search strategy: Standard methods were used to search electronic databases (MEDLINE, EMBASE, SCIENCE CITATION INDEX) and conference proceedings with relevant search terms. A two-step search strategy was utilised. An initial search of these databases was undertaken followed by analysis of the text words contained in the title and abstract, and of the index terms used to describe article. Secondly, the reference list of all identified reports and articles was searched for additional studies.

Selection criteria: Primary areas of interest included: methods available to measure mitochondrial function and mitochondrial DNA, mtDNA cut-off points associated with the risk of clinical and metabolic manifestations.

Secondary outcomes included: mechanisms of ART toxicity, clinical manifestations of mitochondrial DNA depletion (lipodystrophy, hepatic toxicity, myopathy, neuropathy, hematologic toxicity).

Data collection: Two authors assessed each reference for inclusion criteria. The list of keywords included: HIV, ART, mitochondrial DNA, mitochondrial toxicity, mitochondrial depletion, lipodystrophy syndrome.

Results and discussion

According to current opinion, histological examination is the most accurate test for the diagnosis of mitochondrial toxicity.

The following findings indicate a mitochondrial etiology: intracellular microvesicular steatosis, ultrastructural abnormalities of mitochondria, diminished histochemical activities of cytochrome c oxidase, ragged-red fibers in skeletal muscle [1].

Different other methods have been proposed to assess metabolic toxicity and to predict the metabolic risk adequately prior to its onset.

These tools vary from quantitative methods to those based on the analysis of phenotypes for the functions of mitochondria (table I) [3].

Method	Measurement
Southern blot	Molecular weight
Sequencing	Sequence changes
In-house real time PCR	mtDNA/nDNA ratio
Southern blot	mtDNA/nDNA ratio
Retina Mitox	mtDNA/nDNA ratio
Northern blot	mRNA density
Western blot	Protein density
Fluorimetric assays	Oxidative damage
Polarographic	Oxygen consumption
Spectrophotometric	Enzymatic activity

Table I. Methods available to measure mitochondrial function and mitochondrial DNA [3]

Spectrophotometric and polarographic techniques have been developed, which can provide quantitative measurements, using simple instruments. There are two main disadvantages: the necessity of a biopsy and the lack of standardization of the biochemical assays [1].

For current clinical practice, the development of non-invasive tests could be more useful in monitoring and diagnosing mitochondrial toxicity.

We reviewed 14 studies that used different non-invasive methods to assess metabolic toxicity. The studies were too heterogeneous to warrant meta-analyses and some studies did not contain sufficient data for a statistical analysis [5, 7, 8-19].

All the studies examined peripheral blood mononuclear cells (PBMCs) as an accessible source of mtDNA for examining toxicity.

The methods were based on the quantification of both nuclear and mtDNA genes, establishing a ratio between nuclear DNA and mtDNA. This ratio may allow the estimation of the total copy number of mtDNA per cell.

We included 10 studies that used in-house assays, developed to quantify mitochondrial DNA. These methods were based on real-time polymerase chain reaction (PCR) and nucleic acid sequence base amplification (NASBA).

We also reviewed 4 studies that used a commercial assay to quantify mitochondrial DNA [8, 16-18].

Six studies used in-house PBMCs PCR-based quantification assays for ART mitochondrial toxicity monitoring.

Real-time PCR is a standard method for quantification of DNA and RNA in human cells. All the included studies used real-time PCR methods based on the detection of both nuclear and mtDNA genes. All of the PCR-based quantification assays used a calibration curve based on plasmids containing the gene that was amplified and represented either the mtDNA or nDNA molecules.

Several studies showed that blood sample preparation was very important for valid mtDNA quantitation in PBMCs, as platelets contain mtDNA, but not nDNA. Platelet mtDNA contamination may determine higher results, especially when depletion of mtDNA is expected to be found due to NRTI therapy. The quantitation of mtDNA is only minimally affected when the PBMC content is five times higher than the platelet content [5].

In one study, the platelets were sorted using magnetic beads. The samples can also be washed with a medium of phosphate-buffered saline containing 20 mL/L fetal calf serum and subsequently centrifuged for 15 min at 100*g* [5].

Other factors were also found to influence results: difference in nuclear extraction efficiency, differences in calibration and standardization of

the standardization curve, different amplification efficiencies, etc.

We reviewed 4 studies that used nucleic acid sequence based amplification (NASBA) technology for monitoring ART toxicity. NASBA technology was developed as an alternative procedure to standard methods of amplification. NASBA uses fluorescent probes and permits real-time quantification of mitochondrial nucleic acids in relation to nuclear RNA or DNA. The product of NASBA is single-stranded RNA of opposite sense to the original target [6].

One metastudy that evaluated the accuracy of the two techniques (PCR and NASBA), has shown reasonable concordance between the assays and across several labs [7]. Four laboratories were asked to measure and report mitochondrial DNA and nuclear DNA genome copy number, as well as mitochondrial DNA copy number/cell. Concordance between laboratories was assessed by determining the average coefficient of variation of the mean replicate values for each sample. Measurements of mitochondrial DNA and nuclear DNA content for replicate samples varied by an average of less than 6%, and measurements of mitochondrial DNA/cell for replicates varied by less than 12%, with no improvement of precision after standardization. Standardization did significantly improve the concordance of results for measurements of mitochondrial DNA content and mitochondrial DNA/cell [7].

We reviewed 4 studies that used Primagen's Retina™ Mitox™ for mtDNA quantitation. Primagen's Retina™ Mitox™ is the first available commercial assay to quantify mitochondrial DNA. The Retina™ Mitox™ method evaluates the mitochondrial DNA content per cell in a real-time duplex NASBA (Retina) assay [8, 16-18].

In real-time duplex NASBA, both mitochondrial and nuclear DNA targets are simultaneously amplified in a single-tube format. This simultaneous amplification allows the evaluation of mtDNA to nDNA ratio avoiding the external factors that might appear when amplifications are performed separately [5].

Given the range of possible sources of variability, the threshold of mtDNA depletion and association with lipodystrophy varied across the reviewed studies depending on the primers and laboratory protocols used.

For inherited mitochondrial diseases, severe symptoms tend to occur when mtDNA levels decrease 20% or less than normal [3]. We found no study to establish a definite association between the level of mtDNA and the risk of clinical and metabolic manifestations. The lack of studies investigating this association is a significant limitation that prevents the use of mtDNA for the early diagnosis of lipodystrophy syndrome.

One of the most important problems regarding the development of a non-invasive early detection tool of NRTIs toxicities is the fact that changes in mtDNA content in PBMCs may not accurately reflect the changes in other tissues. We could not find any study that evaluated the concordance between PBMCs mtDNA modifications and changes of mtDNA in other tissues. Further studies investigating this concordance are needed, before quantitative mtDNA assays could be implemented in clinical practice.

Conclusions

The monitoring of mtDNA in PBMCs could be an important non-invasive early detection tool of NRTIs toxicities, before the onset of clinical manifestations.

Before quantitative mtDNA assays could be implemented in clinical practice there are important aspects that need to be clarified. The different methods for PBMCs mtDNA assessment have to be further harmonized and validated. There is need for more available commercial tests. Also, further studies are needed, in order to clarify whether physicians can prevent mitochondrial toxicity based on the results of mtDNA measurements. There is yet to establish a threshold for mtDNA reduction, in order to quantify the risk of mitochondrial toxicity.

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