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Short Communication: Transplacental Nucleoside Analogue Exposure and Mitochondrial Parameters in HIV-Uninfected Children

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Abstract

Transplacental nucleoside analogue exposure can affect infant mitochondrial DNA (mtDNA). We evaluated mitochondria in peripheral blood mononuclear cells of children with and without clinical signs of mitochondrial dysfunction (MD) and antiretroviral (ARV) exposure. We previously identified 20 children with signs of MD (cases) among 1037 HIV-uninfected children born to HIV-infected women. We measured mtDNA copies/cell and oxidative phosphorylation (OXPHOS) NADH dehydrogenase (complex I) and cytochrome *c* oxidase (complex IV) protein levels and enzyme activities, determined mtDNA haplogroups and deletions in 18 of 20 cases with stored samples and in sex- and age-matched HIV-uninfected children, both ARV exposed and unexposed, (1) within 18 months of birth and (2) at the time of presentation of signs of MD. In specimens drawn within 18 months of birth, mtDNA levels were higher and OXPHOS protein levels and enzyme activities lower in cases than controls. In contrast, at the time of MD presentation, cases and ARV-exposed controls had lower mtDNA levels, 214 and 215 copies/cell, respectively, than ARV-unexposed controls, 254 copies/cell. OXPHOS protein levels and enzyme activities were lower in cases than exposed controls, and higher in cases than unexposed controls, except for complex IV activity, which was higher in cases. Haplotype H was less frequent among cases (6%) than controls (31%). No deletions were found. The long-term significance of these small but potentially important alterations should continue to be studied as these children enter adolescence and adulthood.

Introduction

Mother-to-child transmission (MTCT) of HIV can decrease from 25% to less than 2% with the use of antiretroviral therapy (ARV) including nucleoside analogues (NA) during pregnancy.[1] However, there is some evidence that *in utero* NA exposure may cause symptomatic mitochondrial dysfunction (MD) in a small number of HIV-uninfected children.[2,3] Recent studies have found significantly higher mtDNA levels in peripheral blood mononuclear cells (PBMCs) of HIV-uninfected infants exposed to NA and to other ARV compared to ARV-unexposed infants at birth and in the first few weeks of life,[4–6] possibly to due compensatory mitochondrial activity in response to NA-induced stress. These findings contradict earlier studies, which reported decreased mtDNA in cord blood and PBMCs in ARV-exposed infants at birth and at 1 and 2 years of age,[7–9] and a recent study that found mtDNA depletion with secondary respiratory chain compromise in placental tissue with ARV exposure.[10] Other findings include significantly lower mitochondrial RNA (mtRNA) levels at birth in NA-exposed vs. unexposed infants[4] and no difference in cytochrome *c* oxidase protein levels.[6] These discrepant findings could be due to differences in inhibitory effects of particular NA on mitochondrial DNA replication or to transcription or differences in mtDNA content of tissues.[11,12] Further studies are needed to elucidate the mechanisms of MD from *in utero* NA exposure, and to identify the significance of mitochondrial variations in children with and without clinical signs of MD.

In a previous epidemiological study we identified 20 children with unexplained clinical signs that could be consistent with MD among 1037 HIV-uninfected children born to HIV-infected women in the U.S. Pediatric AIDS Clinical Trials Group (PACTG) protocols 219 and 219C.[3] Nineteen children

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had neurological abnormalities and one child died of cardiomyopathy. Stored PBMCs of these children provided a unique opportunity to examine mitochondrial parameters in HIV-uninfected children with and without clinical signs of mitochondrial toxicity, and with and without *in utero* NA exposure.

Materials and Methods

Clinical specimens

Of the 20 cases with signs of MD, 18 had stored blood samples and were included, 16 of whom were exposed to ARV and to NA *in utero.* PBMCs drawn at two different time points were assayed: (1) within 18 months of birth and (2) at the time of presentation of MD. The median age of the 18 cases at the time of presentation of signs of possible MD was 16 months (IQR: 9, 20 months), and specimens were obtained a median of 2.8 months (IQR: 0, 6.5 months) from the time of presentation. Ten cases (nine of whom were exposed to ARV *in utero)* had PBMCs drawn within 18 months of age (median age at specimen draw 6.5 months, IQR: 2.9, 9.2 months).

HIV-uninfected children without signs of MD were matched by sex, year of birth, and age ($\pm$12 months) of cases at the time of specimen draw to control for previously identified differences in mitochondrial toxicity due to sex and year of birth.[3] Two sets of HIV-uninfected children were matched to cases at each time point: children exposed to ARV *in utero* ($n = 18$), and children unexposed to ARV *in utero* ($n = 17$).

MtDNA copies/cell quantitation

Analysis of mtDNA copies/cell was conducted by absolute quantitative real-time polymerase chain reaction (PCR) as previously described.[13] Briefly, DNA was extracted from frozen PBMCs using a Qiagen DNA kit (Qiagen, Valencia, CA). Standardization of real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I with the Roche LightCycler instrument (Roche, Indianapolis, IN). A dilution series of the control plasmid[7] containing the 90-bp mtDNA NADH dehydrogenase, subunit 2 and the 98 bp Fas Ligand gene was prepared to set up the standard. Each sample and standard was run in duplicate and the results were analyzed with Version 4.0 LightCycler software.

OXPHOS protein and enzyme activities immunoassays

Protein and enzyme levels of oxidative phosphorylation (OXPHOS) NADH dehydrogenase (complex I) and cytochrome *c* oxidase (complex IV) were determined in duplicate by thin-layer chromatography and immunoassays as described previously.[14] Each vial of viable PBMCs was thawed and washed in 0.5 ml of phosphate-buffered saline (PBS) twice before addition of 0.5 ml of ice-cold extraction buffer [1.5% lauryl maltoside, 25 mM HEPES (pH 7.4), 100 mM NaCl, plus protease inhibitors (Sigma, P-8340)]. Samples were mixed gently and kept on ice for 20 min, and then they were spun in a microcentrifuge at 16,400 rpm at 4°C for 20 min to remove insoluble cell debris. The supernatant, an extract of detergent-solubilized cellular proteins, was then assayed with the OXPHOS immunoassays. All samples were loaded on the immunoassays with equal amounts of total cell protein using an amount previously established with control samples to generate signals within the linear range of the assay. Therefore, the resulting signal was directly proportional to the amount of OXPHOS protein or enzyme activity in the sample. Quantitation of the signal was done by densitometric scanning with a Hamamatsu ICA-1000 reader.

Mitochondrial haplotyping

Mitochondrial haplotype analysis was carried out using PCR amplification and direct sequencing of the first hypervariable region (HVS-1) of mtDNA as described.[15] Sequencing data analysis was performed using Sequencing Analysis Software version 3.4.5 (Applied Biosystems). For haplogroup determination, we employed an open-source research database introduced by Behar *et al.*[16]

We utilized long-range PCR strategy to investigate mtDNA deletions because this methodology requires much less DNA than traditional Southern blotting. Briefly, an aliquot of 100 ng of DNA extracted from PBMCs was amplified with a pair of primers (nt 3066–3099 and nt 780–816 according to the Cambridge reference sequence) using Expand Long Template PCR System kit reagents (Roche Diagnostics Corporation). The amplified PCR products were separated on a 0.8% agarose gel containing ethidium bromide and visualized over ultraviolet light. Large single or multiple deletions can easily be detected because they result in smaller than normal (~16 kb) bands.

Statistics

Characteristics of HIV-uninfected children with and without MD were compared using Fisher's exact tests and Wilcoxon rank sum tests for categorical and continuous variables, respectively. Differences in the median number of mtDNA copies/cell, OXPHOS protein levels, and enzyme activities (complex I and IV quantity and activity) between cases and children without signs of MD were assessed with the Wilcoxon signed rank test in matched analysis and the Wilcoxon rank sum test in unmatched analysis. Cases were compared separately to ARV-exposed and ARV-unexposed children. There was no substantive difference between the matched and unmatched results, thus the unmatched results are presented. MtDNA haplotypes were identified and deletions screened for, and differences according to case status were assessed with the Fisher's exact test. When controlling for ARV exposure, two cases unexposed to ARV *in utero* were excluded in the statistical tests of differences in mitochondrial parameters.

Results

Demographic and clinical characteristics of cases at the time of presentation of symptoms of MD and of matched HIV-uninfected children are shown in Table 1. No significant differences in gender, age, race/ethnicity, year of birth, gestational age at birth, Apgar score, or birth weight were found. As shown, matching ARV unexposed children to the cases' year of birth was difficult given the wide use of perinatal and neonatal ARV to prevent MTCT of HIV in the United States starting around 1994. Thus it was not surprising that ARV-unexposed children were less likely to have used zidovudine prophylaxis in the first 6 weeks of life (47.1% vs. 83.3%). There were no differences in potentially important unmatched characteristics such as particular *in utero* ARV exposures,

Table 1. Characteristics of 18 Cases of Possible Mitochondrial Dysfunction and Both Antiretroviral (ARV)-exposed and ARV-unexposed HIV-uninfected children

	Cases (N = 18)		*ARV-exposed children (N = 18)*			*ARV-unexposed children (N = 17)*[b]		
Characteristic	N	%	N	%	p-*value*[a]	N	%	p-*value*[a]
Sex								
Male	13	72.2	13	72.2	1.00	12	70.6	1.00
Female	5	27.8	5	27.8		5	29.4	
Median age (IQR) at time of specimen draw (months)	18	19 (11, 25)	18	18 (17, 25)	1.00	17	17 (12, 19)	0.69
Race/ethnicity								
Non-Hispanic white	2	11.1	4	22.2	0.19	2	11.8	1.00
Non-Hispanic black	11	61.1	6	33.3		12	70.6	
Hispanic	4	22.2	8	44.4		3	17.6	
Asian/pacific islander	1	5.6	0	0		0	0	
Unknown	0	0	0	0		0	0	
Year of birth								
1992 to 1994	5	27.8	5	27.8	1.00	7	41.2	0.39
1995 to 1997	6	33.3	6	33.3		2	11.8	
1998 to 2000	7	38.9	7	38.9		8	47.1	
Gestational age at birth (weeks)								
<37	12	66.7	16	88.9	0.32	5	29.4	0.19
≥37	3	16.7	1	5.6		5	29.4	
Unknown	3	16.7	1	5.6		7	41.2	
One minute Apgar score								
<7	0	0	3	16.7	0.23	1	5.9	0.32
≥7	17	94.4	15	83.3		7	41.2	
Unknown	1	5.6	0	0		9	52.9	
Five minute Apgar score								
<7	0	0	0	0	-	1	5.9	0.32
≥7	17	94.4	18	100		7	41.2	
Unknown	1	5.6	0	0		9	52.9	
Birth weight (g)								
<2500	4	22.2	2	11.1	0.66	4	23.5	1.00
≥2500	14	77.8	16	88.9		11	64.7	
Unknown	0	0	0	0		2	11.8	
Zidovudine prophylaxis in the first 6 weeks of life								
No	3	16.7	5	27.8	0.69	9	52.9	0.035
Yes	15	83.3	13	72.2		8	47.1	
In utero nucleoside analogue exposure[c]								
Unexposed	2	11.1	0	0	0.49	17	100	-
Exposed	16	88.9	18	100		0	0	
In utero abacavir exposure								
Unexposed	17	94.4	18	100	1.00	17	100	-
Exposed	1	5.6	0	0		0	0	
In utero didanosine exposure								
Unexposed	18	100	17	94.4	1.00	17	100	
Exposed	0	0	1	5.6		0	0	
In utero lamivudine exposure								
Unexposed	8	44.4	9	50	1.00	17	100	-
Exposed	10	55.6	9	50		0	0	
In utero stavudine exposure								
Unexposed	17	94.4	18	100	1.00	17	100	-
Exposed	1	5.6	0	0		0	0	
In utero zidovudine exposure								
Unexposed	2	11.1	0	0	0.49	17	100	-
Exposed	16	88.9	18	100		0	0	
In utero lamivudine/zidovudine exposure								
Unexposed	8	44.4	10	55.6	0.74	17	100	-
Exposed	10	55.6	8	44.4		0	0	
In utero tobacco exposure								
Unexposed	7	38.9	6	33.3	0.71	4	23.5	-
Exposed	6	33.3	8	44.4		5	29.4	

(Table continued →)

Table 1. (Continued)

Characteristic	*Cases* (N = *18*) N	%	*ARV-exposed children* (N = *18*) N	%	p-*value*[a]	*ARV-unexposed children* (N = *17*)[b] N	%	p-*value*[a]
Unknown	5	27.8	4	22.2		8	47.1	
In utero alcohol exposure								
Unexposed	11	61.1	7	38.9	0.13	7	41.2	0.24
Exposed	3	16.7	8	44.4		7	41.2	
Unknown	4	22.2	3	16.7		3	17.6	
In utero cocaine exposure								
Unexposed	11	61.1	12	66.7	1.00	8	47.1	0.42
Exposed	3	16.7	2	11.1		5	29.4	
Unknown	4	22.2	4	22.2		4	23.5	
In utero drug exposure[d]								
Unexposed	11	61.1	11	61.1	1.00	8	47.1	0.42
Exposed	3	16.7	4	22.2		6	35.3	
Unknown	4	22.2	3	16.7		3	17.6	
Median (IQR) highest log maternal HIV RNA in the third trimester	14	3.3 (≤2.6, 4.0)	12	3.6 (2.7, 4.3)	0.61	6	3.5 (3.5, 3.8)	0.71

[a]*p*-value excludes missing observations; calculated from Fisher's exact test except for maternal HIV RNA for which the Wilcoxon rank sum test was used.
[b]A matched noncase specimen unavailable for one case.
[c]Two cases were unexposed to any antiretrovirals *in utero.*
[d]Includes intravenous drugs, cocaine, heroin, marijuana, methamphetamines, and barbiturates.

in utero tobacco, alcohol, or other drug exposure, or maternal HIV RNA levels.

Differences in the mitochondrial parameters of cases and matched children are presented in Table 2. In infant specimens drawn within 18 months of birth (Table 2) no statistically significant differences in mitochondrial mtDNA copies/cell or OXPHOS protein or enzyme activity was observed between cases and matched ARV-exposed or ARV-unexposed children. However, cases had a higher median number of mtDNA copies/cell (269) than exposed (206) or unexposed children (254). In contrast, OXPHOS protein levels and enzyme activities tended to be lower in cases.

In specimens drawn at the time of presentation of signs of MD and in matched HIV-uninfected children, there was no statistically significant difference in the number of mtDNA copies/cell or complexes I and IV quantity or activity. However, cases and ARV-exposed controls had lower mtDNA levels, 214 and 215 copies/cell, respectively, than ARV-unexposed children, 254 copies/cell. OXPHOS protein levels and enzyme activities tended to be lower in cases than ARV-exposed children, and higher in cases than ARV-unexposed children, except for complex IV activity, which was higher in cases.

Differences in the mitochondrial haplotypes of cases and children without signs of MD were assessed irrespective of ARV exposure and timing of specimen draws. Haplotypes A, C, D, H, I, J, L, U, and V were identified. Haplotype L was most common, occurring in 56% of cases and 40% of children without signs of MD. Because of the sparseness of data, for statistical testing haplotypes were grouped as H, L, or other. Although only marginally significant ($p = 0.09$), haplotype H was less common among cases ($N = 1$, 6%) than among children without clinical signs of MD ($N = 15$, 31%). As expected, haplotypes significantly differed by race/ethnicity: haplotype H was most common among non-Hispanic white children (56%), haplotype L was most common among non-Hispanic black children (77%), and all other haplotypes combined were most common among Hispanic children (55.0%). No mtDNA deletions were observed in PBMCs of any children.

Discussion

This is the first study to examine mitochondrial parameters in HIV-uninfected exposed children with and without signs of MD. Descriptive patterns of mitochondrial parameters according to ARV exposure are provided. In specimens drawn within 18 months of age we found higher mtDNA levels and lower mitochondrial enzyme activity in cases than children without signs of MD. The mtDNA trends observed in these 18 month specimens were similar to other recent studies.[4,5]

In specimens drawn at the time of presentation of MD signs and in matched children, mtDNA levels were lower in both cases and ARV-exposed children than ARV-unexposed children while OXPHOS enzymes tended to be higher in cases than ARV-unexposed children. This decreased mtDNA in cases and ARV-exposed children could reflect long-term effects of *in utero* ARV exposure following the initial compensatory increase detected in the 18 month specimens, even in the absence of clinically meaningful differences. The corresponding increase in OXPHOS activity could be a response to the decreased mtDNA replication. However, given the small sample size of our study, random error must be considered. Our small sample size allowed detection of a difference of approximately one standard deviation or more between cases and controls at 80% power.

It is also possible that the effect of *in utero* NA exposure on mitochondrial parameters may be most evident in the neonatal period; return to normal mtDNA levels has been documented in one child with NA-induced mitochondrial toxicity.[17] Our study was based on available PACTG stored

Table 2. MtDNA and OXPHOS Levels in Peripheral Blood Mononuclear Cells in Children with Possible Mitochondrial Dysfunction (Cases) and HIV-Uninfected Children, Unmatched Analysis

	Cases (median IQR)	*ARV-exposed children (median IQR)*	p-*value*[a]	*Antiretroviral-unexposed children (median IQR)*	p-*value*[a]
Specimens drawn within 18 months of age, unmatched analysis[b]					
	($N = 8$)	($N = 8$)		($N = 8$)	
MtDNA (copies/cell)	269 (205, 321)	206 (184, 270)	0.34	254 (171, 299)	0.68
OXPHOS protein levels (optical density)					
Complex I quantity	48.3 (40.9, 72.1)	48.3 (45.6, 60.6)	0.72	57.4 (37.9, 81.4)	0.80
Complex I activity	27.4 (16.3, 50.4)	36.8 (28.6, 50.5)	0.38	36.8 (25.9, 38.4)	0.67
Complex IV quantity	19.0 (6.0, 66.9)	22.7 (18.0, 48.3)	0.88	19.5 (10.3, 36.4)	0.96
Complex IV activity	26.6 (18.9, 47.0)	34.8 (23.7, 51.2)	0.51	33.4 (22.9, 39.2)	0.80
Specimens drawn or matched at the time of presentation of symptoms of mitochondrial dysfunction[c]					
	($N = 16$)	($N = 16$)		($N = 15$)	
MtDNA (copies/cell)	214 (156, 305)	215 (196, 311)	0.80	254 (170, 395)	0.41
OXPHOS protein levels (optical density)					
Complex I quantity	48.0 (38.9, 90.9)	67.4 (44.8, 114.9)	0.30	51.5 (37.9, 72.6)	0.85
Complex I activity	39.2 (17.8, 58.3)	52.4 (26.2, 71.9)	0.38	31.9 (19.4, 57.8)	0.52
Complex IV quantity	37.2 (6.9, 102.9)	40.5 (14.8, 135.3)	0.36	28.6 (5.1, 59.3)	0.66
Complex IV activity	45.7 (23.5, 52.1)	39.3 (28.0, 64.0)	0.80	34.9 (24.3, 45.6)	0.25

[a] *p*-value calculated from Wilcoxon rank sum test.
[b] One case unexposed to ARV *in utero* excluded from mtDNA and OXPHOS analysis, and one case with no protein excluded from OXPHOS analysis.
[c] Two cases unexposed to ARV *in utero* excluded from mtDNA and OXPHOS analysis, and one case with no protein excluded from OXPHOS analysis.

specimens, requiring the use of blood obtained at birth through 18 months of age, and this may have attenuated possible differences between cases and children without clinical signs of MD. Finally, we did not have mitochondrial histological or enzymological studies necessary for definitive mitochondrial disease case identification.[3] However, our findings do provide some support that our cases had signs of possible MD. To date, most studies of MD in HIV-infected children have focused on severe, persistent signs that likely do not characterize the true spectrum of mitochondrial impairment from *in utero* ARV exposure.

This is the first study to examine whether particular mitochondrial haplotypes were associated with mitochondrial phenotypes in a pediatric HIV-exposed cohort. Sequence analyses of mtDNA from different human populations have identified certain stable polymorphic patterns that distinguish major racial groups.[18] Although not statistically significant, in our study haplotype H was less frequent among cases than children without clinical signs of MD. Overall, more cases than noncases were non-Hispanic black and fewer were non-Hispanic white and Hispanic, but this racial distribution did not account for the differences in haplotypes.

The strengths of this study include matching to control for ARV exposure and possible confounders. We also attempted to control for freezer storage by matching on age at specimen draw, and for possible platelet contamination of mtDNA values relative to nuclear genes by measuring OXPHOS proteins and enzyme activity that are not affected by platelets. This first study of mitochondrial genetics and function in HIV-uninfected children with and without clinical signs of mitochondrial toxicity provides further information regarding MD from *in utero* NA exposure. The long-term significance of these small but potentially important alterations should continue to be studied as the population of HIV-uninfected ARV-exposed children enters adolescence and adulthood.

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