

Progressive depletion of mtDNA in mitochondrial myopathy

Abstract—The authors studied seven patients with mitochondrial DNA (mtDNA) myopathy. Over time, there was a progressive depletion of mtDNA, which preferentially affected wild-type mitochondrial genomes. This suggests that loss of wild-type mtDNA is a major feature of mtDNA myopathy, and preventing wild-type mtDNA depletion has treatment implications.

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Mitochondrial DNA (mtDNA) mutations can cause neurologic disease, which affects up to 1 in 8,000 adults. Both point mutations and deletions of mtDNA cause a biochemical defect of the respiratory chain, which often involves cytochrome *c* oxidase (Complex IV, COX). Clinical progression is accompanied by the accumulation of COX-deficient skeletal muscle fibers, and clinical improvement has been associated with a decrease in the frequency of COX-negative fibers.^{1,2}

Most patients with multisystem mtDNA disease harbor both wild-type and mutated mtDNA (heteroplasmy). High percentage levels of mutated mtDNA are associated with a biochemical defect at the cellular level and severe disease in patients. However, it is not clear whether the pathology is primarily due to high amounts of mutated mtDNA,³ low amounts of wild-type mtDNA,⁴ or a combination of both. To develop our understanding of this issue, we followed mtDNA disease progression in seven subjects, correlating the absolute amount of mutated and wild-type mtDNA with the histochemical defect.

Methods. *Patients and histochemistry.* We studied seven patients over a 4- to 14-year period (mean follow-up 7.3 years, 42.8% men; table). All patients were independently ambulant at the time of both biopsies. Four patients had a large-scale single deletion of mtDNA, and three had mtDNA point mutations previously shown

to be pathogenic. Muscle biopsies were taken from different regions of the same muscle and snap frozen in supercooled isopentane and stored in liquid nitrogen at -196°C . Sequential COX-succinate dehydrogenase histochemistry was performed on cryostat sections, and the percentage of COX-deficient fibers and ragged-red fibers (RRFs) was determined from a field of more than 200 muscle fibers.

Molecular genetics. Total genomic DNA was extracted from each biopsy. The percentage deleted mtDNA was determined by Southern blot analysis. PCR amplification of the major arc confirmed that each deletion did not involve the mtDNA *ND1* gene. For the point mutations, the percentage mutated mtDNA was determined by restriction fragment length polymorphism analysis of last-hot cycle radiolabeled PCR products. The relative amount of mtDNA was determined by real-time PCR using iQ Sybr Green on the BioRad ICycler (BioRad, CA) of an mtDNA target template spanning from nt 3459 to nt 3569 in the mtDNA *ND1* gene, and nt 804 to nt 903 of the nuclear DNA (nDNA) reference gene *GAPDH*. Each reaction was optimized and confirmed linear over an appropriate concentration range using genomic DNA standards. Samples were analyzed in triplicate for both assays, enabling calculation of the average mtDNA:nDNA ratio.

Results. There were no major histologic differences between the serial muscle biopsies taken from the same individual (figure E-1 on the *Neurology* Web site at www.neurology.org). There was histochemical evidence of progression in six of the seven patients. In four patients (57.1%), there was an increase in the percentage of COX-negative fibers, and in six (85.7%), there was an increase in the percentage of RRFs. The percentage of mutated mtDNA increased in four individuals and decreased in three. In all subjects, the total amount of mtDNA decreased over time (mean decrease = 5.49% per year, range 0.46% per year to 10.97% per year), even in patients who developed more RRFs. The amount of wild-type mtDNA also decreased in all subjects over time (mean decrease = 7.71% per year, range 1.43% per year to 17.99% per year).

The pattern was more complex for the amount of mutated mtDNA. The amount of mutated mtDNA decreased in six subjects and increased in one. Overall, the decrease in mutated genomes was less marked than the decrease in wild-type genomes, corresponding to the increase in the percentage of mutated mtDNA. However, in the three subjects where the percentage mutated mtDNA decreased over time, an increase in the number of COX-negative fibers was associated with a decrease of wild-type mtDNA. In addition, in the three subjects with minimal change in percentage of mutated mtDNA, clinical progression and a worsening histochemical defect were associated with a larger decrease in the amount of wild-type mtDNA than mutated mtDNA.

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Table Serial histochemical and molecular genetic findings in seven patients with mtDNA myopathy

| Patient sex/ age, y* | mtDNA mutation | Time between biopsies, y | COX-negative fibers, % | RRF, % | Mutated mtDNA, % | Ratio of mtDNA to nuclear DNA per diploid nuclear genome | | | | | |
|-------------------------|-------------------|--------------------------------|---------------------------|-----------|---------------------|--|---|--------------------|---|------------------|---|
| | | | | | | Total mtDNA | Percentage decrease in total mtDNA | Wild-type mtDNA | Percentage decrease in wild-type mtDNA | Mutated mtDNA | Percentage decrease in mutated mtDNA |
| F/18 | 3302A>G | 4 | 30 | 5 | 98 | 2,352.53 | — | 47.05 | — | 2,305.48 | — |
| | | | 30 | 10 | 99 | 1,320.32 | 43.88 | 13.20 | 71.938 | 1,307.11 | 43.30 |
| F/27 | 10010T>C | 5 | 30 | 30 | 91 | 477.71 | — | 42.99 | — | 434.72 | — |
| | | | 50 | 50 | 96 | 466.80 | 2.28 | 18.67 | 56.571 | 448.13 | -3.09 |
| M/13 | 5.2 kb deletion | 6 | 7 | 3 | 65 | 645.08 | — | 225.78 | — | 419.30 | — |
| | | | 7 | 5 | 73 | 330.08 | 48.83 | 89.12 | 60.527 | 240.96 | 42.53 |
| M/48 | 4.9 kb deletion | 12 | 0 | 0 | 23.5 | 1,722.16 | — | 1,317.45 | — | 404.71 | — |
| M/49 | 1 kb deletion | 14 | 0 | 0 | 7.8 | 1,024.00 | 40.54 | 944.13 | 28.337 | 79.87 | 80.26 |
| | | | 15 | 15 | 62.5 | 406.37 | — | 152.39 | — | 253.98 | — |
| F/33 | 5.77 kb deletion | 4 | 20 | 20 | 42.7 | 212.80 | 47.64 | 121.93 | 19.987 | 90.86 | 64.22 |
| | | | 27 | 10 | 67 | 630.35 | — | 208.01 | — | 422.33 | — |
| F/36 | 12320A>G | 6 | 30 | 15 | 71 | 456.14 | 27.64 | 132.28 | 36.408 | 323.86 | 23.32 |
| | | | 5 | 2 | 80 | 322.54 | — | 64.51 | — | 258.03 | — |
| | | | 9 | 3 | 74 | 222.86 | 30.90 | 57.94 | 10.176 | 164.92 | 36.09 |

* Age at first biopsy.

mtDNA = mitochondrial DNA; COX = cytochrome c oxidase; RRF = ragged red fibers.

Discussion. A decrease in the ratio of mtDNA to nDNA could arise through an increase in the number of cell nuclei within muscle fibers, a decrease in mitochondria-rich cells (such as muscle fibers), or an increase in the number of mitochondria-poor cells (such as resting fibroblasts, terminal atrophic fibers, or adipocytes). Careful examination of the muscle histology (figure E-1) did not support these mechanisms, pointing toward a primary alteration of mtDNA content within skeletal muscle fibers.

The proportion of COX-deficient fibers and the percentage mutated mtDNA defect varies within a single muscle in the same individual,⁵ raising the possibility that a difference between two biopsies could be a sampling effect and not a real change over time.⁵ This could explain some unusual findings in this study, such as the decrease in the percentage mutated mtDNA seen in two subjects. However, the overall pattern we observed was histochemical progression of mitochondrial myopathy accompanied by progressive depletion of mtDNA, preferentially affecting wild-type genomes. This suggests that the loss of wild-type mtDNA, rather than an increase in the percentage of mutated mtDNA, is the primary cause of the COX defect that underpins mtDNA myopathy.

Mitochondrial and mtDNA proliferation is a feature of mtDNA disorders, leading to the formation of RRFs in skeletal muscle.⁶ It is therefore intriguing that we saw depletion of mtDNA despite an increase in the percentage of RRFs. It is, however, noteworthy that the most dramatic *increase* in RRF was accompanied by the least dramatic *decrease* in mtDNA levels, in keeping with the view that mitochondrial proliferation attenuates disease progression. In addition, it is difficult to make direct

inference on the level of mtDNA within single cells from DNA extracted from a muscle homogenate, as described here. The most likely explanation is that mtDNA levels are increased in the RRFs but decreased in the other fibers. Further work at the single cell level will clarify these issues.

Muscle deconditioning is common in patients with mitochondrial disease⁷ and also occurs as part of normal aging. Although controversial,⁸ there is evidence that muscle mtDNA levels are reduced in healthy elderly subjects,⁹ and the same process may be contributing to the loss of mtDNA that we have observed in patients with mtDNA myopathy. However, it is difficult to explain the preferential loss of a specific subgroup of mtDNA molecules within the cell (wild-type genomes, rather than mutated mtDNA) solely on the grounds of muscle aging. By demonstrating the importance of wild-type mtDNA in clinical progression, our observations suggest that an increase in the percentage of mutated mtDNA after exercise training should not be a major concern, at least in the short term.¹⁰ The focus of any therapy should be to maintain or increase wild-type mtDNA levels.

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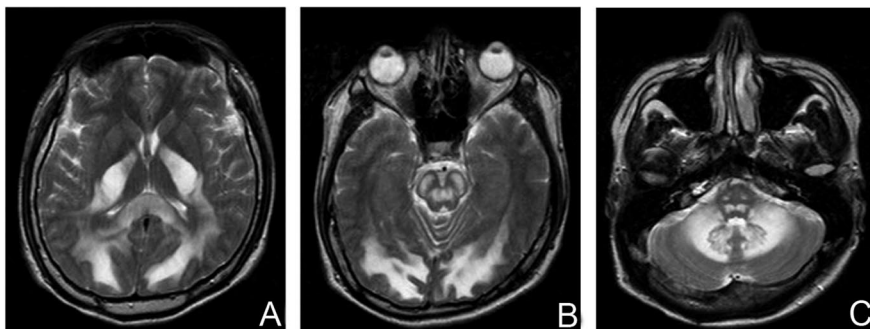


Figure 1. Axial T2-weighted MR images (repetition time/echo time: 4,000/99) show symmetric hyperintensity of the affected posterior cerebral (A), pons (B), and cerebellar (C) white matter. The cortex, basal ganglia, and thalami were spared.

MRI features of spongiform leukoencephalopathy following heroin inhalation

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A 26-year-old man with a history of heroin inhalation presented with impaired motor, speech, and cognitive functions, reduced muscle strength, hyperactive deep tendon reflexes, and intact light-tough, vibration, and pinprick sensitivity.

T2-weighted MRI revealed symmetric hyperintensity in white matter tracts, consistent with spongiform degeneration and demyelination (figure 1).¹ Involvement of these white matter tracts and posterior limb of internal capsule, with sparing of the cortex and basal ganglia, is a characteristic finding in heroin-induced leukoencephalopathy, helping to distinguish it from other causes of leukoencephalopathy. ¹H MRS revealed a decreased N-acetylaspartate/creatinine (NAA/Cr) ratio and a doublet lactate peak (figure 2), indicating mitochondrial dysfunction and neurotoxicity.² MRI may precisely reveal the distribution of white matter abnormalities in patients with heroin-induced leukoencephalopathy, and ¹H MRS, although not essential for the diagnosis, can help further elucidate the condition.

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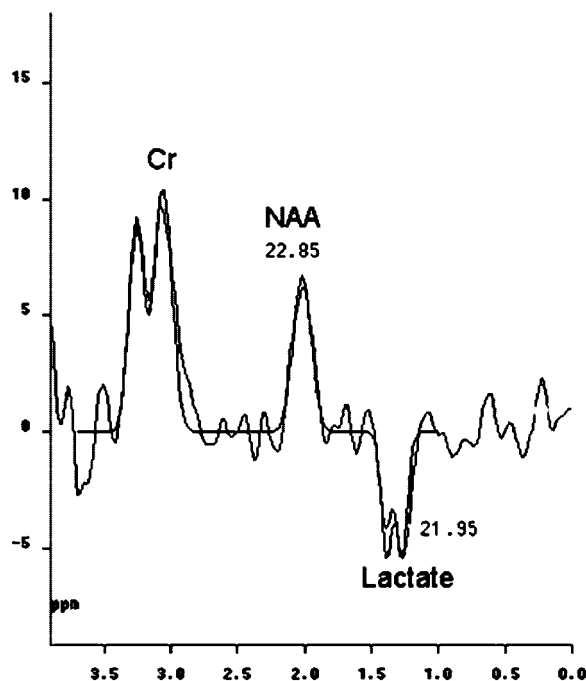


Figure 2. ¹H MRS (repetition time/echo time: 1,500/135) reveals a decreased NAA/Cr ratio and a negative doublet of lactate.

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