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# Contribution of de novo point mutations to the overall mutational burden in mitochondrial DNA of adult rats

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**Abstract**

This study analyzed the incidence of point mutations in mitochondrial DNA of brain and muscle tissues from young (6-month) and old (24-month) male F344 rats. Coding sequence mutations in subunit 5 of the NADH dehydrogenase gene were detected after high-fidelity PCR amplification and cloning by denaturing gradient gel electrophoresis (DGGE) assay followed by sequencing of detected mutants. In total, almost a thousand individual clones were analyzed both in brain and muscle samples. On average, mtDNA from brain tissue showed a 66% increase with age in mutation frequencies ($2.3 \pm 1.9$ vs. $3.8 \pm 4.5 \times 10^{-4}$ mutations/bp, mean $\pm$ SD), which failed to reach statistical significance ($p = 0.45$). Muscle tissues yielded substantially fewer mutants with average mutant frequencies for both young and old rats almost 10 times lower than the corresponding values in the brain tissue ($0.3 \pm 0.4$ and $0.5 \pm 0.6 \times 10^{-4}$, respectively). The difference in mutation accumulation between muscle and brain was highly significant in both the younger group (Chi-squared = 9.7, $p \leq 0.01$) and in older animals (Chi-squared = 10.9, $p \leq 0.001$). Molecular analysis of the mutated sequences revealed that almost half were identical sequences recurring in different samples and tissues. Our findings indicate that the process of mutation accumulation in mitochondria begins in the germ-line and/or during earlier stages of life, contributing up to half of the total mutational burden, whereas de novo spontaneous formation of point mutations in adulthood is far less than was anticipated.


*Keywords:* Mitochondrial DNA; Point mutations; Aging; Rats; Brain; Muscle

## 1. Introduction

Mitochondrial theories of aging have been proposed in which senescence is attributed to a vicious cycle created by the elevated exposure of mitochondria, and in particular, mitochondrial DNA (mtDNA), to free radicals leaked from the electron transport chain (Harman, 1972; Miquel et al., 1980). A portion of oxidative DNA damage is converted into mutations leading to synthesis of defective proteins, which in turn causes ever-increasing production of oxidants. There are several lines of evidence indicating that these organelles may be a weak link in terms of aging. Mitochondria possess several unique features setting them apart from other organelles. Functionally, mitochondria are responsible for production of ATP via oxidative phosphorylation, regulation of apoptosis, and calcium signaling. They are the main site of oxygen consumption and reactive oxygen species (ROS) production. Mitochondria harbor a second genome, which codes for key components of the electron transport system (ETS). By virtue of its close proximity to the ETS machinery and its asymmetric replication mechanism, mitochondrial DNA may be subjected to considerably higher oxidative damage (Richter et al., 1988; Mecocci et al., 1993) repaired less efficiently than similar lesions in the nuclear DNA (Croteau et al., 1999).

These factors combine to produce mutation accumulation in mitochondrial DNA (mtDNA) that is dramatically more severe than in the nucleus. A considerable fraction of the mtDNA collected from old individuals comprised of a variety of truncated molecules (Kovalenko et al., 1998).

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The incidence of specific point mutations tends to increase with age and may accrue to more than 2% of the total mtDNA pool in post-mitotic tissues (Munscher et al., 1993), although this increase is not observed (Pallotti et al., 1996). As a result, mtDNA exhibits 1000–10,000-fold higher mutation frequencies than nuclear DNA, thus affecting the performance of mtDNA-encoded components of enzymatic complexes (Cortopassi and Wong, 1999; Ferrandiz et al., 1994). That aged mitochondria contribute to the deteriorative process is suggested by microinjection experiments in which introduction of mitochondria from senescent cells resulted in the degeneration of young recipient cells (Corbisier and Remacle, 1990).

Organs comprising non-dividing cells may be especially vulnerable to mitochondrial deterioration as dysfunctional cells cannot be replaced. The accumulation of mutations in mitochondrial DNA has been implicated in a range of age-dependent degenerative pathologies including Parkinson's disease (Sherer et al., 2002) and Alzheimer's disease (Castellani et al., 2002). Thus, given the possible role of mitochondria in aging, it would be of interest to know the actual mutational load in mtDNA resulting from accumulated point mutations in post-mitotic tissues.

## 2. Material and methods

### 2.1. Animals

All animal procedures were performed in accordance with institutional guidelines for the care and use of laboratory animals. Male Fisher 344 rats at 6 and 24 months of age (each $n=6$) were purchased from the National Institute on Aging and housed in accredited facilities on a 12:12 light/dark cycle with ad libitum food and water. At the time of sacrifice, rats were anesthetized with ether and exsanguinated by cardiac puncture. Brain and plantaris muscle were dissected, frozen in liquid nitrogen, and stored at −80 °C until DNA isolation and analysis. Rats were assigned identification numbers YM11–YM16 for young, and OM11–OM16 for old males.

### 2.2. Mitochondrial DNA isolation

Total DNA was extracted from cerebral cortex (entire right hemisphere) and whole plantaris muscle using the DNeasy Tissue Kit (Qiagen, Valencia, CA). The presence of mtDNA was verified by electrophoresis on 0.8% agarose gels containing 0.5 μg/ml ethidium bromide. Bands corresponding to full-length mtDNA were excised and extracted using the QIAEX® II Gel Extraction System (Qiagen, Valencia, CA).

### 2.3. PCR amplification, cloning and sequencing

PCR amplifications were carried out with a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) using forward primer CATTCTCAACCTCCCTAG and reverse primer TTGATTAGTCCTTTTTGG (GenBank Accession # NC001665), yielding a 193 bp fragment from the 3′ end of the NADH dehydrogenase gene, subunit 5 (ND5, 13282–13474). The reactions were performed in a 30 μl volume containing 100 ng of mtDNA template, 0.6 μl (1.5 units) of *Pfx* polymerase (Invitrogen, Carlsbad, CA), 25 μM nucleotide triphosphate mix, and the buffer supplied by the manufacturer. Thermocycling consisted of an initial period of denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 68 °C for 2 min, followed by a final extension step at 68 °C for 10 min. Approximately, 5 μl of the PCR products were loaded on a 1% agarose gel, electrophoresed, and visualized after staining with 0.5 μg/ml ethidium bromide. Amplified ND5 fragments were cloned using the Zero Blunt TOPO PCR™ Cloning kit (Invitrogen). Plasmids from the individual bacterial colonies were purified using the Qiagen QIAprep Spin Miniprep kit (Qiagen) and verified for the presence of inserted fragments by electrophoresis on 1% agarose. The plasmids carrying inserts were sequenced using an SEQ8000 DNA sequencer (Beckman Coulter Inc., Fullerton, CA) according to the manufacturer's instructions.

### 2.4. DGGE analysis

The method used for detection of fragments carrying mutations is shown in Fig. 1. After DNA purification, aliquots from 3 to 4 individual plasmids were mixed. These mixes served as templates for the second PCR reaction with GC-clamp forward primer CGCCCGCCGCGCCCCGCG CCCGTCCCGCCGCCCCCGCCCGCATTCTCAACCTC CCTAG using the same PCR conditions. PCR reaction products (approx. 15 μl) were loaded on denaturing gradient (0–60% urea/formamide), 8% acrylamide gels and then electrophoresed at 62 °C, 80 V for 16 h. Bands were visualized using 0.5 μg/ml ethidium bromide under ultraviolet transillumination.

### 2.5. Statistical analysis

All statistical analyses were performed using the Data Analysis facility in Microsoft Excel. The Chi-squared test was used to compare the proportion of mutated fragments in brain vs. muscle for both young and old animals.

## 3. Results

### 3.1. DGGE analysis of mtDNA from brain and muscle samples

A novel aspect of our approach is that we pre-screened pools of mtDNA clones—representing a portion of the coding sequence of NADH dehydrogenase, subunit 5—by denaturing gradient gel electrophoresis (DGGE), and then