FUNCTIONAL AND MOLECULAR CHANGES OF MITOCHONDRIA IN HUMAN AGING: OBSERVATIONS IN DIVIDING TISSUES

A thesis submitted in the fulfillment of Master of Medicine

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July 2000

This thesis is printed on acid-free paper
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DECLARATION

This thesis contains no material which has been submitted for the award of another
degree at any university or institution. All the experiments described in this thesis
were conducted by the candidate except where due acknowledgment has been made,
and no material in this thesis has been previously written or published by another
person.

This thesis contains less than 27,000 words in length excluding figures, tables and
references.

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July, 2000
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SUMMARY

Studies in a number of human tissues have revealed that the activities of mitochondrial respiratory chain enzyme complexes decline during the aging process. Other studies have suggested that aging increases the frequency of mitochondrial DNA (mtDNA) mutation and leads to the accumulation of mutant mtDNA species, mainly those with large deletions and point mutations. Although the mitochondrial theory of aging may be more applicable to post mitotic tissues, abnormalities of mtDNA have also been reported in tissues which retain a mitotic capacity. Fresh tissues from elderly patients are difficult to obtain and only a limited number of studies on biochemical examination of respiratory chain enzyme complex activities have been carried out. Prostate tissue is readily available in elderly male subjects because of the high prevalence of benign prostatic hypertrophy in this sub-group of the population, and endoscopic surgery is routinely performed for excision of the diseased prostate. In this study, mitochondrial respiratory function and the mtDNA mutations in prostate tissues of elderly patients (aged from 56 to 92) were studied in 24 subjects. This included the measurement of the activities of the respiratory chain enzyme complexes and screening for mitochondrial point mutations and deletions at sites commonly affected in neurodegenerative diseases. There was no correlation between increasing age and the respiratory enzyme complex activities from age 56 to over 90 years old as represented by complex I (correlation co-efficiency, r=0.58), complex II & III (r=0.15), or complex IV (r=0.12 ). MtDNA point mutations commonly associated with known diseases were screened. Those included nt 8344A→G, 3243A→G, 3460A→G, and LHON 11778G→A, with negative results in all cases. Three species of deleted mtDNA were identified with polymerase chain reaction and confirmed by primer shift assay. These included the common deletions (4977 bp from nt 8483 to nt 1359; 2/25), and other two large deletions.
These did not occur in an age related manner in prostate tissues. In conclusion, the absence of negative correlation between increasing age and respiratory enzyme complex activities and of a positive correlation between the increased mtDNA mutation and aging observed in this fresh prostatic tissues indicates that mitochondrial respiratory failure is not a universal phenomenon in all aging tissues and that low levels of deleted mtDNA species are a biological phenomenon which neither correlates to advancing age directly nor determines the mitochondrial respiratory function deficiency. These results do not exclude the possibility that mutant mtDNA species accumulate during aging in other tissues and that may still be a factor in acceleration of tissue senescence and induction of age-related mitochondrial diseases.

It was not possible to identify a subgroup of elderly patients with low prostate oxidative phosphorylation (OXPHOS) capacity who may be predisposed to cerebral manifestations of OXPHOS deficiency. My attempt to identify a measure of OXPHOS capacity in elderly people in readily accessible tissues (blood, skin and muscle) may not give useful information about the situation in the central nervous system (CNS) if a similar situation to that found in prostate pertains.


**Weng S**, Kapsa R, Costello A, Jean-Francois B, and Byrne E. Biochemical and molecular studies in ageing human prostate; A window on mitochondrial dysfunction in senescence. (in preparation).
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<td>NARP</td>
<td>Ataxia and retinitis pigmentosa syndrome</td>
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<td>ANT</td>
<td>adenine nucleotide translocase</td>
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<td>B.P.H.</td>
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<td>BIMC</td>
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<td>complex I (NADH)</td>
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<td>LIMD</td>
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<td>Luft’s disease</td>
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<td>OXPHOS</td>
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<td>transfer RNA</td>
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CHAPTER 1

LITERATURE REVIEW

1.1. Structure and distribution of mitochondria

Mitochondria, evolved from free-living aerobic bacteria over a billion years ago, are the major cellular energy plant in both vertebrate and non-vertebrate species. They are semi-autonomous, containing their own genetic system which contributes partially to the cellular regulation of their biogenesis, number, size and function.

Morphologically, mitochondria are small cylinder-like or slender rod-like intracellular micro-organelles which reside in the cytoplasm. Electron microscopic studies have revealed the structure of mitochondria. Figure 1.1. shows the mitochondrial structure in eukaryotic cells. Each mitochondrion consists of two layers of membranous sacs, the inter-membrane space and the matrix. The outer membrane is a thin layer of smoothly contoured ring, the inner membrane runs in parallel to the outer membrane with a series of finger-like enfoldings (also known as cristae) that project into the matrix. This plication of the inner membrane provides an extended area for docking various enzymes and enzyme complexes. The number of cristae in each mitochondrion varies depending on the energy demand of their host cells. There are many more cristae in cells with a high energy turnover such as cardiomyocytes and skeletal muscle cells than in those with a lower rate of metabolism like bone cells. The two membranes differ markedly in terms of their protein and lipid composition. The outer membrane contains proteins that form transmembrane channels while the inner membrane is rich in enzymes and provides an effective permeation barrier to cations
such as protons, potassium and calcium. This property allows the organelle to establish stable ion gradients across the inner-membrane partly for the regulation of ATP synthesis (see below). The matrix contains many mitochondrial enzymes for metabolic functions.


In a cell, the number of mitochondria is positively correlated with the metabolic energy requirements. For example, in heart and skeletal muscle cells, mitochondria are more densely populated while in brain cells, the numbers are much less (Bogenhagen and Clayton, 1974; Velti et al., 1990). During oogenesis, the number of mitochondria increases by approximately a hundred-fold as compared to normal cells (Marinos and Billett, 1981; Nogawa et al., 1988).
1.2. Functions of mitochondria

1.2.1. Bio-energy production

Mitochondria accommodate enzymes for the production of bio-energy in the form of ATP. Fatty acids, pyruvate derived from carbohydrate glycolysis, and amino acids are transported into mitochondria through carriers or translocases which are then further oxidized into acetyl-CoA in the mitochondrial matrix through β-oxidation (fatty acids) or by pyruvate dehydrogenase (pyruvate). The acetyl-CoA is further oxidized via the chain reactions known as tricarboxylic acid (Krebs’) cycles and the reducing equivalents (electrons) produced in the process were translocated along the respiratory chain during the oxidative phosphorylation (OXPHOS) reactions. The ATP molecules are generated (Figure 1.2.).

1.2.1.1. Oxidative phosphorylation

The oxidative phosphorylation pathway, namely the respiratory chain, contains five (I-V) intra-mitochondrial protein-lipid enzyme complexes which are assembled from over a hundred polypeptides. They are complex I (NADH : ubiquinone oxidoreductase), complex II (succinate : ubiquinone oxidoreductase), complex III (ubiquinol : ferrocytochrome c oxidoreductase), complex IV (ferrocytochrome c : oxygen oxidoreductase or cytochrome C oxidase), and complex V (ATP synthetase). All of the enzyme complexes are located in the mitochondrial inner membrane. The OXPHOS reaction starts with collecting electrons from various sources (including NADH and succinate) by complex I or II. The electrons are sequentially transferred to ubiquinone (coenzyme Q10), complex III, cytochrome c, complex IV, prior to reacting
with oxygen, the terminal electron acceptor. The electron equivalents, the protons, are pumped out of the inner mitochondrial membrane which occurs after the reactions by complexes I, III and IV rendering the formation of proton gradients across that membrane. Potential energy stored within these gradients is utilized by complex V to convert ADP into ATP through phosphorylation of the hydroxyl group in the ADP molecule. Exchange of ATP across the inner mitochondrial membrane with ADP is achieved by the adenine nucleotide translocase (ANT) with concomitant release of energy for cellular activity.

![Diagram of cellular energy production via oxidative phosphorylation in mitochondria](image)

**Figure 1.2.** Cellular energy production via oxidative phosphorylation in mitochondria. PDH: Pyruvate dehydrogenase; β-OX: β-oxidation, TCA: tricarboxylic acid cycle (Sottocasa et al., 1967; Takamiya et al., 1986).

Up to 36 molecules of ATP are produced from each molecule of glucose by way of aerobic glycolysis within mitochondria; in contrast, only two molecules of ATP are formed from anaerobic glycolysis in cytosol.
1.2.2. Other functions

Mitochondria also play an important role in regulating calcium homeostasis (Brdiczka Barnard, 1980; Nicholls, 1985; Richter and Frei, 1988) and recently it has been demonstrated that mitochondria play roles in initiating and regulating programmed cell death (Schapira, 1997; Hampton et al., 1998; Kruman and Mattson, 1999).

1.3. The mitochondrial genome

Mitochondria have a limited life span, but they are self-replicating organelles, possessing their own genome which in combination with the nucleic genome, controls their replication and functions. Mitochondria maintain their numbers by a form of division which resembles the binary fission of bacteria.

The mitochondrial genome encodes some of the key components for mitochondrial peptide synthesis and forming part of the respiratory chain. The genome size varies from 15 kb in some animals to over 1000 kb in certain plants such as the muskmelon which has mitochondrial DNA of 1056 kb (Brown et al., 1979; Ward et al., 1981). The human mitochondrial DNA (mtDNA) consists of 16,569 bp. It lacks stretches of non-coding sequence with the exception of a small area, the D-loop. It encodes for the two mitochondrial ribosomal RNA (rRNA) and twenty-two transfer RNA (tRNA), 13 messenger RNA (mRNA) for the polypeptides of OXPHOS (Borst, 1970; Anderson et al., 1981; Clayton, 1992, Figure 1.3.). The two mitochondrial DNA strands are asymmetric in their distribution of guanine and cystine residues which results in different buoyant densities in cesium chloride gradients. The guanine-rich strand is referred to as the heavy (H) chain and the cystine-rich one as the light (L) chain (Clayton et al., 1970; Berk and Clayton, 1974). Each strand has its own
replication initiating site but the two are in opposite directions (Aloni and Attardi, 1971; Murphy et al., 1975; Walberg and Clayton, 1983; Chang and Clayton, 1986). The H-strand encodes polypeptides forming 12 OXPHOS subunits, the 12S and 16S rRNA and 14 tRNA; the L-strand encodes for polypeptides for the remaining 8 tRNA but only one OXPHOS subunit (ND6 of Complex I). Nevertheless, the majority of the OXPHOS components are derived from nuclear genome (up 70 OXPHOS subunits) which encompass all complex II subunits, the remaining subunits for complexes I, III, IV and V, as well as proteins required for mtDNA replication, transcription and translation. Mitochondria respiration relies on a coordinated expression of nuclear and mitochondrially-encoded subunits (Davis et al., 1978; Kholodenko, 1984; Zeviani et al, 1990). Depending on the cellular energy requirement, up to several thousand copies of mtDNA can be present in one cell (Bogenhagen and Clayton, 1974) and on the other hand, some cells only need as low as a few copies such as four copies in platelets (Shuster et al., 1988).
Figure 1.3. The organization of human mitochondrial genome. Genes are represented by differently shaded areas. The inner cycle represents the light strand; the outer cycle represents the heavy strand. OL: light strand origin; OH: heavy strand origin. Single letters are the amino acids whose positions represent the genome locations of their corresponding tRNA genes. (Chomyn et al., 1985; Chomyn et al., 1986; and Anderson et al., 1981).

MtdNA uses a different genetic coding system from nucleic DNA (nDNA) for polypeptide translation. In nuclei, there are 64 codons which require 32 tRNA species to fulfill the protein translation process. Among them 61 are used for the translation of 20 amino acids and 3 for translation terminating signals. However, in mitochondria, there are only 22 mitochondrial tRNA species responsive for polypeptide translation. This is due to the simplified codon-anticodon pairing in mitochondria: One mitochondrial tRNA can recognise a four-member codon family (Benne and Sloof, 1987; Demengeot and Besson, 1996). Furthermore, the mitochondrial stop codons are
also different from those in nucleic DNA (UGA for nDNA and AGA, AGG for mtDNA).

13.1. Regulation of OXPHOS

Despite an apparent functional equivalence of mtDNA in all cell-types, OXPHOS activity exhibits tissue and developmental stage-specificity. In human, following birth and during the first year of life, the OXPHOS activities increase rapidly and reach adult levels by 2 years of age (Chugani et al., 1987). The activities continue to increase and at 3-4 years of age they exceed the adult levels. After 9 years of age, the activities return gradually to adult levels. Such changes can be due to the presence of tissue and developmental stage-specific isoforms of different enzyme complexes (complexes I, II and IV) which regulate the mtDNA replication rate and/or nucleic-cytoplasmic interactions differently (Davis and Davis-ven-Thienen, 1978; Kholodenko, 1984; LaNoue et al., 1986). The OXPHOS activities in different tissues also differ mainly depending on the energy requirement (Tsyganni et al., 1991). However, a recent study of mitochondrial function during human cardiac growth and development demonstrated that there was no change in cytochrome c and complex IV activities at various stages (Marin-Garcia et al., 1998).

Transcriptional controlling elements and environmental factors are also important in regulation of OXPHOS activities. Trans-activators such as nuclear respiratory factor 1 [NRF-1] and nuclear respiratory factor 2 [NRF-2] coordinate the expression of a variety of OXPHOS polypeptides (Evans and Scarpulla 1989, 1990; Virbasius and Scarpulla 1991, 1994; Chau et al., 1992); Mt1, Mt3 and Mt4 sequence element factors
Suzuki et al. 1991) which possess both nuclear and mitochondrial binding sites, determine the tissue-specific regulation. For example, the OXBOX [13 bp] binding factors are only found in myogenic cells and are muscle specific while the REBOX [8 bp] binding factors are found in all cells (Chung et al., 1992). Other factors such as hormone levels can also affect OXPHOS activity by way of regulating one or more OXPHOS subunits. It has been shown that in the rat thyroid hormone triiodothyronine regulates the expression of a selected set of nuclear genes encoding mitochondrial inner membrane proteins. For example, the expression of cytochrome c1 mRNA increased 20- to 50-fold upon the hormone treatment (Satav and Katyave, 1982; Joste et al., 1989). Further studies revealed that triiodothyronine increases the promoter activities of cytochrome c and the adenine nucleotide translocator-2 (ANT2) in adult rat liver since it activates the expression of a reporter gene expression driven by either cytochrome c promoter or the adenine nucleotide translocator-2 (Li et al., 1997). In addition, oxygen supply and ion gradients across the inner mitochondrial membrane also influence OXPHOS activity (Spieckermann et al., 1980; Beal, 1995; Wilson, 1995).

1.3.2. Mitochondrial genetics

The mitochondrial genome is unique not only because of its extra-chromosomal location and the presence of multiple numbers in each mitochondrion, but also because of its distinct characteristics such as maternal inheritance, heteroplasmic tolerance, replicative segregation, threshold expression, developmental and stage-specific isoforms of OXPHOS expression, a high mtDNA mutation rate and a propensity for accumulation of somatic mtDNA mutations with age.
1.3.2.1. Maternal inheritance

In vertebrates, mtDNA is almost exclusively maternally inherited (Dawid and Blackler 1972; Kroon et al., 1978; Avise et al., 1979; Lee and Taylor 1993). During the formation of zygotes, only the maternal mtDNA is transmitted. As a result, most mtDNA encoded mitochondrial diseases are only passed on by mothers (Bacino et al., 1995; Rawson et al., 1996). Paternal mtDNA transmission is very rare and was reported to occur in only 1 in 10000 in mice (Gyllensten et al., 1991; Holme et al., 1995).

1.3.2.2. Replicative segregation and threshold effect

Under normal circumstances the multiple copies of mtDNA in each cell are identical (known as homoplasmic mtDNA; Moore et al., 1977), but mtDNA variants are frequently generated due to the high incidence of pro-mutation environment and the mutant variants can co-exist with the wild-type. This co-existence is known as heteroplasmic mtDNA. During cell proliferation, mitochondria replicate, mtDNA variants are randomly transmitted into newly formed mitochondria which are again randomly segregated into the daughter cells (Lukins et al., 1973; Jenuth et al., 1996). These result in the heterogeneous mtDNA genotype among the daughter cells, varying from cells containing entirely normal to those containing high percentage or exclusively mutant mtDNA (Figure 1.4.).

There is a critical tolerable level for mutant mtDNA co-existing with wide-type mtDNA. The normal mitochondrial functions will be preserved when mutant mtDNA level is below the critical level. mitochondrial dysfunction will occur otherwise (threshold tolerance, Shoffner et al., 1990; Yoneda et al., 1994; Schnopp et al., 1996).
The threshold differs from tissue to tissue. It depends on the metabolic energy requirement, the type and the severity of mutations, the level of heteroplasmy. In post-mitotic tissues requiring high energy if deleterious mtDNA mutations occur in regions which preserve the origins of replication of both mtDNA strands, it appears that these mutants are retained and continue to replicate. In contrast, deletions which disrupt one or more of the protein subunits or tRNAs are less well tolerated. The frequency of reaching the critical level of the mutant mtDNA may also vary in accordance with the rate of cell division. Fixed post-mitotic tissues tend to accumulate mutant mtDNA and are prone to display phenotypic abnormality (Mizusawa et al., 1988; Singh et al., 1989; Bresolin et al., 1991) while rapidly dividing cells appear to dilute the mutant mtDNA after certain cycles of cell division. The mechanisms are unclear but one contributing factor may be the rapid cell division which requires the rapid replication and segregation of mtDNA. That results in the greater mtDNA replication rate than the mutant mtDNA accumulation rate. In that case, the ratio of mutant mtDNA to wild type become smaller as cell dividing. (Blanchard et al., 1993; Biagini et al., 1998; Siregar et al., 1998). A study on the tissue distribution of mutant mtDNA in patients clinical symptoms of mitochondrial encephalomyopathy, lactic acidosis, and stroke-like syndrome (MELAS), demonstrated that the non-dividing skeletal muscle and the low energy requiring tissue hair follicles have easily detectable mtDNA nt3243A→G point mutation while the rapidly dividing tissue blood showed inconsistent results with only 50% of the measured samples containing detectable levels of the mtDNA nt3243A→G mutation (Sue et al., 1998, Figure 1.5.). Similarly, in *in vitro* exhaustive cell growth studies, Siregar et al. (1998) showed that myoblast cells with up to 80% of the mutant mtDNA gradually lose the mutant population cells.
continued to divide resulting in only the wild-type population existing in the cells after the 14th cell passage.

![Diagram of mitochondrial proliferation and random segregation]

Figure 1.4. Schema of random mitochondrial replicative segregation and the threshold effect for phenotype display (Lukins et al., 1973; Shoffner et al., 1990; Yoneda et al, 1994; Schnopp et al., 1996; Jenuth et al., 1996).
Figure 1.5. Schematic representation of the fate of heteroplasmic mtDNA in different cell types. Open circle: wild type mtDNA; filled circle: mutant mtDNA. (a): muscle cells. These cells do not divide. No selection for preferential survival can occur. The mtDNA mutation persists. (b): hair follicles. These cells are low energy requiring cells. The mutant mtDNA has little effect on cell metabolism and there is no survival advantage for those cells with more wild type mtDNA. So, mtDNA mutation persists. (c): blood cells. These cells have high energy requirements and only those with more wild type mtDNA are selectively survived. So, the mtDNA mutation disappears (adapted from Sue et al., 1998).
1.4. Mitochondrial DNA mutations and related diseases

1.4.1. High mutational rate of mitochondrial DNA

MtDNA is in close proximity to oxygen radicals which are generated as by-products during mitochondrial respiration. The mutation rate of mtDNA is about ten times higher than that of nuclear DNA (Fleming et al., 1982; LeDoux et al., 1992). Moreover, there are less protective histones in the mitochondrial genome (Richter and Frei, 1988; Wei et al., 1998) and no associated proteins within the mitochondria to perform nucleotide excision repair as in the nucleus (Clayton et al., 1974; LeDoux et al., 1992). These conditions predispose to mtDNA mutations and to accumulation of the mutant species. Free radicals can cause point mutations, or DNA strand breakage resulting in mtDNA deletion or direct repeats by intra-molecular recombination (Figure 1.6.). Over 30 mtDNA point mutations and 100 mtDNA rearrangements have now been identified as mitochondrial disease related mutations (Cormier et al., 1990, 1991; Brown and Wallace, 1994, Figure 1.7.). Target organs tend to be those with high bioenergetic requirements such as the CNS, heart, skeletal muscle, liver, kidney, and optic nerve.

The production of free radicals in mitochondria can be triggered by the cytoplasmic and the intra-mitochondrial micro-environment. Certain chemicals or radiation are well known inducing agents for free radical generation. Some of those agents can in fact directly cause mtDNA mutations. In mitochondria, the oxidative metabolites, nitric oxides, phospholipid metabolism and proteolytic reactions are potential sources of intracellular free radicals (Simonian and Coyle, 1996). It has been shown that β-amyloid which is abundantly found in Alzheimer's disease (AD) patients can induce
free radical formation (both directly and through the interaction with endothelial cells, Thomas et al., 1996; Schapira, 1996). The oxidative stress caused by the imbalance between the production of free radicals and the ability of the host cell to defend against them is one of the major causes of mtDNA mutation.

1.4.2. The pathophysiological role of mitochondrial DNA mutations

A causal link between mitochondrial dysfunction and human diseases was first noted in 1962 by Luft et al. (1962) who found that a woman with a hypermetabolic state had abnormal uncoupled OXPHOS functions (Luft’s disease). Subsequently defects in mitochondrial functions were implicated in over 100 diseases which were found to be associated with either nuclear or mitochondrial DNA mutations (Shy and Gonatas, 1964; Minchom et al., 1983; Wallace et al., 1988; Tanaka et al., 1990; Tritschler et al., 1991; Lin et al., 1992; van den ouweland et al., 1992; Ballinger et al., 1992). General features common to this group of diseases include myopathy, dementia, movement and other neurological disorders, deafness, blindness, cardiac failure, diabetes, renal dysfunction, and liver diseases. One of the definitive “hallmarks” of mitochondrial disease remains the observation of ragged red fibres (Engel and Cunningham, 1963) which is caused by subsarcolemmal aggregation of abnormal mitochondria shown by Gomori-trichrome staining of muscle biopsy (Figure 1.8.). The potential deleterious effect of a mutation is further compounded by its mitochondrial genome location, the expression level, and the metabolic rate of the tissues (the threshold effect; see section 1.3.2.2.).
Figure 1.6. A slip-replication model results in mtDNA deletions.
Figure 1.7. Schema of the genome location and the variants of commonly occurring mitochondrial DNA mutations, and their associated mitochondrial diseases. * indicates point mutations; H the regions where deletions could occur; # positions of possible duplications. OH: open reading frame for heavy strand replication; OL: open reading frame for light strand replication; HSP: heavy strand promoter; LSP: light strand promoter (Goto et al., 1990a; Wallace et al., 1988; Brown et al., 1992a, 1992b; Moraes et al., 1989; Singh et al., 1989; Rotig et al., 1989; Kleinle et al., 1997).
Figure 1.8. Illustration of ragged-red fibers (RRF, white stars) in muscle from a patient with mitochondrial myopathy using modified gomori trichrome staining for succinate dehydrogenase. x250 (DiMauro, 1993).

Mitochondrial diseases can be classified into four groups according to the locality and the pattern of gene mutation.

Class I - Disorders of the nuclear-encoded genes (Table 1.1.)
Class II - MtDNA point mutations (Table 1.2.)
Class III - MtDNA deletions and duplications (Table 1.3.)
Class IV - Disorders of unknown inheritance (Table 1.4.)
Table 1.1. Class I mutations  
Disorders due to Mutations of the Nuclear OXPHOS Genes

<table>
<thead>
<tr>
<th>Diseases</th>
<th>References</th>
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<tbody>
<tr>
<td>Benign Infantile Mitochondrial Myopathy (BIMM)</td>
<td>Tritschler et al., 1991.</td>
</tr>
<tr>
<td>Lethal Infantile Mitochondrial Disease (LIMD)</td>
<td>Minchom et al., 1983.</td>
</tr>
<tr>
<td>Benign Infantile Mitochondrial Myopathy and Cardiomyopathy (BIMC)</td>
<td>DiMauro et al., 1980; Bolhuis et al., 1991.</td>
</tr>
<tr>
<td>Lethal Infantile Cardiomyopathy: X-Linked Cardioskeletal Myopathy (Barth syndrome)</td>
<td>Barth et al., 1983.</td>
</tr>
</tbody>
</table>
| Chronic External Ophthalmoplegia Plus (KS/CPEO) Syndromes:| (a) Cormier et al., 1991  
(b) Mizusawa et al., 1988. |
| a) Autosomal Dominant Inheritance                         |                                                |
| b) Autosomal Recessive Inheritance                         |                                                |
| Inherited Exertional Myoglobinuria                        | Ohno et al., 1991.                            |
| Leigh Disease (Subacute Necrotizing Encephalopathy)       | Miranda et al., 1989.                        |
| Mitochondrial Myopathy                                    | Hudgson et al., 1972; Mechler et al., 1981. |

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<tr>
<th>Diseases</th>
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<tr>
<td>Leber’s Hereditary Optic Neuropathy (LHON)</td>
<td>a) Singh et al., 1989; Newman et al., 1991; Nakamura et al., 1992;</td>
</tr>
<tr>
<td>a) MTND4*LHON11778</td>
<td>b) Huoponen et al., 1991; Brown et al., 1992a;</td>
</tr>
<tr>
<td>b) MTND1*LHON3460</td>
<td>c) Brown et al., 1992b, 1994; Wallace et al., 1992</td>
</tr>
<tr>
<td>c) MTND1*LHON7444</td>
<td></td>
</tr>
<tr>
<td>d) Multi-sites mutation</td>
<td></td>
</tr>
<tr>
<td>LHON and Multiple Systems Degeneration</td>
<td>Howell et al., 1991</td>
</tr>
<tr>
<td>Myoclonic Epilepsy and Ragged Red Fiber Disease (MERRF)</td>
<td>Wallace et al., 1988</td>
</tr>
<tr>
<td>Hypertrophic Cardiomyopathy and Myopathy</td>
<td>a) Tanaka et al., 1990.</td>
</tr>
<tr>
<td>a) Infantile onset</td>
<td>b) Zeviani and Antozzi, 1992; Antozzi and Zeviani, 1997.</td>
</tr>
<tr>
<td>b) Adult onset</td>
<td></td>
</tr>
<tr>
<td>Ataxia and retinitis pigmentosa (NARP) syndrome</td>
<td>Holt et al., 1990; Parfait et al., 1999</td>
</tr>
<tr>
<td>Leigh’s Disease (Subacute Necrotizing Encephalomyopathy)</td>
<td>Degoul et al., 1995.</td>
</tr>
<tr>
<td>Alzheimer’s and Parkinson’s Diseases</td>
<td>Lin et al., 1992, Shoffner et al., 1993; Davis et al., 1997b.</td>
</tr>
<tr>
<td>Maternally Inherited Sensorineural Deafness</td>
<td>Jaber et al., 1992; Gardner et al., 1997; Hyslop et al., 1997.</td>
</tr>
<tr>
<td>Diabetes</td>
<td>van den Ouweland et al., 1992; Suzuki et al., 1997; Kameoka et al., 1998;</td>
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<td></td>
<td>Shiotani et al., 1998.</td>
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Table 1.3. Class III mutations
Disorders due to MtDNA Deletions and Duplications

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<tr>
<th>Diseases</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Kearns-Sayre and Chronic Progressive External Ophthalmoplegia Syndromes due to mtDNA deletions</td>
<td>Moraes et al., 1989; Schon et al., 1989; Larsson et al., 1990; Goto et al., 1990b.</td>
</tr>
<tr>
<td>Kearns-Sayre and Chronic Progressive External Ophthalmoplegia Syndromes due to duplications</td>
<td>Kleinle et al., 1997.</td>
</tr>
<tr>
<td>Pearson’s Syndrome due to deletions</td>
<td>Rotig et al., 1989; Cormier et al., 1990; Superti-Furga et al., 1993.</td>
</tr>
<tr>
<td>Migraines and Stroke (Malignant Migraine)</td>
<td>Bresolin et al., 1991.</td>
</tr>
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Table 1.4. Class IV mutations
Disorders of Unknown Inheritance

<table>
<thead>
<tr>
<th>Diseases</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Lethal Infantile Cardiomyopathy (LIC)</td>
<td>Kauffman et al., 1972.</td>
</tr>
<tr>
<td>Idiopathic Torsion Dystonia (ITD)</td>
<td>Fletcher et al., 1990; Benecke et al., 1992.</td>
</tr>
<tr>
<td>Myoneurogastrointestinal Disorder and Encephalopathy (MNGIE)</td>
<td>Bardosi et al., 1987.</td>
</tr>
<tr>
<td>Luft’s Disease</td>
<td>Luft et al., 1962; Shy and Gonatas, 1964; DiMauro et al., 1976; Gobernado et al., 1980; Luft, 1992.</td>
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</table>
Improving mitochondrial ATP production is one of the key aims of metabolic treatment intended to prevent clinical deterioration in these diseases.

1.5. Mitochondrial dysfunction in aging

Aging is a multi-factorial process which involves a progressive decline in almost all physiological functions of various organs. Notably, the aging process involves disturbance of the endocrine, neuroendocrine and immune systems, as well as abnormalities of gene composition and function with repression of proliferation genes and reciprocal expression of anti-proliferation genes (Goldstein, 1990). A decline in mitochondrial function with age results in an inadequate ATP supply leading to functional impairment of multi-organs and tissues. In human, aging causes skeletal muscle fiber loss and atrophy which coincides with an exponential rise in the number of cytochrome-oxidase deficient fibers (Byrne and Dennett, 1992; Cortopassi & Wong, 1999). Apart from undergoing various other morphological changes, mitochondria in old organs and cultured cells after many divisions are reduced in number but increased in size (Kiessling et al., 1973; Flannery et al., 1989). Kiessling et al. (1973) demonstrated a 20% increase in mitochondrial volume in aged men which was entirely contributed to by an increase in organelle size. This indicates that aging may be associated with a diminished capacity to replace damaged mitochondria which results in compensatory increasing in size.

1.5.1. Mitochondrial respiratory activity in ageing

Mitochondrial respiratory functions decline with normal aging. The activities of COX I, III and IV were reported to be reduced by 50% in aged Drosophila subobscura
(Morel et al., 1995). Increased OXPHOS failure was also confirmed in the
senescence-accelerated mouse (SAM) strain (Mori et al., 1998). In aged rats
mitochondrial mRNA levels in brain and heart tissues were declined which was
associated with the reduction in transcription rates (Biggs et al., 1991; Hudson et al.,
1998; Nicoletti et al., 1998). In humans, aging is associated with an increase in the
numbers of COX-negative fibres in heart and skeletal muscles (Muller-Hocker, 1990;
Muller-Hocker et al., 1992; Byrne and Dennett, 1992), and a decrease in respiratory
complex I and IV activities in skeletal muscle and liver (Trounce et al., 1989; Yen et
al., 1989; Cooper et al., 1992; Morel et al., 1995). A small but significant decline in the
activity of the nuclear encoded complex II has also been detected in polarographic
studies, indicating that both nuclear and mitochondrial gene expression are altered,
each thereby contributing to age-related respiratory dysfunction (Trounce et al., 1989).
Other studies show that the activities of NADH dehydrogenase, rotenone-sensitive
NADH-cytochrome reductase, succinate-cytochrome c reductase, and cytochrome c
oxidase were also shown to be reduced (Weindruch et al., 1980; Lemeshko and
Belostotskaya, 1992; Muller-Hocker et al., 1992; Lenaz et al., 1997).

1.5.2. Mitochondrial DNA variants in ageing

An accumulation of mutant mitochondrial DNA (Miquel et al., 1983; Lee et al., 1994;
Yen et al., 1994; Liu et al., 1997; Miquel, 1998) nuclear DNA encoding for
mitochondrial polypeptides (Hayashi et al., 1994) with age is evident. An age related
deficiency in gene repair may be a contributing factor in accelerating the aging process
(Mozzhuikhina et al., 1991).
The increase in free radical production in advanced age accelerates the rate of mtDNA mutations (Nohl, 1993; Lu et al., 1999). Accumulated N-methyl-D-aspartate excitatory amino acid receptors and increased intracellular calcium levels are among the major cellular factors to increase the production of free radicals (Beal, 1995). Electron microscopic studies in aged insects showed that senescence is associated with mtDNA breakdown (Miquel, 1998). Rats and mice show age related accumulations of a wide range of MtDNA mutations such as deletions (Piko et al, 1988; Linnane et al, 1989; 1990; Tanhauser and Laipis, 1995; Muscari et al., 1996), dimeric catenated mtDNAs (Murray & Bacalvage, 1982; Piko et al, 1984), high molecular weight forms of mtDNA that have been covalently cross-linked by lipid peroxidation (Hruszkewycz, 1992), single-stranded and double stranded breaks (Richter et al., 1988; Katsumata et al., 1994; Ozawa, 1997), and possible covalent cross-linking of the mtDNA with mitochondrial proteins (Oleinick et al., 1987). Studies on non-dividing tissues in humans have identified age-associated accumulation of heterogeneous mtDNA mutations (deletions and base substitutions) which are accompanied by a decline in OXPHOS activity (Lezza et al., 1994). The mtDNA deletions observed in aged tissues ranges in size from 3 - 7.4 kb, and are often characterized by break points flanked by direct repeat sequences (Cortopassi & Arnheim, 1990; Corral-Debrinski et al., 1991; Yen et al., 1994, Lee et al., 1994). A "common" 4977 bp deletion between nucleotides 8483 and 13460 of the mitochondrial genome seen in patients with KSS and CPEO increases in abundance in post-mitotic tissues such as the brain, heart and skeletal muscle (Linnane et al., 1989; Sugiyama et al., 1991; Soong et al., 1992; Zhang et al., 1997). Furthermore, there are regional variation patterns in the accumulation of the 4977 bp deletion in the aged brain (Corral-Debrinski et al., 1992), the accumulation being especially apparent in those regions with high oxygen consumption (Figure
1.9). In human brain, the mutational levels of mtDNA are generally less than 0.1% (Corral-Debrinski et al., 1994). In ovarian tissues, mitochondria tend to accumulate the deleted mtDNA species after menopause (Suganuma et al., 1993; Kitagawa et al., 1993). Some point mutations frequently seen in mitochondrial cytopathies are also seen to accumulate in aging tissues, such as the tRNA<sub>leu(UUR)</sub> 3243<sup>A→G</sup> mutation normally seen in lactic acidosis and stroke-like episodes (MELAS) patients (Zhang et al., 1993) and the tRNA<sub>lys</sub> 8344<sup>A→G</sup> mutation which mainly occurs in myoclonus epilepsy and ragged-red fibres (MERRF) (Münscher et al., 1993). In addition, oversized mtDNAs due to multiple repeats in the D-loop region have also been detected (Kovalenko et al., 1997). It is not clear whether these mutations play potential roles in the loss of OXPHOS capacity in aging brain, although the accumulation of low levels of mutated mtDNA in peripheral tissues with age may be tolerated. The common view is that normal aging can be accompanied by a gradual accumulation of pathogenic mtDNA mutations in the absence of any overt mitochondrial disease. (Trounce, et al., 1989; Boffoli et al., 1994) (Figures 1.9. & 1.10.).
Figure 1.9. Age-related accumulation of mtDNA damage in normal brain (Murray and Bacalvage, 1982; Soong et al., 1992; Rosenberg et al., 1993).
1.5.3. OXPHOS failure and mtDNA variants in age-associated neurodegenerative diseases including Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD).
which are generally insidious in onset and run a gradually progressive inexorable course which can continue over many years (Beal, 1995). It has been proposed that the accumulation of mtDNA mutations during aging results in a progressive impairment of OXPHOS. The finding of lower levels of qualitatively similar pathologic lesions in the normal aged brain to those found in AD and PD suggests that there may be some overlap between these disease processes and the aging process. In addition, a fall-off in Purkinje cell numbers is also observed in normal aging (Sturrock, 1990; Fattoretti et al., 1998). The cerebellar Purkinje cell pool, which is very sensitive to hypoxia, is also involved in mitochondrial cytopathies.

The etiology of these conditions is still unclear and it is possible that several different pathologic processes, both inherited and acquired, may contribute to the pathogenesis. It is also possible that several of these factors may co-exist in a single case ("convergence hypothesis"). Mitochondrial respiratory dysfunction is highly regional specific in each disease. In preliminary studies on AD, a reduced complex IV activity is seen in the cortex while in PD, complex I activity is specifically decreased in substantia nigra (Mann et al., 1992). Somatic mtDNA mutations observed in AD (Corral-Debrinski et al., 1994) and PD (Ikebe et al., 1990; Kapsa et al., 1996) patients have the potential to act synergistically with inherited nuclear gene defects, hastening the onset of diseases in a similar way to homoplasmic secondary mtDNA mutations in Leber’s Hereditary Optic Neuropathy (Shoffner et al., 1993). In particular, candidate mutations of mtDNA such as nucleotide substitutions at positions 3196 AD/PD: G→A (Shoffner et al., 1993), 3397 AD/PD: A→G (Brown et al., 1996), 4336 AD/PD: T→C (Mayr-Wohlfart et al., 1997) and 5460 PD but not AD: G→T/A (Kosel et al., 1994; Schnopp et al., 1996; Janetzy et al., 1996), and an insertion between positions 956-965 have been identified in
patients with late onset of AD or PD (Shoffner et al., 1993). These results are, however, often circumstantial and controversial, with different observations by different research groups. The increase in accumulation of the "common" 4977 bp mtDNA deletion in the substantia nigra of PD patients (Ikebe et al., 1990) compared with age-matched controls suggests that the mtDNA damage in substantia nigra is highly specific to PD patients (Schapira et al., 1992; Lezza et al., 1999). This supports our own observations of non-conservative polymorphisms at evolutionarily conserved sites in the mtDNA-encoded ND subunits of substantia nigra in PD and aged controls (Kapsa et al., 1996). On the other hand, Loeffler et al. (1996) found that coeruloplasmin, the major plasma anti-oxidant and copper transport protein, are increased in many brain regions of AD and PD patients as compared with normal aged people, indicating a local compensatory response to increased oxidative stress.

Mitochondria-mediated apoptotic cell death also contributes to tissue senescence and organ failure. Recent studies indicate that mitochondria are important sensors of apoptosis. The release of cytochrome c and mild oxidative stress are potent stimuli of caspases mediated apoptosis (Hampton et al., 1998; Ozawa, 1998).

1.6. The use of prostate tissue as a model for mitochondrial dysfunction study in aging

The question whether the decline in OXPHOS capacity with age can in turn accelerates the aging process and the development of age-related neurodegenerative diseases is an attractive hypothesis which has raised much interest amongst researchers. However, results are diverse and often inconsistent. This may be attributed to the different experimental conditions and the different tissues or cells
used in various studies. Among the published work involving human tissues, mainly autopsy samples were used which may confound accuracy in the measurement of respiratory enzyme complex activities because of problems related to post-mortem delay. Because of this there has been some interest in identifying more easily accessible tissues (blood, skin) which may reflect the biochemistry of the CNS.

The hypothesis that cumulative mtDNA mutations plays a role in human aging would be greatly supported if a correlation could be demonstrated between mutation level and OXPHOS activity. That is to show whether aging is associated with an increase in mtDNA mutation rate and a decrease in OXPHOS activity. Such a study requires a biopsy approach and is ethically impossible in the CNS. In this study, human prostatic tissue was selected for evaluation as it is commonly excised in elderly male subjects with prostatic hypertrophy and provides an opportunity to use fresh human tissues to study the correlation of respiratory chain function and mtDNA genotype. Prostate tissue has a potential disadvantage in that it is a dividing tissue. It is generally accepted that accumulation of mutant mtDNA occurs more frequently in non-dividing tissues than dividing tissues due to the replicative segregation effect. If, however, mtDNA mutations increase markedly with age, the chance of detecting this should also be high even in dividing tissues. It might also be the case that oxidative stress with age may have a profound effect on certain dividing tissues because some dividing tissues have a higher metabolic rate than non-dividing tissues. This would result in higher rates of mtDNA mutation. It is of interest therefore to find out whether aging prostate tissues exhibit an impaired respiratory function or a high rate of mtDNA mutation.
Fresh human prostate tissue obtained by biopsy has made it possible to accurately analyze the relationship of mtDNA mutations and enzyme activities among aged populations.

1.6.1. Structure and functions of human prostate

The prostate is adjacent to the bladder and it is transpierced by the urethra and ejaculatory ducts. It also serves as the pedestal of the bladder. The bladder rests upon the base of the prostate and the conical apex of the prostate sits upon the deep layer of the urogenital diaphragm.

The adult prostate is a firm, elastic gland with the shape of a chestnut, that is, flattened anteroposteriorly. It varies in size with an average length of 3.4 cm, width of 4.4 cm, thickness of 2.6 cm and weight of 20 g. It contains five lobes and is composed of glandular tissue and smooth muscle. In aged man, the incidence of benign prostatic hypertrophy (B.P.H.) and prostatic cancer (P.C.) is high.

A relationship between the B.P.H. and P.C. has been appreciated since the beginning of the century. 75% of the patients with P.C. would have suffered from B.P.H. and 5-25% of patients with B.P.H. would develop P.C. In Caucasians, the incidence of these diseases is high, however it is rather low in Orientals. The incidence of B.P.H. increases with age and reaches as high as 95.9% in the 9th decade in autopsy studies. According to clinical surveys, as many as 23% of the men at age of 40 suffer from some symptoms of urethral obstruction, and in the 60-85 age range, the incidence
increases to 78%. Some degree of prostatic hypertrophy is an integral part of the aging process.

1.6.2. Age-associated changes in the prostate gland

Following the initial 6 - 7 week post-natal period of development, there is little change in the prostate gland until puberty at which time the gland grows under the stimulus of testosterone, but the growth slows down again with little change inside up to 50 years of age. With subsequent advancing years, the gland may start to undergo progressive atrophy due to a decreased production of testosterone; alternatively, it may gradually increase in size until death (benign hypertrophy). In the latter case, which often occurs in older men, B.H.P. may impede the passage of urine by encompassing and distorting the prostatic urethra, requiring surgical removal of the prostate gland in some cases. The prostate gland can become cancerous. The cancerous cells are stimulated in their growth by testosterone. Growth arrest of these cells can be achieved by the removal of testosterone and the administration of oestrogen.

1.6.3. Aims of this study

This study examined the suitability of fresh human prostate tissue (dividing tissue) as a means of studying the age related functional changes in mitochondrial respiration and mitochondrial genome integrity. If the results were consistent with those found in brain or muscle tissues, studies using prostate tissue could provide with knowledge in further understanding the roles of mitochondria during aging process in other tissues such as the CNS and heart. Using fresh human prostatic tissue from patients of various ages, the correlation of respiratory enzyme activities (complex I, II and IV
respectively) with age was studied. In addition, a search was made for mtDNA mutations and this was correlated with both age and OXPHOS activity.

Specific questions were (1) Whether a fall in OXPHOS with age is seen in human prostate; (2) Whether key mtDNA mutations occur with age in human prostate; (3) Whether there is a correlation between OXPHOS efficiency and mtDNA mutations. (4) Whether very low respiratory activity can be identified in a subgroup. Such a subgroup may be predisposed to organ dysfunction caused by ATP depletion.
CHAPTER 2
MATERIALS AND METHODS

2.1. Sources of human fresh prostate tissue

2.1.1. Recruitment of patients

Twenty three male patients ranging in age from 57 to 90 years and suffering from either benign prostatic hyperplasia (B.P.H.) or prostate carcinoma (P.C.) were selected for this study. All were in-patients attending the Department of Urology at St. Vincent’s Hospital, Melbourne, Australia during 1993. Patient selection for inclusion in this study was on a sequential basis after obtaining informed consent. The project was approved by the ethics committee at St. Vincent’s Hospital, Melbourne. Most of the patients had B.P.H., three had areas of neoplastic transformation, and one had combined B.H.P. and basal cell carcinoma. Table 2.1 outlines the clinical conditions of each patient included in this study.

2.1.2. Prostate tissue collection

Fresh prostate tissues were taken from the biopsies of human transurethral resections. They were excised primarily for pathological examinations, the remaining parts were collected for this study. The collection weight of samples ranged from 0.23g to 15.5g.
### Table 2.1. Age and clinical conditions of the selected patients

<table>
<thead>
<tr>
<th>ID* No:</th>
<th>Age (years)</th>
<th>Final diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>P93/1</td>
<td>75</td>
<td>P.C.</td>
</tr>
<tr>
<td>P93/2</td>
<td>78</td>
<td>1) B.P.H. with acute urine retention, 2) Hypertension, 3) Right hemiplegia, 4) Epilepsy, 5) Dysphasia, 6) Severe reflex esophagitis</td>
</tr>
<tr>
<td>P93/3</td>
<td>75</td>
<td>1) B.P.H., 2) Urinary tract infection, 3) Non-insulin dependent diabetes (NIDDM)</td>
</tr>
<tr>
<td>P93/5</td>
<td>87</td>
<td>1) Adenocarcinoma of the prostate, 2) Acute urine retention, 3) Vitreous hemorrhage</td>
</tr>
<tr>
<td>P93/6</td>
<td>71</td>
<td>1) Acute urine retention due to prostatic hypertrophy, 2) Adenocarcinoma of the prostate, 3) NIDDM</td>
</tr>
<tr>
<td>P93/7</td>
<td>77</td>
<td>1) Haematuria secondary to prostatic hypertrophy, 2) Hypertension</td>
</tr>
<tr>
<td>P93/8</td>
<td>60</td>
<td>B.P.H.</td>
</tr>
<tr>
<td>P93/9</td>
<td>87</td>
<td>1) B.P.H., 2) Basal cell carcinoma, 3) Hypertension, 4) Right cerebellar vascular infarction, 5) Paget's disease of the skull</td>
</tr>
<tr>
<td>P93/10</td>
<td>71</td>
<td>1) B.P.H., 2) Hypertension</td>
</tr>
<tr>
<td>P93/11</td>
<td>81</td>
<td>1) B.P.H., 2) Constipation, 3) Hypertension</td>
</tr>
<tr>
<td>P93/12</td>
<td>74</td>
<td>1) B.P.H., 2) Hypertension</td>
</tr>
<tr>
<td>P93/15</td>
<td>67</td>
<td>1) B.P.H., 2) Right inguinal hernia, 3) Hypertension</td>
</tr>
<tr>
<td>P93/16</td>
<td>68</td>
<td>1) B.P.H., 2) Arthritis, 3) Hypertension</td>
</tr>
<tr>
<td>P93/17</td>
<td>63</td>
<td>B.P.H.</td>
</tr>
<tr>
<td>P93/18</td>
<td>75</td>
<td>B.P.H.</td>
</tr>
<tr>
<td>P93/19</td>
<td>90</td>
<td>1) Tumour and prostatic obstruction, 2) Haematuria with clot</td>
</tr>
<tr>
<td>P93/20</td>
<td>57</td>
<td>B.P.H. with prostatism</td>
</tr>
<tr>
<td>P93/21</td>
<td>69</td>
<td>1) B.P.H., 2) Haematuria, 3) Clot retention, 4) Hypertension</td>
</tr>
<tr>
<td>P93/22</td>
<td>61</td>
<td>B.P.H.</td>
</tr>
<tr>
<td>P93/23</td>
<td>65</td>
<td>Prostatic obstruction</td>
</tr>
<tr>
<td>P93/24</td>
<td>62</td>
<td>B.P.H.</td>
</tr>
<tr>
<td>P93/25</td>
<td>59</td>
<td>1) B.P.H., 2) Bladder calculus</td>
</tr>
<tr>
<td>P93/26</td>
<td>65</td>
<td>1) Prostatic obstruction, 2) Right inguinal hernia, 3) Hypercholesterolaemia</td>
</tr>
</tbody>
</table>

*ID: Identification

### 2.1.3. Tissue processing and storage

Under aseptic conditions, the fresh prostatic tissues were trimmed to remove connective and adipose tissues. The tissue was then cut into blocks of about 0.2
cm³, and transferred into pre-chilled microfuge (1.5ml) tubes for either immediate use or storage at -80°C.

2.2. Biochemical studies on mitochondrial respiratory chain function

2.2.1. Extraction of mitochondria from prostate tissues

The prostate tissue was minced coarsely with a pair of scissors and then transferred into a glass homogeniser (5 ml, Dounce). Approximately 9 x tissue volumes of Mannitol -EDTA -Sucrose (MES: 225 mM mannitol/ 75 mM EDTA/ 0.1 mM sucrose) buffer was added and the tissue homogenised manually with a tight-fitted pestle (fitting B) using 8 gentle strokes. The homogenate was transferred into another 10 ml tube and the homogeniser washed with 1.0 ml of MES and another 8 gentle strokes with a loose-fitting pestle (fitting A) to recover the remaining homogenised sample. The homogenised sample was then aliquoted into two microfuge tubes. To eliminate tissue debris, two quick spins each at 12,900 x g for 7 seconds at 4°C (13,000 rpm, Biofuge 13, Radiometer Pacific, Melbourne) were applied. The supernatant was then spun again at 12,900 x g at 4°C for 10 min to precipitate mitochondria (mitochondria enriched fraction). The supernatant was then aspirated and 1 x tissue volume of MES buffer added to the pellet. The extracted mitochondrial fraction was kept at 4°C for immediate experiments or stored at -80°C.
2.2.2. Measurement of protein concentration

The concentration of protein in the extracted mitochondrial fraction was estimated using modification of Lowry’s method (Lowry et al., 1951). A standard curve was constructed using bovine serum albumin (BSA, fraction V, Sigma) ranging from 0 to 40 µg (0, 4.0, 8.0, 12.0, 16.0, 24.0, 32.0 and 40 µg/ml) for inclusion in each assay. Duplicated samples and standards were used for all measurements.

To a 5 ml plastic tube, 10 µl of either the sample or the standard was added which was followed by the addition of 1 ml of a Na\(^+\) & Cu\(^{2+}\) mixture (5.0% CuSO\(_4\)·5H\(_2\)O in 10% sodium citrate: 2% Na\(_2\)CO\(_3\) in 0.4% NaOH = 1:500) and 0.1 ml of Folin-Cioclateu reagent (May & Baker, Australia). Mixtures were vortexed vigorously after each addition and kept in the dark for 60 min to ensure that the colour reaction was completed. The optical density (OD) was measured at 750 nm using a Hitachi U-2000 spectrophotometer. The standard curve was plotted with a built-in software and the sample concentrations calculated accordingly.

2.2.3. Measurement of the activities of the respiratory chain enzyme complexes

2.2.3.1. Cytochrome c oxidase (EC 1.9.3.1): Complex IV

Cytochrome c oxidase catalyses the following reaction:

\[
\text{Cytochrome c + H}^+ \xrightarrow{\text{Cytochrome c oxidase}} \text{cytochrome c + oxygen + H}_2\text{O} \\
\text{(reduced form) \hspace{1cm} (oxidised form)}
\]
The activity of cytochrome c oxidase can therefore be expressed by the rate of reduction in the concentration of cytochrome c (reduced form) as the reaction proceeds forward. The reduced cytochrome c concentration can be determined by measuring the OD at 550 nm.

The mitochondrial suspension (prepared as in section 2.2.1.) was sonicated on ice 3 times with 15 second bursts using a 100 W ultrasonicator ([MSE, London, TOSC], 20 kc/sec at maximum output).

**Preparation of a reduced cytochrome c stock solution**

200 mg of lyophilised horse heart cytochrome c (oxidised form, Sigma type VI, C7752) was dissolved in 1.0 ml distilled H$_2$O followed by addition of 1.0 ml 0.1 M L-ascorbate (BDH). The mixture (reduced cytochrome c, ascorbate and dehydroascorbate) was separated on a Sephadex G-25 resin column (1 x 20 cm, Pharmacia, Uppsala, Sweden) which was pre-washed and equilibrated with 50 ml of de-gassed MES buffer. The middle two-thirds of the red cytochrome c (reduced form) eluate was collected in glass ampules (~20 x 200 µl) and stored at -80°C after sealing.

**Assay procedures**

Distilled H$_2$O was used as the control blank and its OD at 550 nm was adjusted to zero. To a glass cuvette, 1.0 ml of the reaction buffer (containing 0.1 M 2-(N-morpholinethanesulfonic acid and 10 µM EDTA, nMES) and 6.0 µl of reduced cytochrome c (final concentration of 20 µM) were added. The cuvette was placed in the thermostatic chamber (30°C) for at least 2 min, then 8 to 10 µl of the sonicated
mitochondria suspension was added to initiate the reaction. The reactants were quickly mixed by inversion, and the OD at 550 nm was continuously recorded for at least 2 min by using both a chart recorder and a built-in auto-recorder. To examine the specificity of complex IV action, at the end of the 2 min recording a complex IV inhibitor, 1.5 µl of 200 mM KCN, was added, mixed and the OD at 550 nm recorded. The rate of change of OD at this stage was expected to diminish if the reaction was complex IV specific. After each measurement, the cuvette was rinsed three times with distilled water. The above procedures were repeated once to ensure the accurate readings.

Calculations

As described by Yonetani and Ray (1965), the reaction rate is linear for approximately one minute from the start, and is proportional to the complex IV activity of the samples to be assayed. The enzyme activity can therefore be determined by the change in the substrate (reduced cytochrome c) concentration. This was extrapolated to calculate the enzyme activity by using the extinction coefficient of 18.5 mM⁻¹cm⁻¹. The equation is shown below (the activity was expressed as nmol/min/mg mitochondrial protein).

\[
\frac{d\text{OD}}{dt} \text{(mim}^{-1}) \times 1000 \times 1000
\]

Complex IV activity = \[
\frac{18.5 \text{ (mM}^{-1}.\text{cm}^{-1}) \times \text{mitochondria protein used (µg)}}{18.5 \text{ (mM}^{-1}.\text{cm}^{-1}) \times \text{mitochondria protein used (µg)}}
\]
2.2.3.2. Complex II & III: Succinate-cytochrome c reductase (EC 1.3.99.1)

Succinate-cytochrome c reductase complex catalyses the following reaction:

\[
\begin{align*}
\text{Succinate} & \xrightarrow{\text{Complex II coenzyme Q}} \text{cytochrome c (reduced)} \\
2H^+ & \quad 2H^+ \quad 2e^- \\
\text{cyanide} & \quad \times \\
\text{cytochrome c (oxidised)}
\end{align*}
\]

As the reaction proceeds forward, the concentration of cytochrome c (reduced) increases if the further oxidation reaction from reduced cytochrome c to oxidised cytochrome c is blocked as shown in above diagram.

A modification of the method of Sottocasa et al. (1967) was used to determine succinate-cytochrome c reductase activities. In the presence of 50 μl cyanide (7.2 mM KCN), the absorbance at 550 nm increases as reduced cytochrome c increases.

**Assay procedures**

Distilled H₂O was used as a blank and the OD at 550 nm was adjusted to zero. The temperature controlled chamber was maintained at 30°C. The cuvette was pre-warmed to 30°C in the chamber as described above. To each 10 μl of sonicated mitochondria suspension (protein concentration 7 to 14 μg/μl), 0.4 ml of 150 mM KH₂PO₄ (pH 7.7, final concentration: 50 mM); 50 μl of 7.2 mM KCN (final concentration 0.3 mM), 50μl of rotenone (final concentration 1.5 μM ) and 10 μl of
2.4 mM cytochrome c (final concentration: 0.1 mM) and water were added sequentially to a final volume of 1.2 ml. The mixture was incubated at 30°C for at least 3 min. The reaction was initiated by adding 50 μl of 240 mM potassium succinate (pH 7.7, final concentration 10 mM) and the reactants mixed gently with several inversions. The OD was continuously recorded for 3 min. The procedure was repeated three times, and the average of the three measurements was taken as the final result.

During the experiment, while other reagents could be left at room-temperature, the cytochrome c solution should be kept on ice.

**Calculation**

Similarly, the activities of succinate-cytochrome c reductase are indicated by the rate of the formation of reduced cytochrome c. The equation is shown below (extinction coefficient: 18.5 mM⁻¹. cm⁻¹; the activity was expressed as nmol/min/mg mitochondrial protein).

\[
\frac{d\text{OD}}{dt} (\text{mm}^{-1}) \times 1000 \times 1000
\]

Complex II & III activity = \[
\frac{18.5 (\text{mM}^{-1} \cdot \text{cm}^{-1}) \times \text{mitochondria protein used (μg)}}
\]
2.2.3.3. **NADH-cytochrome c reductase (complex I rotenone-sensitive, EC 1.6.5.3.)**

The assay was based on the method by Moreadith et al. (1984). The activities of complex I were reflected by the rate of NADH consumption based on the following reaction.

\[
\text{NADH} \quad \xrightarrow{\text{complex I}} \quad \text{coenzyme QH}_2
\]

\[
\text{rotenone (\text{-})} \quad 2e
\]

\[
\xrightarrow{\text{X}} \quad \text{coenzyme Q}
\]

The following solutions were used: 62.5 mM potassium phosphate buffer pH 8.0, prepared by titrating 62.5 mM KH$_2$PO$_4$ against 62.5 mM K$_2$HPO$_4$.3H$_2$O containing EDTA (0.625 mM) and sucrose (250 mM); 200 mM KCN; 10% (w/v) dodecyl-β-D-maltoside (Hoechst, USA); 5 mM CoQ$_1$ (in ethanol or distilled H$_2$O); 0.5 mM rotenone (in ethanol); 10 mM NADH (Boehringer-Mannheim) made fresh and kept on ice during the assay.
**Assay procedures**

To each of two quartz cuvettes, 0.8 ml of potassium phosphate buffer (final concentration of 50 mM with 0.5 mM EDTA and 200 mM sucrose), 10 μl of KCN (final concentration 2 mM), 10 μl of CoQ₁ (final concentration 50 μM), and 10 μl of dodecyl-β-D-maltoside (final concentration 0.1%) were added sequentially, and the mixture was placed in the temperature controlled chamber (30°C) for at least 3 min to warm up the reactants. To one cuvette, water was added as a blank. To the other, 10 μl of sonicated mitochondrial suspension was added and mixed thoroughly by inversion. The mixture was equilibrated at 30°C for further 3 min before the final reagent, 10 μl NADH, was added and mixed. The change of OD at 340 nm was recorded immediately and continuously for up to 10 min. At the end of the recording, 10 μl of 0.5 mM rotenone was added to inhibit the complex I activity, and the OD value was monitored for a further 2 min. Any enzymatic activity measured thereafter should be considered as background and subtracted from the previously measured values.

**Calculation**

Complex I activity (rotenone sensitive) was expressed by the difference of NADH consumption rates per microgram of mitochondrial protein in the absence and presence of rotenone, and calculated by using the extinction coefficient of 6.22 mM⁻¹ cm⁻¹. The equation is shown below (the activity was expressed as nmol/min/mg mitochondrial protein):
difference of NADH consumption rates \( \text{min}^{-1} \times \text{min}^{-1} \times 1000 \)

Complex I activity = ~~~~~~~~~~~~~~~~~~~~~~~~~~

\( 6.22 \text{ (mM}^{-1} \text{.cm}^{-1}) \times \text{mitochondria protein concentration (\mu g)} \)

2.3. Mitochondrial genome studies

Prostate tissues from the 23 patients were screened for possible abnormalities of the mitochondrial genome including mtDNA point mutations and large size common deletions.

2.3.1. Total DNA extraction

Total DNA was extracted from the prostate tissues of the 23 patients and skeletal muscles from either normal (as negative controls) or CEPO patients known to harbor the common mtDNA 4977 bp deletion. Skeletal muscle biopsies were obtained under ethics approval in conjunction with another project conducted in the same laboratory. The method for DNA extraction was based on that of Davis et al. (1986) with a few modifications (Blok et al., 1995). Tissues (10-20 mg) were thawed on ice. Under sterile conditions, each tissue was transferred into a 2 ml all glass homogeniser (Duall 20, Konetes Glass Co. New Jersey) to which 300 \( \mu l \) of pre-chilled MES was added. Tissues were homogenised manually using a tight-fitting glass pastel, then 1 ml of lysis buffer (10 mM Tris, PH 7.5, 400 mM NaCl and 2 mM EDTA), 17 \( \mu l \) of 20\%(W/V) SDS and 130 \( \mu l \) of proteinase K (5 mg/ml, Promega) were added sequentially and mixed thoroughly (see 2.3.5. below). The samples were incubated at 50\(^\circ\)C overnight. After the digestion, 500 \( \mu l \) of 6 M NaCl
was added and mixed vigorously for 15 seconds, followed by centrifugation at 700g
(3000 rpm, Biofuge 13, Radiometer Pacific, Melbourne) at 4°C for 15 min. The
supernatant was transferred to a clean 10 ml tube to which 3.3 ml of cold absolute
ethanol (stored at -20°C) was added and the tube contents mixed gently by
inversion. The mixture was aliquoted into 3 microfuge tubes and the DNA was
allowed to precipitate at -20°C for overnight or by leaving on dry ice for at least 30
min, then the precipitated DNA was pelleted by centrifugation at 13,060 x g
(13,000 rpm, Biofuge 13) for 10 min at 4°C, followed by washing with 500µl of
pre-chilled (at -20°C) 70% ethanol and spun immediately at 13000 rpm for 5 min.
The supernatant decanted, the DNA pellet air dried and resuspended in 80 µl of
deionised water. The DNA concentration was determined by measuring the OD at
the wave length of 260 nm on a spectrophotometer (QuantaGene, The Australian
Chromatography Company) at an appropriate dilution. The quality was determined
by the OD ratio of λ at 260/280 nm (OD at λ=280 nm indicates the extent of
protein contamination). An OD ratio of λ at 260/280 greater than 1.6 was
considered as a batch of good quality of DNA preparation.

2.3.2. DNA preparation for mitochondrial genome studies

For pilot experiments, Purer DNA was prepared from the DNA preparations of four
patients (P93/4, P93/5, P93/10 and P93/16) using above mentioned method.

To each total DNA pellet prepared as above, 80 µl distilled H2O, 20µl digestion
buffer (10 x, 500mM Tris, PH 8.0, 10 mM EDTA, and 5% Tween 20), and 100 µl
of proteinase K (7.5 mg/ml) were added and mixed thoroughly. DNA was digested
at 37°C overnight and the reaction ceased by heating the mixture in a water bath at 92°C for 20 min. To prevent evaporation, a thin layer of oil was laid on the top of the digested product before heating at 92°C. Samples were then spun at 13000 rpm for 2 min. The supernatant was transferred to a clean tube and the concentration measured in a spectrophotometer at \( \lambda \) 260 nm, protein contamination was checked at 280 nm.

### 2.3.3. DNA quality examination

The extracted mitochondrial DNA samples were used as templates for a designated PCR to examine whether the purity and quantity were sufficient to carry out further studies. The PCR amplification was at the regions of mtDNA where deletions are unlikely to occur, such as the region between nt 2826 and nt 3728 [corresponding primer pair was B\(_1\)F\(_1\)(nt 2826-2849) and B\(_1\)R\(_1\)(nt 3728-3705), for primer sequences, see Table 2.4.]. The PCR thermal cycles are described in section 2.3.4 and the ingredients for PCR are shown in Table 2.2.

### 2.3.4. Detection of the mtDNA\(^{4977}\) common deletion

MtDNA\(^{4977}\) common deletion in human tissues occurs between nt 8482 and nt 13459. The primer pair chosen which flanked this region was L\(_{790}\) and H\(_{1365}\) (Table 2.4.). The expected sizes would be 5750 bp for wild-type and 773 bp for those mtDNA with one 4977 bp deletion. To validate the results, size matching tests by primer pair shifting PCR (L\(_{790}\) & G\(_1\)R\(_1\) and L\(_{644}\) & H\(_{1365}\)) were also carried out (for primer pair details see Table 2.4.).
**PCR conditions**

The typical PCR reaction was performed in a total volume of 25 µl. and occasionally 50 and 100 µl reactions were used depending on the efficiency of PCR. Table 2.2. lists the reagents and the amount used for a standard 25 µl reaction. Larger PCR reactions were scaled up accordingly.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Volume used(µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR 10 x buffer B (Promega, Mg^{2+} free)</td>
<td>2.5</td>
<td>1 x</td>
</tr>
<tr>
<td>10 mM dNTP(dATP, dCTP, dGTP, dTTP)</td>
<td>0.5</td>
<td>200µM</td>
</tr>
<tr>
<td>25 mM MgCl2</td>
<td>1.5</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>40 pmol/µl 5' Primer</td>
<td>0.25</td>
<td>10 pmol</td>
</tr>
<tr>
<td>40 pmol/µl 3' Primer</td>
<td>0.25</td>
<td>10 pmol</td>
</tr>
<tr>
<td>5U/µl TaqDNA Polymerase(Promega)</td>
<td>0.13</td>
<td>0.65 U</td>
</tr>
<tr>
<td>10 or 20 ng/µl DNA template</td>
<td>2.0</td>
<td>20 or 40 ng</td>
</tr>
<tr>
<td>Milli Q (deionised and distilled) water</td>
<td>17.87</td>
<td>N/A</td>
</tr>
<tr>
<td>Total volume</td>
<td>25.0</td>
<td></td>
</tr>
</tbody>
</table>

As specified in Table 2.2., to each PCR tube, an appropriate amount of sample DNA and PCR reagents were added and mixed thoroughly. A thin layer of ultra pure paraffin oil (Fluka) was overlaid on the top of the reactants in each tube. PCR thermal cycles for primer pair L_{790}/H_{1365} were as follows: hot start at 92°C for 3 min, followed by 30 cycles of denaturing at 92°C for 1 min, annealing and extension at 65°C for 5 min. For primer pair shifting PCR (L_{790} / G_{1}R_{1} and L_{644} / H_{1356}), the thermal cycles
were modified as follows: hot start at 94°C for 3 min, followed by 30 cycles of
denaturing at 94°C for 30 seconds and annealing and extension at 65°C for 7 min.

**Electrophoresis of PCR products**

PCR products were separated on a 1% agarose-TAE gel (Promega, TAE: 0.4 M Tris-
acetate 0.01 M EDTA) and visualized under UV light (Ultra-Lum, Inc., U.S.A.) after
staining with 0.1 μg/ml of ethidium bromide. Each 10 μl PCR product was mixed
with 3 μl of 6x loading buffer (40% w/v sucrose containing 0.25% bromophenol blue)
and loaded into each well of the gel, a molecular weight marker (lamda DNA digested
with Hind III, 100 ng/μl) was run in parallel at 4.0 volts/cm for 1 hour. The DNA
fragments were separated according to their molecular weights. The gels were
photographed using a Polaroid Instant Camera System (Polaroid MP 4+, Model 44-16,
Cambridge, MA, U.S.A.).

2.3.5. Identification of mtDNA deletions by Southern hybridization

2.3.5.1. Optimising the experimental conditions

**Selection of restriction enzymes**

Sequence analysis of the entire mtDNA genome revealed that there is only one
BamH I restriction site which lies outside the region of the common deletion.
Therefore, both wild type mtDNA and mtDNA with common deletion can be cut be
linearized by BamHI but the common deletion can be easily distinguished from
wild type mtDNA on the basis of a size difference.
Optimal DNA quantity

Different amounts of total DNA 1 and 5 μg (extracted according to methods in Section 2.3.1.) and the further purified total DNA (10 and 50 ng (as described in Section 2.3.2.) were used in restriction digestion. The results (not shown) indicated that 5 μg total DNA was sufficient for this purpose.

5 μg of total DNA was digested at 37°C with 2 μl BamH I (10 U/μl, Promega) in the presence of 2 μl of 10 x buffer E (Boehringer Mannheim) in a total of 20 μl reaction volume for overnight.

Agarose gel electrophoresis

To check whether the restriction digestion was successful, a pre-run test was carried out on a much smaller scale using a method based on the books of Sambrook et al., 1989. A 0.6% agarose-TAE gel containing 0.2 mg/100 ml g of ethidium bromide was used to separate the above restriction digestion products. Four microlitres (20% of the total digestion, equivalent to 1 μg of total DNA) of the digested product was mixed with 1μl of 6 x loading buffer and loaded into each well. Electrophoresis was carried out at a constant voltage of 4.7 volts/cm for 1 hour. DNA markers: λHind III (100 ng/μl) and φ174 x Hae III(100 ng/μl) were run parrel. If the DNA had digested completely, the remaining reaction product was combined with 5 μl of 6x loading buffer and run on a 0.6% agarose gel (15 x 25 cm) for approximately 18 hours at 40 volts.
Transferring DNA from agarose gel to nylon membranes

The Bam HI digested DNA fragments separated on an agarose gel were transferred to a nitrocellulose membrane using the method modified from those of Southern 1975 and Sambrook et al., (1989). Briefly, at room temperature, the gel was firstly de-ureated in 0.25 M HCl for 30 min on a rocking platform, followed by denaturing the gel for another 30 min in 1.5 M NaCl/0.5M NaOH and subsequent neutralization in 1 M Tris-HCl containing 1.5 M NaCl, pH 7.5 for 30 min. The DNA was transferred on to a nitrocellulose membrane in the presence of a high salt solution 20x SSC (3 M NaCl and 0.3 M sodium citrate, PH 7.0). The transfer was facilitated as described below. Briefly, a container which was filled with 20x SSC was overlaid with a clean glass plate. Two layers of pre-wet rectangular 3 MM Whatman filter paper were placed on the plate with both ends hanging down in the 20x SSC reservoir. Sequentially, the gel (inverted), a sheet of pre-soaked gel size nylon membrane (Hybond N, Amersham), two more sheets of pre-soaked gel size filter paper and a stack of Kimsoft interfold paper towers (Kleenex, Australia) cut to the same size were overlaid. A light weight (about 100 g) was placed on the top of the stack. To increase the transfer efficiency, the four edges along the outline of the gel were taped with laboratory film (Parafilm, “M”, U.S.A.). The DNA was allowed to transfer overnight (approximately 18 hours), after which the filter was washed in 2x SSC buffer for 5 to 10 min and briefly air-dried. Permanent attachment of the DNA to the nylon membrane was achieved by baking the membrane in an 80°C oven for 2 hours in a sandwich setting: filter paper-membrane-filter paper.
Hybridization with [α-32P]dATP labeled mtDNA probes

Radio-labeled probes encompassing the entire mtDNA genome were kindly provided by Dr. R. Blok. Briefly, they were generated by incorporating [α-32P]dCTP to a specific activity of approximately 10^9 cpm/mg into DNA fragments using a random priming method (Promega DNA labeling kit). 50 - 100 ng of mtDNA was denatured at 100°C for 5 min and then quenched on ice. Five microlitres of [α-32P]dCTP, 1 μl of (5 U) klenow DNA polymerase, and 500 nmol of each dGTP, dATP and dTTP were used for a 50 μl reaction. The reaction was allowed to proceed at 37°C for 60 min. The labeled probes were denatured by boiling for 5 - 10 min before hybridization.

The DNA-bound nylon membrane was pre-hybridized in 50% formamide, 10% dextran sulfate, 1 M NaCl, 1% SDS, 50 mM Tris (pH 7.4), 0.2% BSA, 0.2% Ficoll type 400 (Sigma), 0.2% polyvinylpyrrolidone(PVP) type 40 (Sigma) and 0.1% sodium pyrophosphate at 42°C for 2 hours and then hybridized with the probes for overnight at 42°C. The hybridized membrane was rinsed with 2x SSC/0.1% SDS at room temperature. Subsequent post-hybridization washes were sequentially performed for 30 min each at 65°C with agitation: (i) 1x SSC/0.1% SDS; (ii) 0.5x SSC/0.1% SDS; (iii) 0.25x SSC/0.1% SDS. This was followed by a final wash in 0.1x SSC/0.1% SDS at room temperature for 5 min. The membrane was then placed within a plastic wrap. Autoradiography (Kodak XAR5 film) was at room temperature using intensified screens.
2.3.6. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP was used to identify a potential point mutation which leads to either the loss or the creation of a specific restriction enzyme recognition site or creating one. Thus this point mutation can be distinguished by a change in the digestion pattern of a particular restriction enzyme. In practice, it refers to amplifying a segment of mtDNA by PCR followed by a restriction digestion. A few such point mutations in mtDNA have been identified with PCR-RFLP. Table 2.3. lists the possible sites at which a stringent nucleotide sequence is necessary for designated restriction enzyme digestion; and point mutation changes the nature of these restriction sites.
Table 2.3. Point mutations detected within RFLP regions in mtDNA

<table>
<thead>
<tr>
<th>MtDNA position</th>
<th>Mutation</th>
<th>Affected genes</th>
<th>Disease</th>
<th>*Primers</th>
<th>Restriction enzymes</th>
<th>Post-RFLP fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nt3243</td>
<td>A → G</td>
<td>tRNA - Leu</td>
<td>MELAS</td>
<td>B₁F₁</td>
<td>Apa I</td>
<td>902</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B₁R₁</td>
<td></td>
<td>485</td>
</tr>
<tr>
<td>nt8344</td>
<td>A → G</td>
<td>tRNA - Lys</td>
<td>MERRF</td>
<td>Sense</td>
<td>Bgl I</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MRF-2</td>
<td></td>
<td>73</td>
</tr>
<tr>
<td>nt3460</td>
<td>G → A</td>
<td>ND 1</td>
<td>LHON</td>
<td>B₁F₁</td>
<td>Hsp 921</td>
<td>634</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B₁R₁</td>
<td>or Acy I</td>
<td>268</td>
</tr>
<tr>
<td>nt11778</td>
<td>G → A</td>
<td>ND 4</td>
<td>LHON</td>
<td>L₁₁₁²</td>
<td>Bcl I</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ND₁₁H</td>
<td></td>
<td>143</td>
</tr>
</tbody>
</table>

- the details of each primer pair are in Table 2.4.

The primer pairs to be chosen for these purposes should also comply with the following requirements: 1) the chances of primer pair mismatching should be minimal; 2) a convenient fragment size should be amplified using the selected primer pair. Table 2.4 lists the oligonucleotide sequences of each primer used in the PCR reactions. The PCR components were the same as those listed in Table 2.2. Table 2.5 summarize the thermal cycles used for amplified mtDNA regions potentially with known point mutations.
### Table 2.4. Oligonucleotide Primers Used for PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide Sequence/Number</th>
<th>3' Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>B1P1</td>
<td>2826</td>
<td>GAG</td>
</tr>
<tr>
<td>B1R1</td>
<td>3728</td>
<td>GAG</td>
</tr>
<tr>
<td>L644</td>
<td>6441</td>
<td>CAA</td>
</tr>
<tr>
<td>L799</td>
<td>7901</td>
<td>TGA</td>
</tr>
<tr>
<td>Sense(L493)</td>
<td>8278</td>
<td>CTA</td>
</tr>
<tr>
<td>MRF-2</td>
<td>8385</td>
<td>GTA</td>
</tr>
<tr>
<td>L177</td>
<td>11728</td>
<td>CTC</td>
</tr>
<tr>
<td>NDH(H)</td>
<td>11919</td>
<td>GTA</td>
</tr>
<tr>
<td>H106</td>
<td>13050</td>
<td>GGG</td>
</tr>
<tr>
<td>G1R1</td>
<td>13028</td>
<td>CTA</td>
</tr>
</tbody>
</table>
Table 2.5. Thermal cycles for each PCR reaction

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Thermal Cycles</th>
<th>DT: 95°C AT: 55°C ET: 72°C AT &amp; ET: 65°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense &amp; MRF-2</td>
<td>30</td>
<td>1'</td>
</tr>
<tr>
<td>B1F1 &amp; B1R1</td>
<td>22</td>
<td>1' 1' 2'</td>
</tr>
<tr>
<td>L1172 &amp; ND4H</td>
<td>25</td>
<td>1' 1' 2'</td>
</tr>
</tbody>
</table>

*1): DT: Denaturation temperature; AT: Annealing temperature; ET: Extension temperature; AT & ET: Sharing the same temperature for annealing and extension

2): A hot start at 92°C for 3 min was undertaken before commencing the thermal cycles for each PCR reaction. 1% agarose gel was used for separating PCR products amplified by B1F1 and B1R1; the rest of the products were separated on a 2.5% agarose gel.

2.3.7. Restriction length polymorphism and selection of restriction enzyme

The appropriate restriction enzyme used to cut PCR fragments and the ingredients and the quantities used for each PCR are illustrated in Table 2.6..

Table 2.6. Restriction digestion of PCR fragments with potential RFLP sites

<table>
<thead>
<tr>
<th>Disease related mtDNA point mutation</th>
<th>MERRF 8344 A → G</th>
<th>MELAS 3243 A → G</th>
<th>LHON 3460 G → A</th>
<th>LHON 11778 G → A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactants</td>
<td>Buffer D: 2.0μl</td>
<td>Buffer A: 2.0μl</td>
<td>Buffer F: 2.0μl</td>
<td>Buffer C: 2.0μl</td>
</tr>
<tr>
<td>G → G</td>
<td>Bgl I: 1.0μl</td>
<td>Apa I: 1.0μl</td>
<td>Hsp 921: 1.0μl</td>
<td>Bcl I: 1.0μl</td>
</tr>
<tr>
<td>DNA: 17.0μl</td>
<td>Total: 20.0μl</td>
<td>BSA: 2.0μl</td>
<td>BSA: 2.0μl</td>
<td>DNA: 17.0μl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNA: 15.0μl Total: 20.0μl</td>
<td>DNA: 15.0μl Total: 20.0μl</td>
<td></td>
</tr>
<tr>
<td>Working condition</td>
<td>37°C, O/N</td>
<td>37°C, O/N</td>
<td>37°C, O/N</td>
<td>50°C, O/N</td>
</tr>
</tbody>
</table>

All the buffers and BSA were 10x concentrated, and enzymes were 10 U/μl (Promega).
Each 10 μl of digested product was mixed with 3.0 μl loading buffer (6x) and separated on an agarose gel. The molecular marker used was either 2.0 μl of λHind III (100 ng/μl) or the same amount of φ174 x Hae III (100 ng/μl) or both, depending on the sizes of the expected products. The gel and gel running conditions varied according to the sizes of the digested DNA fragments. The details are listed in Table 2.7.

Table 2.7. Electrophoresis condition for separation of the PCR-RPLP digestion products

<table>
<thead>
<tr>
<th>Diseases</th>
<th>MERRF 8344 A → G</th>
<th>MELAS 3243 A → G</th>
<th>LHON 3460 G → A</th>
<th>LHON 11778 G → A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker used</td>
<td>φ174 x He III</td>
<td>φ174 x He III</td>
<td>φ174 x He III</td>
<td>φ174 x He III</td>
</tr>
<tr>
<td></td>
<td>λHind III</td>
<td>λHind III</td>
<td>λHind III</td>
<td>λHind III</td>
</tr>
<tr>
<td>Agarose gel</td>
<td>2.5 %</td>
<td>1.0 %</td>
<td>2.5 %</td>
<td>2.5 %</td>
</tr>
<tr>
<td>Voltage for</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.0</td>
</tr>
<tr>
<td>electrophoresis (volts/cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
CHAPTER 3

MITOCHONDRIAL RESPIRATORY ENZYME ACTIVITIES IN AGING HUMAN PROSTATIC TISSUES

3.1. Introduction

An age associated decline in mitochondrial respiratory capacity, most markedly in complex I and complex IV activity, has been noted in a number of tissues, especially in post-mitotic tissues such as skeletal muscle (Trounce et al., 1989; Cooper et al., 1992; Boffoli et al., 1994), heart (Zeviani and Antozzi, 1992), and brain (Chagnon et al., 1995; Davis et al., 1997a, b). The impact of OXPHOS impairment on the aging process and on the associated senescent changes warrants further study. One of the major impediments however is the difficulty in obtaining human biopsy specimens. What is available are the tissues from autopsy. Autopsy samples inevitably introduce variable errors when used for enzymatic assay because enzyme activities depend largely on the biochemical viability of the tissues and the tissue condition at the time of collection. The time delay to post-mortem is among the key factors affecting the assay accuracy. The desired material for accurate enzyme kinetic assays is therefore, an ante-mortem biopsy specimen. Compared with other tissues, fresh prostatic tissues can be readily obtained because prostate excision is a common surgical practice conducted in patients with prostate hypertrophy. Moreover, such prostatic conditions are more common in the aging population. This allowed me to obtain fresh tissue specimens in very old subjects. Although prostatic tissue represents a dividing tissue model, it is interesting to investigate whether the OXPHOS profile changes with age, especially in this very old age group. The aims of this part of the study were to determine the respiratory activities in freshly obtained human prostate tissues in aging
subjects and to assess the suitability of prostate tissue for the study of aging associated decline in OXPHOS capacity.

3.2. Protocols and methods

The detailed methods for extracting mitochondria and the mitochondrial component from tissues and for measuring respiratory enzyme activities [i.e. NADH-cytochrome c reductase (complex I), succinate-cytochrome c reductase (complex II & III), and cytochrome c oxidase (complex IV)] are described in Chapter 2.] Briefly, in the presence of excess respective substrates and under the conditions optimal for each enzyme reaction, complex I activity was determined by the rate of NADH consumption, complex II & III by the rate of the accumulation of reduced cytochrome C and complex IV by the rate of decline in reduced cytochrome C.

The enzyme activities for complex I and IV were estimated in 23 and complexes II & III in 21 prostatic samples. The correlation of each enzyme complex activity with age was determined by the correlation coefficient $r$ (or $R^2$) of the linear regression curves which were constructed by using the Minitab statistic programme (Minitab release 10.1 Htra, USA). To examine whether the freezing and thawing process affected complex I activity, selected prostatic tissues were frozen for 7 days and thawed before the enzymatic assay was carried out. The values were compared with those obtained from corresponding fresh samples.
3.3. Results

3.3.1. The correlation of NADH-cytochrome C reductase (complex I) activity in human prostate biopsy tissue with age

The complex I activities in prostate tissues were much lower than in skeletal muscle (1.1±0.02 nmol/min/mg mitochondrial protein in prostatic tissues, vs 157 nmol/min/mg mitochondrial protein in skeletal muscle (Trounce, PhD thesis, 1990). The activities measured in the 23 patients only varied slightly (less than 2 fold) regardless of age or clinical symptoms. The results indicate that none of the patients exhibited significantly low enzyme activity of complex I. A linear regression analysis indicated that there was no correlation between complex I activity and age since the correlation co-efficient was low (r=0.18) and the correlation constant, the slope, close to zero (k=0.05, Figure 3.1.).
Figure 3.1. Correlation between respiratory chain complex I activity in prostatic tissue and age. Patients with ( ● ) and without ( □ ) mitochondrial DNA common deletion (see Chapter 4). The regression curve was constructed from the data of all patients.
Figure 3.1. Correlation between respiratory chain complex I activity in prostatic tissue and age. Patients with (●) and without (☐) mitochondrial DNA common deletion (see Chapter 4). The regression curve was constructed from the data of all patients.

3.3.2. The correlation of enzyme succinate-cytochrome c reductase (complex II & III) activity in human prostate biopsy tissue with age

The values measured for Complex II&III activities were also significantly lower than those reported in skeletal muscles ([1.5 vs 108 nmol/min/mg mt protein (Trounce, PhD thesis, 1990)]. The data in the studied group varied considerably with a 7-fold difference between the high and low levels, but the linear regression analysis indicated that there was no apparent correlation of complex II & III activity with age (k=0.012, r=0.15, Figure 3.2.).
y = 0.74 + 0.012x  \quad R^2 = 0.022

**Figure 3.2.** Correlation between respiratory chain complex II/III activity in prostatic tissues and age. patients with (●) and without (□) mitochondrial DNA common deletion (see Chapter 4). The regression curve was constructed from the data of all patients.

### 3.3.3. The correlation of enzyme cytochrome c oxidase (complex IV) activity in human prostate biopsy tissue with age

Activity of complex IV was also lower than in skeletal muscle [18 vs 926 nmol/min/mg mt protein (Trounce, PhD thesis, 1990)]. The values among patients varied slightly with a 2-fold difference between the high and the low levels. A linear regression analysis indicated that there was no correlation between the complex IV activity and age (k=0.08, r=0.014, Figure 3.3.).
Figure 3.3. Correlation between respiratory complex IV activity in prostatic tissues and age. Patients with (●) and without (□) mitochondrial DNA common deletion (see Chapter 4). The regression curve was constructed from the data of all patients.

The respiratory complex activities (complex I, II&II, and IV) assayed one week after being frozen at -70°C displayed inconsistent results compared with data obtained from corresponding fresh tissues. There was a general trend towards a decline in enzyme activity although not all samples showed this (figure 3.4.).
Figure 3.4. The influence of freezing and thawing procedure on the activity of respiratory chain enzyme complexes. The enzyme activities of complexes I, II&III, and IV were assayed in fresh and frozen prostate tissues.

3.4. Discussion

Studies indicate that decline in OXPHOS is associated with advanced age and the OXPHOS failure is in turn one of the contributing factors in accelerating both tissue or organ senescence and in age-related degenerative diseases. However, tissue response to the aging process is not uniform, and even with the same organ, different results were obtained in different studies. Post-mitotic organs such as skeletal muscle, the central nervous system and the heart are among organs mostly affected by advanced age and their post-mitotic status is also the propensity for mitochondrial diseases to harbor to these tissues. Trounce et al. (1989) were among the first groups to publish comprehensive data showing a negative correlation of respiratory activity with age in human skeletal muscle; the decrease in complex I and IV activities were over 50% among patients over 70 years of age as compared with 20 year olds. In
support of this age-related impairment, morphological studies showed that cytochrome oxidase negative fibers in human diaphragmatic skeletal muscle were increased 10-fold in elderly subjects (in their 90s) compared with middle aged people in their 40s (Byrne and Dennett, 1992). In brain, significantly decreased complex I activity in the substantia nigra was observed in patients with late onset of PD (Mann et al., 1994; Singer et al., 1995). It was also reported that in aged mouse and rat brain tissues, the different enzyme complex activities of the respiratory chain were significantly decreased, although to different extents, as compared with adult controls (Ferrandiz et al., 1994; Davis et al., 1997b).

The more recent studies using large sample sizes and different means of data normalization such as by considering physical activities or health conditions demonstrated that there was no correlation in declining OXPHOS capacity with advanced age in human skeletal muscle (Barrientos et al., 1996; Chretien et al., 1998). In an in vitro nuclear transfer study, mitochondrial DNA from (mtDNA)-less HeLa cells (r0-Hela cells) was transferred to human skin fibroblast cell lines from both aged subjects and fetus. Their nuclear hybrid clones were isolated. the mitochondrial translation and respiration properties of the nuclear hybrid clones were compared. No respiration deficiencies were observed in any nuclear hybrids irrespective of whether their mtDNAs were exclusively derived from aged or fetal donors (Isobe et al., 1998; Hayashi et al., 1994). OXPHOS activity may be influenced by the sample size, the evaluation methods and the sources of specimen (environmental factors, or the health conditions of each individual included in the studies).
In dividing tissues, mitochondrial respiratory dysfunction happens less frequently due to the replicative segregation phenomenon discussed in chapter 1. But in certain dividing tissues, such as the liver and hair follicles (Kotsimbos et al., 1994; Muller-Hocker et al., 1997; Sue et al., 1998). Defects in complex II and IV have been reported to occur in 87% of people over 50 years of age (Muller-Hocker et al., 1997). In this study, I chose to use human prostate tissue, another dividing tissue, to study the correlation of mitochondrial respiratory chain activity with age because this tissue is readily available, especially among elderly people. In 23 subjects with ages ranging from 53 to 90 years, there was no age-associated decline in respiratory enzyme activities in the prostate. The results are consistent with its dividing tissue status. Like other dividing tissues such as the skin, blood and platelets, instead of accumulating during the aging process, the abnormal mtDNA species which probably determine the respiratory dysfunction are diluted or even eliminated by continuous replication and segregation during rapid cell division (Bourgeron et al., 1993; Sue et al., 1998). This was demonstrated clearly by in vitro cell exhaustive growth studies in myoblast culture from patients with mtDNA MELAS or CPEOS mutations; a progressive elimination of mutant mtDNA was accompanied by improvement of respiratory chain activity, that is, the significant improvement of complex I and complex IV activities in cells after a few passages (Siregar et al. 1998).

Our results further support a diversity of mitochondrial function changes during the aging process within different tissues. Prostate tissue is not an appropriate tissue model to project the age associated mitochondrial functional changes in other organs especially the CNS. The same consideration is likely to apply to other dividing tissues such as blood, hair follicles or skin (Sue et al., 1998; Thorns et al., 1998; Birch-Machin et al., 1998).
CHAPTER 4

EXAMINATION OF MITOCHONDRIAL DNA MUTATIONS IN AGED HUMAN PROSTATIC TISSUES

4.1. Introduction

Mitochondrial DNA (mtDNA) encodes 26 polypeptides which encode some of the key components of respiratory chain enzyme complexes, and tRNAs and ribosomal RNAs essential for mitochondrial peptide synthesis. The increased generation of oxygen free radicals during oxidative stress and the decreased DNA repair capacity in aging subjects (Sohal et al., 1990a, 1990b; Sohal and Sohal, 1991) may accelerate the accumulation of mutant mtDNA. If such changes occur, they could contribute to the development and progression of age-associated neurodegenerative diseases. There is a possibility that some individuals may be prone to high levels of mtDNA mutation with aging and if so, this could contribute to both normal senescence and pathological aging. Using aged human prostate tissues, this part of my study examined the incidence of several mtDNA mutations, namely the common deletion and certain point mutations known commonly to cause cytopathies.

4.2. Protocols and methods

4.2.1. Preparation of DNA samples for PCR and Southern hybridisation, and determination of their quality
The method for total DNA extraction from each prostate tissue is described in chapter 2. The extracted DNA samples were used as either templates for PCR or for Southern hybridization studies.

To check the quality of the extracted DNA samples, a simple PCR designed to amplify a fragment from human mtDNA nt 3728 to nt 2826 was performed in all prostate samples and a 903 bp PCR product was appeared in each sample preparation (data not shown).

4.2.2. PCR screening for the mtDNA\textsuperscript{4977} common deletion

The presence of mtDNA\textsuperscript{4977} common deletion (from nt 8482 to nt 13459) was assessed by PCR co-amplification of wild type and the deleted mtDNA in 23 prostate tissues. With the chosen primer pair: 5' primer flanking from nt 7901 of the light chain and 3' primer flanking from nt 13650 of the heavy chain, the expected size for amplified mtDNA was 773 bp with common deletion and 5750 bp for intact mtDNA. To validate the results, primer pair shift PCR with two additional pairs of primers was also conducted to confirm the existence of mtDNA deletion or duplication in this region.

4.2.3. Detection of mtDNA deletions by Southern hybridisation

In order to estimate the ratio of the deleted mtDNA populations to the wild type populations, Southern hybridization was performed in 25 prostatic DNA samples. 5µg of total DNA was digested with BamH I, and the post-digestion products were separated on a 0.6% agarose gel at 4.7 volts/cm for 1 hour. The separated DNA fragments were transferred to a nitrocellulose membrane, and radio-labeled mtDNA
probes were used to probe mtDNA fragments. The appearance of bands smaller than 5.75 kb indicated the existence of mtDNA deletion. A 773 bp band indicates the common deletion.

4.2.4. Screening for mtDNA point mutation using PCR-RFLP

PCR-RFLP was used to identify point mutations at nt 8344 A→G, 3243 A→G, 3460 A→G and 11778 G→A. mtDNA fragments encompassing the above positions were amplified by PCR, and this was followed by restriction enzyme digestion.

- 8344 A→G: PCR amplified fragment would be 108 bp; further digestion with Bgl I would generate two smaller bands (78 and 35 bp) in the presence of the mutation.
- 3243 A→G: PCR amplified fragment would be 902 bp; further digestion with Apa I would generate two smaller bands (417 and 485 bp) in the presence of that mutation.
- 3460 A→G: PCR amplified fragment would be 902 bp; further digestion with Hsp921 would normally lead to two further bands (634 and 268 bp), but if the mutation occurs, a portion of 902 bp band would be retained.
- 11778 G→A: PCR amplified fragment would be 193 bp; further digestion with Bgl I would generate two smaller bands (50 and 143 bp) in the presence of that mutation.

4.3. Results

The quality of both mitochondrial DNA and total DNA samples extracted from the prostate tissues were compatible with the criteria for further PCR and Southern hybridization studies. Figure 4.1a. & b. showed that an expected PCR band was obtained from each sample with little non-specific bands being generated.
Figure 4.1a. Examination of the quality of the mitochondrial DNA and total DNA extracted from human prostate biopsy using the polymerase chain reaction.
The expected PCR fragment is 903 bp. M: λHind III digested marker; Lanes 1-4, extracted mitochondrial enriched DNA* (40 ng); lanes 5 (20 ng) & 6 (40 ng): positive control (total DNA from previous studies); lanes 7-10, extracted mitochondrial enriched DNA* (20 ng); lanes 11 (162 ng) & 12 (123 ng), positive control (mitochondrial enriched DNA from previous studies); lanes 13 & 14, milli Q H₂O as the negative control.

* the extracted mitochondrial enriched DNA samples were from patients P₄, P₅, P₁₀ and P₁₆ respectively.
Figure 4.1b. Examination of the quality of total DNA extracted from human prostate biopsy tissues using the polymerase chain reaction. The expected PCR fragment is 903 bp. M: \(\lambda\)Hind III digested marker; Lanes 1, 3, 5 & 7: total DNA* (20 ng); lanes 2, 4, 6 & 8: total DNA* (40 ng); lanes 9 (162 ng) & 10 (123 ng): positive control (mitochondrial enriched DNA from previous studies); lanes 11 (20 ng) & 12(40 ng): positive control (total DNA from previous studies); lanes 13 & 14, milli Q H\(_2\)O as the negative control.

* the total DNA samples were from patients P\(_4\), P\(_5\), P\(_{10}\) and P\(_{16}\) respectively.

4.3.1. Results of the survey from common deletion by PCR

Three out of 23 patients contained a small percentage of mutant mtDNA with the common deletion in prostatic tissues (Figure 4.2.). These results were confirmed by primer shift PCR (Figure 4.3.). In addition, there were other undefined large deletions in these three subjects. No correlation of the accumulation of mtDNA deletion load with age was found, nor was a correlation seen between the deletion load and the respiratory chain enzyme activities (Figure 3.1.).
Figure 4.2. Screening of mtDNA 4977 common deletion by PCR. Primer pairs used were L790 & H1365 which amplify the mtDNA fragment from nt 7901 to nt 13650. Samples and markers used:
M1: λ Hind III (1 μg, Promega);
M2: φ × 174 Hae III (2 μg, Promega);
C+: total DNA (50 ng) from the skeletal muscle of a CPEO patient;
C−: total DNA (50 ng) from normal skeletal muscle;
1 to 26: prostatic total DNA (50 ng);
W: milli Q H2O.

Summary of the results:

<table>
<thead>
<tr>
<th></th>
<th>C+</th>
<th>C−</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4977 common deletion</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>multiple deletion</td>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>
Figure 4.3. Validation of mtDNA deletion with primer pair shift PCR analysis.

Primer pairs used:
- lane a: from nt 7901 (L_{790}) to nt 13650 (H_{1365});
- lane b: from nt 7901 (L_{790}) to nt 13928 (G_{1}R_{1});
- lane c: from nt 6448 (L_{644}) to nt 13650 (H_{1365}).

Samples and the marker used:
- M: 1 kb DNA extension ladder (2 μg, Life Technologies)
- A: Negative control: total DNA (20 ng);
- B: Positive control: total DNA (20 ng) from a CPEO patient;
- C: Positive control: total DNA from tissue culture;
- D: Total DNA (40 ng) from *P_3 which was extracted in 1995;
- E: Total DNA (40 ng) from P_5 which was extracted in 1995;
- G: P_{10}'s Mitochondria enriched DNA (162 ng) from P_{10};
- H: Mitochondria enriched DNA (123 ng) from P_{16};
- I: milli Q H_2O.

*patient
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* "Aa" means sample "A" was amplified by the primer pair "a".

4.3.2. Results for mtDNA deletions by Southern hybridisation

All 26 total DNA samples from prostatic tissues showed a distinctive band corresponding to the full size mtDNA(16,569 bp), however, there were no smaller bands in any of the samples being examined (Figure 4.4.). The result indicates that the portions of the common deletion detected in 3 patients with PCR were very low which below the threshold for Southern hybridization.
Figure 4.4. Southern analysis for detecting large deletion in human prostate mtDNA. A hybridised band corresponding to the wild type mtDNA species was apparent in each sample, but there was no detectable smaller size band in any of the samples indicating the low abundance of the mtDNA population bearing the common deletion or any other large fragment deletion.

4.3.3. Screen for point mutations in mtDNA

Four point mutations (nt 8344 A→G, 3243 A→G, 3460 A→G and 11778 G→A) were examined using PCR-RFLP. No point mutation of any kind was detected in 23 prostatic mtDNA samples (Figures 4.5., 4.6., 4.7., 4.8.).
Figure 4.5. Screening for the mtDNA 8344 A → G point mutation in human prostate tissue by PCR-RFLP. A 108 bp mtDNA fragment was amplified using primer pair SNS(8278) and MRF-2(8385). The product was then digested with Bgl I and two fragments of 73 and 35 bp were expected in samples with that point mutation.
Figure 4.6. Screening for mtDNA 3243 A → G point mutation in human prostate tissue by PCR-RFLP. A 902 bp mtDNA fragment was amplified using primer pair B1F1(2826) and B1R1(3728). The product was then digested with Apa I and two fragments of 405 and 417 bp were expected in each sample but a 902 bp band should be retained with that point mutation.
Figure 4.7. Screening for the mtDNA 3460 G → A point mutation in human prostate tissue by PCR-RFLP. A 902 bp mtDNA fragment was amplified using primer pair B1F1(2826) and B1R1(3728). The product was then digested with Apa I and two fragments of 635 and 228 bp were expected in each sample but a 902 band should be retained with that point mutation.
Figure 4.8. Screening of mtDNA 11778 G → A point mutation in human prostate tissue by PCR-RFLP. A 146 bp mtDNA fragment was amplified using primer pair L1172(11728) and ND4H(11919). The product was then digested with Bcl I and two fragments of 96 and 50 bp were expected in samples with that point mutation.
4.4. Discussion

MtDNA mutations have been considered to be sensitive predictive markers for OXPHOS failure in human tissues. As described in chapter 1, the frequency of mtDNA mutation is significantly higher than in nDNA due to the high oxygen free radical environment within mitochondria. This is further exaggerated with age since aging is associated with increased oxidative stress and reduced DNA repair capacity (Lezza et al., 1994; Kovalenko et al., 1998). Age-associated accumulation of mutant mtDNA can be very high in post-mitotic tissues such as skeletal muscle, the CNS, and cardiac tissues (Kovalenko et al., 1998; Zhang et al., 1999). Mutant mtDNA accumulation has also been detected in some non-post-mitotic tissues such as liver and skin (Birch-Machin et al., 1998; Lu et al., 1999), but not in others such as blood including platelets or hair follicles (Sue et al., 1998; Biagini et al., 1998).

In this study, the mtDNA 4977 bp common deletion together with other un-defined deletions were detected by PCR in human prostate tissues in 3 patients out of 23, but there was no correlation of the load or presence of mtDNA deletion with age. The percentages of such deletions were low since they could not be detected by Southern blot. The results indicate that mtDNA deletions occur at a low frequency in aged prostatic tissues. The fact that no other common point mutations were detected in any of the patients. This is in line with the finding of unchanged enzyme activities with age described in chapter 3. Prostate tissue therefore represents a typical dividing tissue model, and is not a suitable model for studies on mtDNA involvement in tissue senescence and progression of age-related diseases as commonly seen in the CNS,
skeletal muscle and the cardiovascular system. A reliable and representative human tissue model would be helpful for further understanding of the roles of mitochondria in aging and disease progression. It is probable that mitochondrial studies in the aging brain will require direct study of the CNS. In this regard, early post-mortem specimen may be more revealing. In addition, human skeletal muscle biopsies may also be used as a surrogate.
CHAPTER 5

GENERAL DISCUSSION

An age-associated decline in respiratory chain activity which was reflected by the reduced activities in complex I, III and IV in human skeletal muscle mitochondria was first reported in 1989 (Trounce et al., 1989). Subsequent studies revealed that only the activities of complex I and IV were significantly reduced. A similar fall has been documented in human liver mitochondria (Yen et al., 1989). Evidence from histochemical studies suggest that the decline in the respiratory capacity with age is patchy, with the development of an energy mosaic (Muller-Hocker, 1989, 1990; Byrne and Dennett, 1992). These data support the hypothesis that partial failure of oxidative phosphorylation may contribute to the aging process (Fleming et al., 1982, Byrne et al., 1991). The recognition that mitochondrial DNA mutations are central in certain rare disease processes together with the knowledge that mtDNA is prone to mutation led to the proposal that cumulative mutation of the mitochondrial genome may play a major role in aging (Linnane et al., 1998). This led to a search in aged subjects for deletional mutations commonly identified in mitochondrial cytopathies. The 4977 bp common deletion from nt 7901 to nt 13650, the most prevalent deletion identified by Holt et al.(1988) in mitochondrial diseases, involves several subunits of mitochondrial complexes: I, IV and V, and several tRNA genes. This deletion has been found in some post-mitotic tissues from aged subjects, notably in the heart and in certain brain regions. In some subjects, this deletion level reached very high (Arnheim and Cortopassi, 1992). Other species of large deletion (i.e. an 7436 bp deletion) have also been found in aged skeletal muscles (Wei, 1992). An accumulation of deletional
species has also been reported in ischaemic heart (Zhang et al., 1999). In most of these studies, the validity of the deletions has been confirmed using primer shift PCR.

Although the presence of deletions in aged tissue is now incontestable, their significance is far from clear as even at the highest level reported they account for only a small percentage (less than 1%) of all mtDNA present. Given the fact that much higher levels of deletional mutation (typically greater than 70% of mtDNA) are encountered in the CPEO syndromes (Zeviani et al., 1988) and that there is probably an up regulation of mtDNA copy number in aged tissues (Gadaleta et al., 1994), this small amount of the common deletion is unlikely to affect mitochondrial OXPHOS. However, given the large numbers of point and deletional mutations now identified in human disease, the possibility arises that the common and other deletions maybe a marker for mtDNA abnormality in general.

A cumulative effect of a number of mutations, each representing only a small percentage of the total mitochondrial copy number, could then be envisaged as leading to mitochondrial respiratory chain function decline with aging. This is an attractive theory as a series of random mutational events with a cumulative effect best fits what we know of mitochondrial DNA biology in the aging cell. This hypothesis would be on stronger ground if it were possible to demonstrate that a) progressive accumulation of deletional species is seen in extreme senescence and b) there is a strong correlation between mitochondrial respiration efficiency and the appearance of deletional species. In this study, human prostate was selected as a tissue that could be obtained ethically in amounts suitable for both biochemical and DNA studies in ante-mortem biopsy material.
With Complex I and IV respiratory activity, an age span of 40 year was studied. This is comparable to that uncovered in the skeletal muscle and liver tissue in other studies although exact comparison with those studies is difficult as the age range studied was much wider but with a smaller percentage of very elderly patients (Trounce et al., 1989; Yen et al., 1989; Cooper et al., 1992). Prostatic specimens were not available from younger subjects as benign prostatic hypertrophy is exclusively a condition of the elderly with the result that only the last period of the aging process was examined. Because of the elderly presentation of prostatic pathology, a much larger number of very old subjects were studied than in any other previous study. Although deletional mutations, sometimes multiple, were found in 3 out of 23 patients, there was no age preference.

These findings suggest that the detection of low levels of the common deletion in elderly subjects does not predict a less efficient mitochondrial respiratory capacity, and by inference that the presence of the common deletion does not predict the accumulation of multiple small deletions or point mutations in the genome which impair respiratory capacity. These findings do not disprove the mtDNA theory of aging, but simply reinforce the need for great caution in interpreting the significance of the common or similar large deletional mutations. The absence of any point mutations with a clear link to mitochondrial disease in aged prostatic tissue is also of interest and suggests that if cumulative mutations do occur in the mitochondrial genome, they have no particular prediction for the positions most clearly linked to mitochondrial disease in younger individuals. These findings do not exclude the possibility of cumulative mutations with random distribution within the mitochondrial
genome with aging which have potentially deleterious effect on mitochondrial respiratory function. However, no such decline was seen in mitotic tissue over the 40 years of age span covered.

Study of non-cerebral tissue represents an attractive means to detain information on whole body and indirectly on brain OXPHOS capacity. Tissues that are readily available (blood, skin, or platelets) are all mitotic, and the discordance between the results identified here in prostate, a dividing tissue with no age related fall and in the same laboratory, studies in non-dividing tissues (brain, skeletal muscle, heart tissue) where an age related fall occurs suggests that great care must be taken in drawing general conclusions from results in one tissue. It was hoped that a subgroup of patients with low OXPHOS capacity in prostate might be identified but this was not realized.

The primary aim of this study was to check the suitability of this tissue as a window to explore further mitochondrial involvement in aging. The outcome was negative. However, prostatic tissue can be readily accessed and may be useful in other studies of senescent mechanisms.
Bibliography


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Wei Y.H. Oxidative stress and mitochondrial DNA mutations in human aging.


APPENDIX

Reagents and Buffers

a) Salting out lysis buffer: 1 ml of 1 M Tris pH 7.5
   8 ml of 5 M NaCl
   400 μl of 0.5 M EDTA
   make up to 100 ml by dH₂O, autoclave.

b) MES buffer: 225 mM Mannitol
   75 mM Sucrose
   0.1 mM EDTA

c) Proteinase K: Prepare a 5 mg/ml or 7.5 mg/ml stock in sterile water
   Store frozen in aliquots at -20°C.

d) 6 M NaCl: 35.06 g NaCl into 100 ml dH₂O. Autoclave. This is a
   saturated solution of NaCl.

e) Digestion buffer(10x): 500 mM Tris, pH 8.0
   10 mM EDTA
   5% Tween 20

f) PCR buffer B(10x): 20 mM Tris-HCl(PH 8.0)
   100 mM KCl
   0.1 mM EDTA
   1 mM DTT
   50% glycerol
   0.5% Nonidet
   0.5% Tween 20

g) TAE buffer(10x): 0.4 M Tris-acetate
   0.01 M EDTA

h) Loading buffer(6x): 0.25% Bromophenol Blue
   0.25% Xylene Cyanol
   40% (W/V) Sucrose in dH₂O

i) λ Hind III (0.25 μg/μl):
   200 μl Stock λ (375 ng/μl)
   10 μl Hind III(10 U/μl)
   40 μl Buffer E (10 x)
   150 μl dH₂O

   37°C in water bath for 2.5 hours,
   added 100 μl of Loading Buffer(6 x)

j) 20 x SSC buffer: 3 M NaCl
0.3 M sodium citrate, pH 7.0

k) Hybridization solution: 50% Formamide (stock concentration: 100%)
   (for Southern blot) 6 x SSC (stock concentration: 20 x)
   0.01 M EDTA (stock concentration: 0.5 M)
   5 x Denhardts (stock concentration: 50 x)
   0.5% SDS (stock concentration: 20%)
   2% Casein (Skim milk powder, freshly made)

l) Washing solution: (for Southern blot)
   (a) 1x SSC
       0.1% SDS
   (b) 0.5x SSC
       0.1% SDS
   (c) 0.25x SSC
       0.1% SDS
   (d) 0.1x SSC
       0.1% SDS
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Author/s: 
Weng, Shan

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