

Mini review

Role of mitochondrial DNA in toxic responses to oxidative stress

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Received 9 February 2005
Available online 4 May 2005

Abstract

Mitochondria are at the crossroads of several crucial cellular activities including: adenosine triphosphate (ATP) generation via oxidative phosphorylation; the biosynthesis of heme, pyrimidines and steroids; calcium and iron homeostasis and programmed cell death (apoptosis). Mitochondria also produce considerable quantities of superoxide and hydrogen peroxide (H₂O₂) that in conjunction with its large iron stores can lead to a witch's brew of reactive intermediates capable of damaging macromolecules. Mitochondrial DNA (mtDNA) represents a critical target for such oxidative damage. Once damaged, mtDNA can amplify oxidative stress by decreased expression of critical proteins important for electron transport leading to a vicious cycle of reactive oxygen species (ROS) and organellar dysregulation that eventually trigger apoptosis. Oxidative stress is associated with many human disorders including: cancer, cardiovascular disease, diabetes mellitus, liver disease and neurodegenerative disease. This article reviews the evidence that oxidative damage to mtDNA can culminate in cell death and thus represents an important target for therapeutic intervention in a number of human diseases.

Published by Elsevier B.V.

Keywords: DNA repair; Hydrogen peroxide; Mitochondrial DNA; Oxidative stress; Quantitative polymerase chain reaction

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Abbreviations: AP, apurinic/aprimidinic; APE, apurinic/aprimidinic endonuclease; EndoIII, endonuclease III; EndoVIII, endonuclease VIII; ExoIII, exonuclease III; FAD, flavine adenine dinucleotide; H₂O₂, hydrogen peroxide; MMS, methyl methanesulfonate; MPG, *N*-methylpurine DNA glycosylase; mRNA, messenger RNA; mtDNA, mitochondrial DNA; NAD, nicotinamide adenine dinucleotide; nDNA, nuclear DNA; 3-NPA, 3-nitropropionic acid; OGG-1, 8-oxodG glycosylase; ROS, reactive oxygen species; QPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcriptase polymerized chain reaction; TCA, tricarboxylic acid

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1. Introduction

Mitochondria normally are associated with generation of ATP through oxidative phosphorylation. However, these organelles also participate in a wide variety of essential cellular functions such as homeostasis of calcium and iron, as well as biosynthesis of heme, pyrimidines and steroids. By releasing several proteins that incite programmed cell death, mitochondria act as the “executioners” in apoptosis (for a recent review see [1]).

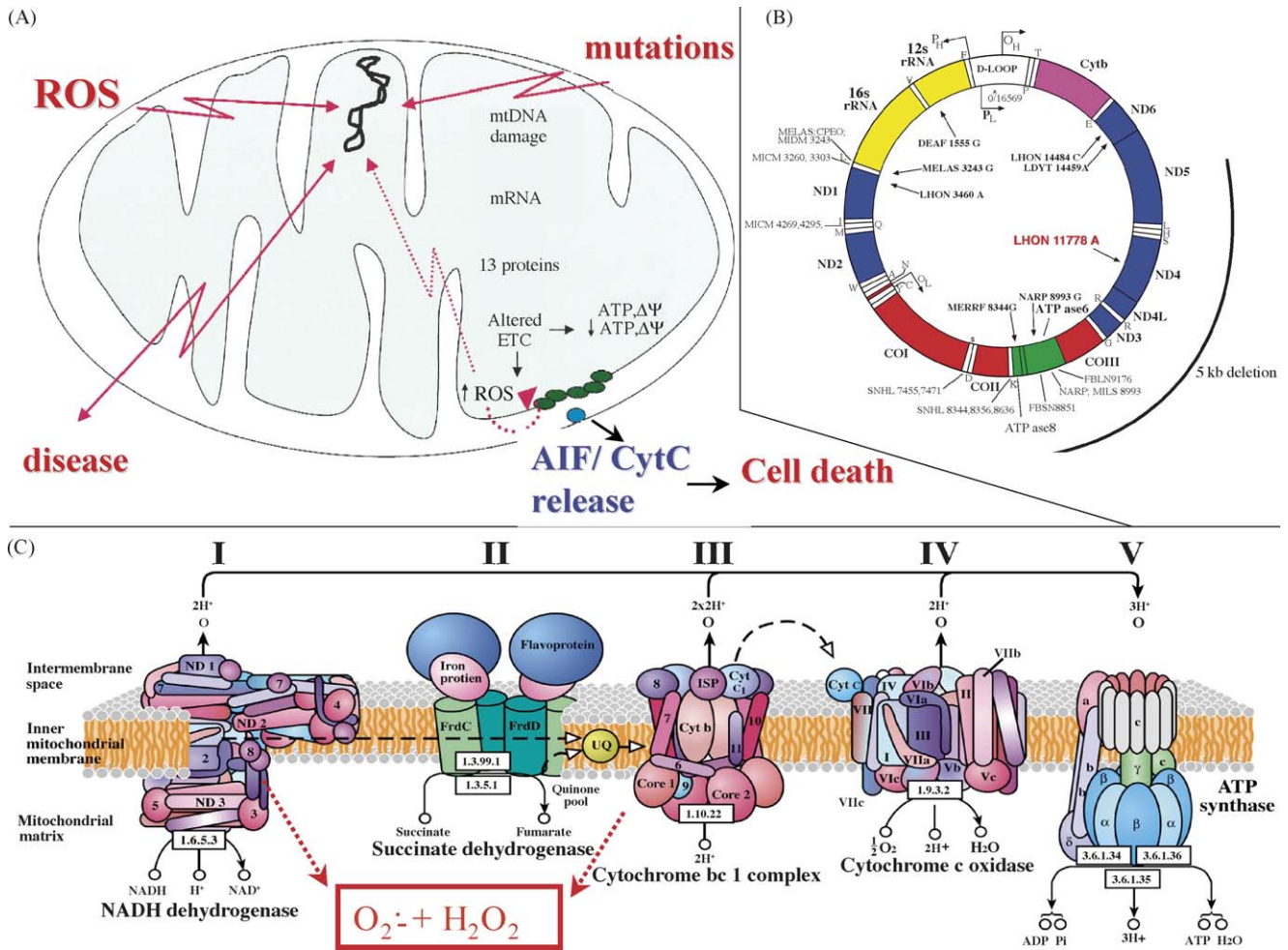


Fig. 1. (Panel A) Role of mtDNA in oxidative stress-induced injury. mtDNA damage can lead to loss of expression of mitochondrial polypeptides, subsequent decrease in electron transport and increase in ROS generation, loss of mitochondrial membrane potential and release of signals for cell death, such as CytC and AIF. (Panel B) Mitochondrial genome from human cells indicating, the genes, and some mitochondrial mutations associated with human disease. (Panel C) Electron transport chain showing path of electrons, direction of proton movement, site of ATP synthesis and the two major sites of superoxide generation.

Mitochondria possess both an outer and inner membrane, the latter of which is impermeable to all molecules, including charged ions. The complex process of ATP synthesis occurs at the inner mitochondrial membrane through the donation of electrons by nicotinamide adenine dinucleotide (NAD) or flavine adenine dinucleotide (FAD) equivalents generated by the tricarboxylic acid (TCA) cycle (Fig. 1). These electrons are passed along a series of molecular complexes known as the electron transport system. Simultaneously, protons are transferred across the inner membrane at complexes I, III and IV to establish a trans-membrane gradient of electrical charge known as the membrane potential. Complex V (F₀F₁ ATP synthase) harnesses this membrane potential as chemical energy in the form of ATP. The final electron acceptor is molecular oxygen, which is reduced through a four electron addition to water. However, a significant portion of electrons may escape the electron transport chain as superoxide anions. Superoxide, in turn, may spawn the production of H₂O₂ spontaneously or through the action of manganese superoxide dismutase (MnSOD) ([2]; reviewed in [3]). H₂O₂ is

normally broken down in mitochondria by glutathione peroxidase, but depletion of glutathione or excessive H₂O₂ production may allow H₂O₂ to react with Fe²⁺, yielding hydroxyl radicals. The highly reactive hydroxyl radical can damage macromolecules within mitochondria, including lipids, proteins and DNA.

In human cells, each mitochondrion has approximately 10–15 copies of a small genome consisting of 16,569 base pairs (Fig. 1B). This mtDNA encodes 13 polypeptides, 22 transfer RNAs and 2 ribosomal RNAs, all of which are essential for electron transport and ATP generation and consequently for normal cellular physiology. While Attardi and co-workers have shown that cells depleted of mtDNA (rho⁰) by ethidium bromide treatment can survive and grow, these cells must maintain adequate supplies of ATP via glycolysis (reviewed in [4]). Surprisingly, some of this ATP is hydrolysed by F₀F₁ ATP synthase (complex V) to maintain the proton gradient across the inner mitochondrial membrane. Many specialized cells in the body, such as neurons, cannot sustain adequate ATP levels through glycolysis and thus loss

Table 1
Human diseases associated with oxidative stress and mitochondrial dysfunction

Disease	Comments	References
Asbestosis/mesothelioma		[8,9]
AZT	HIV therapy	[10]
Cancer		[11]
Breast		[11]
Colorectal		[11]
Gastric	<i>H. pylori</i> infection	[11,12]
Hereditary paragangliomas	Mutations in SD	[13]
Phaeochromocytomas/ paraganglioma	Mutations in SD	[13]
Papillary renal cell	SD mutations	[11,13]
Uterine leiomyomata/skin leiomyomata	Mutations in FH	[13]
Cardiovascular disease		
Atherosclerosis		[14]
Progressive heart failure		[15,16]
Diabetes mellitus		[17,18]
Ischemia-reperfusion injury		[19]
Liver disease		
Alcoholic hepatitis		[20–22]
Hemochromatosis		[23]
Neurodegenerative diseases		[24,25]
Alzheimer's disease		[26–28]
Amyotrophic lateral sclerosis		[29]
Friedreich's Ataxia		[30–32]
Huntington's disease		[33–35]
Parkinson's disease		[36–39]

of oxidative phosphorylation leads to cell death. Various human diseases are associated with mutations in mitochondrial genes (reviewed in [5,6]; see also Fig. 1B). During the last three decades, numerous human diseases also have been associated with ROS generation and the ensuing mitochondrial dysfunction that results in cell death (reviewed in [7] and see Table 1 and references therein). mtDNA therefore represents a critical cellular target for oxidative damage that could lead to lethal injury through the loss of electron transport, mitochondrial membrane potential and ATP generation. The following criteria should be fulfilled to conclude that oxidative mtDNA damage is a direct cause of cell death:

- mtDNA rather than nuclear DNA (nDNA) lesions should correlate with cell death;
- mtDNA damage should precede the ATP depletion, loss of electron transport and failure of mitochondrial membrane potential that culminate in cell death;
- enhancement of mtDNA repair should confer protection from cell death, whereas;
- loss of mtDNA repair should promote cell death.

The following review explores a body of experimental evidence that meets these criteria, thereby supporting the hypothesis that oxidative injury to mtDNA is an important mediator of cell death and disease. As such, mtDNA represents an important potential target for intervention during the course of disease.

2. mtDNA damage is more severe than nDNA damage following oxidative stress

Early studies by Ames and co-workers suggested that mtDNA might be more prone to oxidative damage than nDNA [40]. However, a decade later it appeared that assays measuring oxidation of 8-oxo-guanosine in DNA using high performance liquid chromatography with electrochemical detection were in error due to high levels of background oxidation inflicted during mitochondrial isolation and DNA purification [41]. These problems inspired my laboratory to develop an assay that does not rely upon segregation of mtDNA from nDNA during purification. The quantitative polymerase chain reaction (QPCR) we developed allowed us to show that mtDNA suffers 3–10-fold more damage than nDNA following oxidative stress in numerous cell types from yeast, mouse, rats and humans ([42,43]; reviewed in [44]).

3. Loss of mtDNA repair is associated with cell death

In the mid-1970s, Clayton and Friedberg found that UV-induced pyrimidine dimers are not repaired in mtDNA of human cells. This phenomenon often is cited as demonstrating that mitochondria have no DNA repair capacity [45]. Over the past 15 years, it has become clear that mitochondria lack the necessary enzymes for nucleotide excision repair, the major pathway for the removal of DNA adducts induced by UV-irradiation, carcinogenic polycyclic aromatic hydrocarbons and chemotherapeutic agents like cisplatin. However, base excision repair, the principal pathway for the removal of oxidative DNA damage, has been well characterized in the mitochondria of various organisms. mtDNA repair is beyond the scope of this article and the reader is referred to a number of excellent reviews [46–52]. All the mtDNA repair machinery is encoded in the nucleus and the corresponding gene products are subsequently transported into the mitochondria through the membrane potential and a mitochondrial targeting sequence, which comprises 20–25 N-terminal amino acids that are cleaved off during the transport process.

Repair of base damage in mitochondria was first reported by, Wilson, Ledoux and co-workers [53,54]. Since that time mitochondria have been shown to efficiently repair oxidative DNA damage by a number of strategies (see reviews above). We documented complete repair of both nDNA and mtDNA lesions in SV-40 transformed human fibroblasts within 1.5 h of a 15 min treatment with H₂O₂ (200 μM) [42]. Furthermore, after this recovery period, DNA of both genomes in treated cells had fewer lesions than the DNA of control cells. This observation was apparently due to increased expression of repair enzymes responsible for removal of oxidative DNA damage [55]. Surprisingly, we found that extending the H₂O₂ exposure period to 60 min led to persistent mtDNA lesions that were refractory to repair up to 24 h after treatment cessation (even though >90% of the H₂O₂ had been metabolized by 60 min), while in contrast, nDNA repair occurred within

90 min after H₂O₂ withdrawal [42]. In addition, persistent mtDNA adducts resulting from prolonged H₂O₂ exposure correlated with loss of mitochondrial function and eventual cell death. These findings demonstrate two important principles: (1) although mitochondria have DNA repair machinery, mtDNA is more susceptible to oxidative damage than nDNA. This is likely due to the characteristically high iron content of mitochondria that mediates free radical formation and (2) lesions in mtDNA block RNA polymerase thereby preventing mtDNA transcription. This, in turn, results in the loss of key mtDNA-encoded electron transport proteins and the initiation of a vicious cycle of ROS propagation and mtDNA oxidation. The cascade instigated by oxidative mtDNA damage that leads to faulty gene expression, deficiency of key electron transport enzymes, subsequent ROS generation and ultimately, cell death, is known as the mitochondrial catastrophe hypothesis (Fig. 1). Experimental evidence in support of this hypothesis is outlined below.

4. mtDNA damage leads to loss of membrane potential, ATP synthesis and cell death

Ballinger and Runge, working with our group, found that human umbilical vein endothelial cells treated with H₂O₂ or peroxynitrite suffered more mtDNA than nDNA damage [56]. This mtDNA damage was associated with decreased steady-state levels of messenger RNA (mRNA) encoded by the mitochondrial genome and reduced concentrations of all 13 polypeptides translated in the mitochondria. Diminished mitochondrial protein expression was accompanied by lowered ATP production, a decreased inner mitochondrial membrane potential, and generation of secondary ROS within mitochondria [56]. Ayala-Torres (unpublished observation) obtained similar results following H₂O₂ treatment of SV-40 fibroblasts, which showed a rapid decline in steady-state levels of mitochondrial mRNA accompanied by a loss of ATP. Analysis of mRNA by reverse transcriptase polymerase chain reaction (RT-PCR; a quantitative amplification of mRNA) showed that most of the mRNA could not be reverse-transcribed, suggesting that considerable oxidative damage to mRNA causes degradation and loss of protein synthesis.

Previously, we had observed that overexpression of the anti-apoptotic protein Bcl2 did not prevent H₂O₂- or peroxynitrite-induced damage in rat Bcl2-transfected PC12 cells. Nevertheless, cells overexpressing Bcl2 exhibited more rapid rates of mtDNA repair and increased cell survival relative to controls [57]. Working in our group, Mandavilli used the fungal toxicant 3-nitropropionic acid (3NPA) to investigate whether ROS derived directly from mitochondria could promote mtDNA damage, loss of ATP and subsequent cell death [58]. Ingestion of 3NPA can cause symptoms mimicking Huntington's disease in rodents and humans. Effects of 3NPA are believed to be mediated by direct inhibition of succinate dehydrogenase (complex II), leading to diminished electron transport and increased ROS generation, followed by

inhibition of the TCA cycle. Treatment of PC12 cells with 3NPA resulted in rapid ROS induction, as well as, damage to mtDNA, but not nDNA, followed by a reduction in ATP synthesis. In contrast, PC12 cells overexpressing Bcl2 generated 50% less ROS after 3NPA treatment and maintained higher levels of ATP. Most importantly, Bcl2 expression completely protected cells treated with 3NPA from mtDNA damage, resulting in increased cell survival [58].

Santos, another member of our group, found that telomerase-immortalized fibroblasts treated for 60 min with 200 μM H₂O₂ suffered large amounts of mtDNA damage, but no detectable nDNA damage [59]. Although these cells repaired about 50% of their mtDNA damage, cell death began to ensue at 24 h post-treatment. Cell demise was characterized by caspase 3 activation consistent with apoptosis. Twenty-four hours post-H₂O₂ treatment, analysis revealed that approximately 70% of the cells had low mitochondrial membrane potential [59]. FACS sorting of cells according to their mitochondrial membrane potential, followed by QPCR analysis of mtDNA damage, showed that 30% of cells had high membrane potential and no mtDNA damage, whereas cells that lost mitochondrial membrane potential (70%) had substantial DNA damage. Moreover, cells with low membrane potential had elevated levels of ROS as compared to those with high membrane potential. These studies demonstrate that (1) mtDNA damage precedes loss of membrane potential and that cells with low membrane potential generate increased amounts of ROS and (2) mitochondria can exhaust their ability to repair mtDNA damage due to oxidation of mtDNA repair proteins and/or compromised ability to import newly formed repair proteins (encoded in the nucleus) into the mitochondrial matrix. Thus, extinction of mitochondrial membrane potential promulgates persistent mtDNA damage and apparently condemns the cell to death.

One potential challenge to this concept is that rho⁰ cells (cells lacking mtDNA) can still undergo apoptosis after certain agents [4]. While these experiments suggest that mtDNA is not essential for apoptosis, they do not speak to the issue of whether mtDNA damage can enhance the rate of cell death. Oliveira and co-workers have shown that functional mitochondria and normal mtDNA are required for cell death induced by beta-amyloid, which produces intracellular hydrogen peroxide [60,61]. More recently the same group has shown that rho⁰ cells are actually more sensitive to hydrogen peroxide-induced caspase activation and cell death than cells containing mtDNA and functional mitochondria [62]. This study seems to underscore that fact that mitochondrial dysregulation enhances ROS-induced cell death and is consistent with other results presented in this review.

5. Enhanced mtDNA repair protects cells from cell death

If mtDNA damage is responsible for cell death, then enhancement of mtDNA repair should improve cell survival.

Table 2
Alterations in mitochondrial DNA repair leads to changes in cell survival after damage

Enzyme	Oxidant	Lesion	Cells	Increased DNA repair	Increased cell survival	References
OGG-1	Menadione	8-oxo-dG	HeLa	++	++	[63]
OGG-1	Hypoxanthine/XO	8-oxo-dG	Rat pulmonary artery endothelial cells	++	++	[64]
OGG-1	Asbestos	8-oxodG	Rat pleural mesothelial	ND	++	[8]
OGG-1	Menadione	8-oxo-dG	Oligodendrocytes	++	++	[65]
Exo III	Hypoxanthine/XO	Abasic sites	Human mammary adenocarcinoma	--	--	[66]
MPG	MMS	Methylpurine	Human mammary adenocarcinoma	-/+	--	[67]
EndoIII/EndoVIII	Menadione	Oxidized pyrimidines	HeLa/TETon	++	++	[68]
OGG-1	Hypoxanthine/XO	8-oxo-dG	Rat pulmonary artery endothelial cells	++	++	[69]
APE	Hypoxanthine/XO or hyperoxia	Oxidized bases	Oxidant resistant hamster fibroblasts	++	++	[70]

Abbreviations: OGG-1: 8-oxodG glycosylase; ExoIII: exonuclease III, a major apurinic/aprimidinic endonuclease from *E. coli*; MPG: *N*-methylpurine DNA glycosylase; MMS: methyl methanesulfonate, a methylating agent; EndoIII: endonuclease III, contains both a glycosylase and AP lyase activity; EndoVIII: endonuclease VIII, contains both a glycosylase and AP lyase activity; APE: apurinic/aprimidinic endonuclease; ND: not determined.

Oxidative DNA damage produces a wide variety of DNA lesions including oxidation of purines or pyrimidines, abasic sites and single-strand breaks. During base excision repair, 8-oxodG glycosylase (OGG-1) is responsible for the initial excision of 8-oxo-guanine lesions. In an elegant series of experiments, Wilson, Ledoux and co-workers directed OGG-1 into mitochondria through attachment of a mitochondrial leader sequence [63]. They noted that control HeLa cells were very inefficient at removing menadione-induced mtDNA damage, whereas cells transfected with OGG-1 containing a mitochondrial leader sequence had rapid mtDNA repair and enhanced cell survival. Subsequent studies by their and other laboratories have repeated this observation among additional cell types, DNA damaging agents and repair enzymes (see Table 2 for references).

It is interesting to note two cases in which supplementation of mtDNA repair enzymes actually decreased repair resulting in cell death [66,67]. Wilson, Ledoux and co-workers found that targeting the bacterial protein, exonuclease III (ExoIII), to the mitochondria in mammary adenocarcinoma cells uncoupled repair of oxidative DNA damage causing an increase in cell death [66]. ExoIII has two major enzymatic activities; it degrades DNA 3' → 5' and also is the major apurinic/aprimidinic endonuclease (APE). The authors attributed this ExoIII-dependent increase in DNA damage and cell death due to the formation of abasic sites that cannot be further processed by repair activities within the mitochondria. Furthermore, ExoIII could also digest in a 3' direction widening the repair gaps to be filled, lead to more uncoupling of BER. Kelly, Fishel and coworkers [67] found that human mammary adenocarcinoma cells transfected with a human *N*-methylpurine DNA glycosylase (MPG) gene construct that targets MPG to the mitochondria exhibited rapid removal of methylated purines in mtDNA, but also displayed excess AP sites and an increased lethality in response to methyl methanesulfonate (MMS). Moreover, simply targeting MPG

to mitochondria increased cell mortality in the absence of MMS treatment, suggesting that methylated bases arise spontaneously in mtDNA and that if these lesions are only ameliorated by MPG there is a consequent escalation in cell mortality. In both control and MMS-treated MPG-transfected cells, cell death appeared to be due to apoptosis. However, in control MPG-transfected apoptosis was caspase-3 independent (reviewed in [71]).

By fulfilling the last two of the aforementioned criteria, these studies illustrate that while augmented mtDNA repair can enhance cell survival, uncoupled mtDNA repair exacerbates cell death. More recently, Wilson, Ledoux and co-workers [70] have taken this one step further. They chronically subjected cells to elevated, as well as, incrementally increasing amounts of ROS by culturing them in the presence of hypoxanthine/xanthine oxidase, H₂O₂, or 95% oxygen. Cells that adapted to elevated ROS levels had higher concentrations of AP endonuclease in their mitochondria. This amplified level of APE was associated with lower steady-state levels of mtDNA damage and increased repair rates after oxidant challenge.

Chronic exposure to ROS might also provoke the opposite response, by compromising mtDNA repair capability and elevating steady-state levels of mtDNA damage. Indeed, one popular theory of aging, the free radical theory of aging [51], suggests that aging is characterized by an increase in ROS production in the face of a decreased ability to cope with ROS. Since mitochondria are an important source of ROS it is possible that a cardinal feature of aging is a decline in mtDNA repair capacity that instigates a vicious cycle of ROS proliferation. Two recent reports suggest that aging is correlated with a decline of APE activity in the mitochondria as a consequence of impaired mitochondrial translocation of APE encoded by nDNA [72–74].

One final study that strongly implicates mtDNA damage in aging was recently published by Larsson and co-workers,

who demonstrated that 3-exonuclease deficient DNA polymerase mice age prematurely [75]. DNA polymerase is responsible for DNA replication and gap-filling during DNA repair of mtDNA. The 3' exonuclease activity imparts fidelity to the enzyme, ensuring the removal of mispaired bases. Accordingly, mice deficient in this activity exhibit relatively higher rates of mispairing and mutation in their mtDNA.

6. Conclusion

Oxidative stress is associated with many human diseases, including: cancer, cardiovascular disease, diabetes mellitus, liver disease and neurodegenerative disease. Data presented in this review have established that mtDNA represents an important target for oxidative damage, and if not repaired, can lead to mitochondria dysregulation and cell death. This association between oxidative stress and mitochondrial dysfunction provides an opportunity for therapeutic interventions that prevent or alleviate a wide range of human diseases. Such therapies might include maximization of anti-oxidant status through nutrition and supplement management. In addition, mtDNA damage might provide an important biomarker for monitoring disease progression and efficacy of therapies.

Acknowledgements

The authors would like to thank Drs. Leroy Worth and Joe Wachsman for their critical comments. BVH has had the privilege to work with many gifted colleagues over the years, much of whose work was cited in this review. These scientists include: Drs. Sylvette Ayala-Torres, Scott Ballinger, Carl Cotman, Gagmin Deng, Bhaskar Mandavilli, Brooke Mossman, Marshal Runge, Janine Santos, Dennis Sawyer, Samuel H Wilson and Michael Yakes.

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