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OXIDATIVE STRESS AND ITS BIOCHEMICAL CONSEQUENCES IN MITOCHONDRIAL DNA MUTATION-ASSOCIATED DISEASES: IMPLICATIONS OF REDOX THERAPY FOR MITOCHONDRIAL DISEASES

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3.1 INTRODUCTION

Mitochondrial diseases are a heterogeneous group of disorders characterized by mitochondrial dysfunction. Defects in any of the numerous mitochondrial pathways including electron transport chain and oxidative phosphorylation (OXPHOS) can be caused by mutations in either mitochondrial DNA (mtDNA), which has only 37 genes, or nuclear DNA (nDNA), which encodes most of the mitochondrial proteins and all other proteins required for mitochondrial metabolism [1]. Because most of the mitochondrial diseases involve tissues with high energy demand such as brain and skeletal muscle, respiratory chain disorders caused by mtDNA mutations deserve special attention. Human cells contain hundreds or thousands of mitochondria, and each mitochondrion contains approximately 2–10 copies of mtDNA, which are distributed randomly among the daughter cells at cell division. When a mutation occurs in the mitochondrial genome, it usually affects only a portion of mtDNA and thereby results in the coexistence of two populations of mtDNA (wild type and mutant) within a cell, tissue, or individual, and this phenomenon is termed heteroplasmy [2, 3]. Therefore, clinical manifestation of a pathogenic mtDNA mutation is determined in large part by the relative proportion of normal and mutant mtDNAs in different affected tissues including muscle, blood, and hair follicles. Up until now, mutations in the mitochondrial genome have been mostly documented in a heterogeneous group of disorders in which the nervous system and the skeletal muscle are predominantly affected [4, 5].

The majority of mitochondrial diseases are maternally inherited because mtDNA is transmitted from the maternal lineage [6]. A mother carrying an mtDNA mutation can pass it to all her children (boys and girls), but only her daughters will transmit the mtDNA mutation to their children [7]. However, evidence has been obtained to show that there can be paternal inheritance of mtDNA, but such an event appears to be quite rare [8]. Identification of pathogenic mtDNA mutations were first reported in 1988 [9, 10], which provided the basis for modern molecular genetics and the classification of mitochondrial diseases. In the past two decades, more than 250 pathogenic mtDNA mutations have been detected in the affected tissues of patients with mitochondrial diseases [11]. Most of the affected individuals harbor mutations in the transfer RNA (tRNA) or protein-coding genes in mtDNA and display a cluster of clinical features that fall into discrete clinical syndromes. The notable syndromes include mitochondrial encephalomyopathy, lactic

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acidosis and strokelike episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), neurogenic muscle weakness, ataxia, retinitis pigmentosa (NARP), Leber's hereditary optic neuropathy (LHON), and Leigh syndrome. In addition, the deletion and duplication of mtDNA are associated with a wide spectrum of mitochondrial diseases including chronic progressive external ophthalmoplegia (CPEO), Kearns–Sayre syndrome (KSS), diabetes mellitus, deafness, and hypertrophic or dilated cardiomyopathy [12–14]. Specific mutations of mtDNA have been identified to cause or be associated with these mitochondrial diseases. In addition, some mtDNA mutations have also been documented in a number of genetic disorders and neurodegenerative diseases, including Parkinson disease (PD) [15], Alzheimer disease (AD) [16], amyotrophic lateral sclerosis (ALS) [17], and Wilson disease [18], and also as one of the contributory factors for aging [14, 19]. It was estimated that as many as 1 in 10,000 people have clinically manifesting mitochondrial disease and a further 1 in 6000 people are at risk [20]. Importantly, the correlation between the phenotype (clinical symptom) and genotype (mtDNA mutation) is rather poor for mitochondrial diseases [21]. In addition, the molecular mechanisms that underlie the complexity and diversity of the pathophysiology of mitochondrial diseases remain unclear.

3.2 MITOCHONDRIAL DNA MUTATION-ELICITED OXIDATIVE STRESS

Mitochondria are the powerhouse of human cells and are responsible for the supply of the majority of ATP by the OXPHOS machinery, which is located on the inner membranes of mitochondria. The structure of the mitochondrion is bounded by a double membrane that divides the mitochondrion into four distinct parts: the outer membrane, intermembrane space, inner membrane, and matrix. The mitochondrion has its own DNA molecules in the mitochondrial matrix. Human mtDNA is a 16,569-bp circular, double-stranded DNA molecule, which encodes 13 polypeptides that constitute the OXPHOS system as well as 2 ribosomal RNAs (rRNAs) and 22 tRNAs, which are essential for protein synthesis in mitochondria [22]. The mitochondrial electron transport chain and OXPHOS system comprise five multisubunit enzyme complexes: complex I [nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase], complex II (succinate-ubiquinone oxidoreductase), complex III (ubiquinol-cytochrome *c* oxidoreductase), complex IV (cytochrome *c* oxidase), and complex V (ATP synthase) [22]. In addition, two mobile electron carriers, ubiquinone and cytochrome *c*, are also involved in the elaborated electron transport process.

3.2.1 Generation of Reactive Oxygen Species in the Electron Transport Chain

The electron transport chain of mitochondria is involved in a series of redox reactions. After the reducing equivalents are transferred to the respiratory chain from NADH via complex I or from reduced flavin adenine dinucleotide ($FADH_2$) via complex II, electrons pass down to complex III and IV via ubiquinone and cytochrome *c*, respectively, and finally reduce molecular oxygen (O_2) to form water (H_2O). This series of electron transfer reactions accompany the proton pumping from matrix to intermembrane space to establish an electrochemical H^+ gradient, which is then used by complex V to drive ATP synthesis. Under normal physiological conditions, most of the tissue oxygen (over 90%) is consumed by mitochondria in human cells [23]. Although the electron transport process efficiently shuttles electrons to O_2, about 1–5% of the consumed O_2 is transformed to superoxide anions ($O_2^{\bullet -}$) via "electron leak," particularly at complex I (at the bound FMN on the matrix side) and complex III (at the ubiquinol oxidation side) [24]. The $O_2^{\bullet -}$ can be rapidly metabolized into hydrogen peroxide (H_2O_2) in mitochondria by manganese-dependent superoxide dismutase (Mn-SOD), and then H_2O_2 can be detoxified by catalase (CAT) or glutathione peroxidase (GPx) into H_2O. However, if $O_2^{\bullet -}$ or H_2O_2 is not promptly and efficiently removed, highly reactive peroxynitrite ($ONOO^-$) or hydroxyl radical ($HO^{\bullet}$) could be produced in the presence of nitric oxide (NO) or Fe^{2+} and Cu^+, respectively [24, 25]. It has been reported that the production of intracellular reactive oxygen species (ROS) is directly proportional to the rate of mitochondrial oxygen consumption of the mammalian cells [26]. Therefore, while the mtDNA mutations exist in affected tissue cells, the rate of mitochondrial ROS production would be significantly increased in the mutant cells, as expected.

3.2.2 Vicious Cycle of Mitochondrial Oxidative Stress

Since mitochondria are the major source of ROS and mtDNA is not protected by binding with histonelike proteins, human mtDNA is inflicted with more oxidative damage compared with nDNA in mammalian cells [22, 27]. In addition, the mutation rate of human mtDNA was reported to be about 20 times higher than that of nDNA because of the lack of an efficient DNA repair system in mitochondria [28]. The accumulation of mtDNA mutations in brain tissues is thought to lead to the neurodegenerative disease-associated decline of mitochondrial function [19, 29]. Indeed, defective mitochondrial respiratory enzymes have been frequently observed in the affected tissues of patients with mitochondrial diseases caused by mtDNA mutations (Table 3.1). Therefore,

TABLE 3.1 Clinical phenotype and mtDNA mutations with mitochondrial dysfunction in affected tissues or cells from patients with mitochondrial diseases

Patients	Biopsies	mtDNA mutation	RRF	OXPHOS dysfunction	Reference
MERRF	Muscle	A8344G (RNA^{Lys})	+	Complexes I, III, and IV	57, 56
MERRF	Muscle	G611A (RNA^{Phe})	+	Complex IV	66
MERRF	Cybrids	A8344G (RNA^{Lys})	NA	O_2 consumption rate (83%↓)	67
MELAS	Muscle	A3243G (RNA^{Leu})	+	Complexes I and IV	56, 68
MELAS	Muscle	A3243G (RNA^{Leu})	+	Complex IV (95%↓)	69
MELAS	Myoblasts	A3243G (RNA^{Leu})	NA	Complexes I (80%↓) and IV (37%↓)	70
MELAS	Cybrids	A3243G (RNA^{Leu})	NA	O_2 consumption rate (55%↓)	67
LHON	Muscle	G11778A (ND4)	−	Complex I (50%↓)	71
LHON	Lymphoblasts	G11778A (ND4)	NA	Complex I (20%↓) and O_2 consumption rate (36%↓)	72, 73
LHON	Lymphoblasts	14484C (ND6)	NA	O_2 consumption rate (15%↓)	73
CPEO	Muscle	4,977-bp deletion	+	Complexes I, I+III, II+III, and IV	74
CPEO	Muscle	4,977-bp deletion	+	Complex IV	75, 76
CPEO	Cybrids	4,977-bp deletion	NA	Complex I+III (3%↓), II+III (18%↓), and IV(48%↓)	76, 77
KSS	Muscle	4,977-bp deletion	+	Complexes I, III and IV	78
KSS	Myoblast	4,977-bp deletion	+	Complexes I (26%↓) and IV (56%↓)	70
NARP	Muscle	T8993G (ATPase 6)	−	Complexes I and V	79
NARP	Lymphoblasts	T8993G (ATPase 6)	NA	Complex V (58%↓)	80
Leigh syndrome	Muscle	T8993G (ATPase 6	−	PDH and Complex IV	81
Leigh syndrome	Skin fibroblasts	T8993G (ATPase 6)	NA	ATP synthesis rate (80%↓)	82

Patients with mitochondrial diseases were aged from 30 to 55 years. "↓" indicates % of decrease in enzymatic activity; "+" indicates positive results; "−" indicates negative results. Abbreviations: RRF, ragged red fibers; PDH, pyruvate dehydrogenase; NA, not available.

mtDNA mutation-induced inefficiency of mitochondrial respiration could further produce more ROS, which in turn further enhance oxidative damage to various biomolecules in mitochondria. This "vicious cycle" is propagated in typical mitochondrial diseases and results in the widely observed accumulation of oxidative damage and mutation of mtDNA, which ultimately leads to a progressive decline in the bioenergetic function of affected tissue cells in patients with mitochondrial diseases [30, 31]. We have hypothesized that increase of oxidative stress and oxidative damage are involved in the deterioration of bioenergetic function of the affected tissues in patients with mitochondrial diseases [32–34]. Recent studies conducted in other laboratories have provided compelling evidence to support the notion that oxidative stress elicited by impairment of the respiratory chain in the affected tissues of patients plays a role in the pathophysiology and progression of mitochondrial diseases [35–38].

3.3 MITOCHONDRIAL DNA MUTATION AND MITOCHONDRIAL DISEASES

The striking feature of mitochondrial disorders caused by mtDNA mutations is their clinical heterogeneity, ranging from single-organ involvement to severe multisystem disorders [39]. The same mutation of mtDNA or different mutations in the same mtDNA genes may present with different clinical features, while the same clinical phenotype may arise from different mutations of mtDNA. In addition, the onset of clinical symptoms and phenotypic variability for mitochondrial diseases caused by the pathogenic mtDNA mutations are governed by a number of factors, including the threshold effect and mitotic segregation [11]. Most importantly, mitochondrial disorders caused by mtDNA mutations are mostly transmitted by maternal inheritance, but not all the children display symptoms, and some are even spared.

3.3.1 Heteroplasmy of mtDNA Mutations

The majority of mtDNA mutations in affected tissues are heteroplasmic, and the ratio of wild-type to mutant mtDNA may determine the onset of clinical symptoms, which is called the threshold effect of an mtDNA mutation [40]. A certain proportion of mtDNA with a pathogenic mutation is required to cause mitochondrial defects in affected tissues, and this threshold level differs with different tissues. Generally, it is suggested that the threshold level is lower in tissues that are highly dependent on aerobic metabolism than in tissues that can rely on anaerobic glycolysis for supply of ATP [41]. However, solid evidence is lacking to support a good correlation between clinical severity and the proportion of a pathogenic mtDNA mutation. On the other hand, the phenomenon of mitotic segregation can also partly explain the markedly different levels of mutated mtDNA in different

members of the same family and among different tissues of an affected individual [42]. During mitosis, heteroplasmic mutations of mtDNA may be randomly segregated in an inter- or intramitochondrial manner to each daughter cell, in which the proportion of mutant mtDNA can thus be shifted. The mitotic segregation of mtDNA mutation can explain the differential spectrum of phenotypic manifestation of the mutated mtDNA between different tissues and at different stages of life.

3.3.2 mtDNA Mutation-Related Mitochondrial Diseases

The presence of a particular heteroplasmic mutation of mtDNA strengthens the argument that the specific mutation of mtDNA is pathogenic [43]. Pathogenic mtDNA mutations include base substitutions (point mutations) and rearrangements (deletion and duplication) in the mitochondrial genome. More than 250 mutations of mtDNA have been reported to be associated with mitochondrial diseases including MERRF, MELAS, LHON, NARP, and CPEO syndromes [11, 44]. Mutations in protein-coding genes of mtDNA have been mainly documented in patients with LHON and NARP or Leigh syndrome. LHON is characterized by acute or subacute blindness that presents in early adulthood and usually affects males [45]. Over 20 point mutations of mtDNA have been associated with LHON, with most of the gene products constituting complex I. The primary mutations include the G14459A and the T14484C transitions in the *ND6* gene, the G11778A transition in the *ND4* gene, and the G3460A transition in the *ND1* gene. On the other hand, NARP is characterized by neurogenic muscle weakness, sensory neuropathy, ataxia, and retinitis pigmentosa and may associate with learning difficulty or dementia [46]. The most common mutation associated with this disease is the T8993G transversion or T8993C transition in the *ATP6* gene, which impairs the assembly of ATP synthase (complex V). It is noteworthy that the heteroplasmy of T8993C/G mutation of mtDNA would result in different clinical phenotypes depending on the mutation load [47]. The clinical features of NARP are observed with a good correlation between mutant load and clinical severity when the proportion of mutated mtDNA is lower than 75%. However, when the T8993G mutation reaches higher than 95%, it can cause Leigh syndrome, an often early-onset and lethal disorder associated with ataxia, hypotonia, spasticity, developmental delay, optic atrophy, and ophthalmoplegia [48].

The mtDNA mutations that affect overall mitochondrial protein synthesis include point mutations in tRNA genes or large-scale deletions of mtDNA, which remove one or more tRNA genes and structural genes [49]. The most common A3243G mutation in the $tRNA^{Leu}$ gene is associated with MELAS [50], and the A8344G mutation in the $tRNA^{Lys}$ gene is responsible for the pathogenesis of MERRF [51]. MELAS is characterized by mitochondrial encephalopathy, repeated strokelike episodes, seizures, and lactic acidosis, while MERRF is characterized by myoclonus, seizures, mitochondrial myopathy, cerebellar ataxia, peripheral neuropathy, and multiple lipomas. Although the clinical features and biochemical defects associated with the MELAS- and/or MERRF-specific mutations of the tRNA genes have been determined, it is still unclear as to why and how they result in such different clinical phenotypes [52]. On the other hand, diseases associated with mtDNA rearrangements mostly refer to CPEO [53] and KSS [54]. A most common defect in mtDNA is a single large-scale deletion (4,977-bp deletion), which removes one or more tRNA genes and mitochondrial protein-coding genes. Patients with CPEO and KSS present with early-onset ophthalmoplegia, ptosis, and retinitis pigmentosa, cerebellar ataxia, cardiac conduction block, renal dysfunction, and diabetes mellitus as common symptoms. Importantly, the proportion of mtDNA with a large-scale deletion has been found to increase over time in affected skeletal muscle and to accompany the progression of the disease [55].

3.4 BIOCHEMICAL CONSEQUENCES OF mtDNA MUTATION IN MITOCHONDRIAL DISEASES

Defects in the structure or function of mitochondria caused by mtDNA mutation have been shown to be associated with a wide spectrum of clinical phenotypes. There are well-documented reports of mitochondrial dysfunction in affected tissues of patients with mitochondrial diseases. In addition, mitochondrial dysfunction-elicited oxidative stress has been proven to be involved in the pathogenesis and progression of mitochondrial diseases. Furthermore, defects in glucose metabolism and the biosynthesis of heme and iron-sulfur (Fe-S) clusters, have recently been revealed in some mitochondrial diseases. Accordingly, we summarize the findings from our own and other laboratories about the relationship between the clinical phenotype and mitochondrial dysfunction in patients with mtDNA mutation-related mitochondrial diseases (Table 3.1).

3.4.1 Defects in the OXPHOS System

In a muscle pathology laboratory, defects in mitochondrial function have been determined by histological and histochemical techniques based on dye staining for specific mitochondrial enzyme activities. Alterations in the activities of cytochrome *c* oxidase (COX, complex IV) and

succinate dehydrogenase (SDH, complex II) are most often detected in affected muscles [56, 57]. Usually, the SDH activity stain clearly reveals the subsarcolemmal accumulation of mitochondria (SDH positive), and the activity assay of COX is particularly useful in the evaluation of mitochondrial myopathies (COX negative) [58]. In addition, the so-called ragged red fibers (RRF) can be found in the affected muscle fibers by Gomori trichrome stain, which shows the subsarcolemmal accumulation of abnormal mitochondria [59, 60]. On the other hand, assay of the rate of mitochondrial oxygen consumption with a Clark oxygen electrode and measurement of specific activities of the respiratory enzyme complexes spectrophotometrically have been widely applied in the diagnosis of mitochondrial diseases and in the study of the biochemical consequences of a pathogenic mutation of mtDNA [61, 62]. More recently, the Seahorse XF24 Extracellular Flux analyzer has been employed to measure metabolic profiles [oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)] and to monitor the switch of aerobic metabolism to anaerobic metabolism as a response to mitochondrial dysfunction in cells harboring a pathogenic mtDNA mutation [63]. We have suggested that the decrease of ATP generation caused by defective mitochondria can induce a compensatory upregulation of the glycolytic pathway, thus increasing the dominance of glycolysis in the cellular ATP production. It has been reported that a decrease in mitochondrial respiration and oxidative phosphorylation with a concomitant increase of glycolysis represents a hallmark of mitochondrial diseases [64, 65].

3.4.2 Oxidative Damage to Affected Tissues

A great deal of research results has suggested that mtDNA mutation-elicited ROS and oxidative damage play an important role in the pathogenesis and progression of mitochondrial diseases (Table 3.2). For example, in patients with MELAS, MERRF, LHON, or CPEO syndromes the intracellular levels of H_2O_2 and oxidative damage to DNA and lipids have been found to be increased in the primary culture of skin fibroblasts [83–86]. Majora and colleagues [87] also reported that the level of mitochondrial ROS in skin fibroblasts of KSS patients was 2.5-fold higher than that of normal control subjects. In addition, the oxidative damage to DNA in muscle of CPEO patients is much more extensive than that in muscle of patients with other types of diseases that are not associated with neuromuscular disorders [88, 89]. Moreover, Piccolo and colleagues [90] reported that lipid peroxides and fluorescent adducts of organic aldehydes with plasma proteins in blood of patients with CPEO syndrome were elevated compared with those of normal subjects. Similarly, in blood cells of patients with MELAS-related mitochondrial disorders and patients with LHON, telomere was found to be shortened by ROS, which suggests that systematic oxidative stress is elevated in patients with mitochondrial diseases [91]. Notably, another study revealed that the skeletal muscle with a significant increase of 8-hydroxy-2′-deoxyguanosine (8-OHdG) content displayed a large amount of RRFs in patients with KSS or CPEO syndrome and that there was an excess amount of hydroxyl radicals and aldehydic lipid peroxidation products in cultured skin fibroblasts [92]. Recently, van Eijsden et al. [93] reported that oxidative damage to proteins and the content of superoxide anions in the muscle biopsies of patients with MELAS syndrome were significantly increased compared with those of age-matched normal subjects.

To understand the biochemical consequences of a pathogenic mtDNA mutation, a promising cell model has been established through the fusion of enucleated cytoplasts (derived from patients with mtDNA mutations) with immortalized human cell lines that are devoid of endogenous mtDNA (Rho zero cells, ρ^0) [94]. Many lines of research have suggested that cybrids are an excellent tool for studying the biochemical and pathological consequences of varying proportions of a specific mtDNA mutation [95–97]. Recently, we investigated oxidative modification to mitochondrial proteins in cybrid cells harboring the A8344G mutation of mtDNA from an MERRF patient by two-dimensional gel and proteomic techniques [34]. A total of 16 carbonylated mitochondrial proteins were identified in the MERRF cybrids compared with the wild-type cybrids. It is worth mentioning that the voltage-dependent anion channel (VDAC), aconitase, and prohibitin (PHB) were quite susceptible to oxidative damage in the MERRF cybrids [34]. VDAC is a major component of the permeability transition pore complex on the outer mitochondrial membrane, which regulates the transport of ions and metabolites in and out of the mitochondria. We speculate that the accumulated oxidative damage to VDAC may cause a loss of bidirectional fluxes of ions and metabolites across mitochondrial membranes, which in turn leads to bioenergetic breakdown and elicits the pathological changes and clinical manifestations of MERRF syndrome [98]. Under normal physiological conditions, PHB acts as a chaperone to prevent misfolding of mitochondrial proteins, and thus damage to the PHB protein could inhibit its function and aggravate the mitochondrial dysfunction [99].

3.4.3 Deficiency in Iron Metabolism and Heme Synthesis

The involvement of mitochondria in the biosynthesis of heme and iron-sulfur (Fe-S) clusters underscores the

TABLE 3.2 Accumulated oxidative damage and alterations of antioxidant enzymes in affected tissues or cells from patients with mitochondrial diseases

Patients	Biopsies	Oxidative damage	Redox status and antioxidant enzymes	Reference
MERRF	Muscle	NA	Mn-SOD↑[c]; GSH↑[c]	115
MERRF	Blood	Plasma F_2-isoprostane (1.6-fold↑)	NA	136
MERRF	Cybrids	Carbonylated VDAC (2.0-fold↑)[b]; PHB (1.9-fold↑)[b]	Mn-SOD↑[b]; Cu,Zn-SOD (−)[b]	34
MERRF	Cybrids	H_2O_2(1.7-fold↑)	Mn-SOD (1.7-fold↑)$_a$; CAT (1.7-fold↑)[a] Cu,Zn-SOD (1.5-fold↑)[a]	121
MERRF	Skin fibroblasts	Mitochondrial aconitase (40%↓)[a]; heme *c* (70%↓)[b]	NA	106, 107
MELAS	Muscle	8-OHdG↑[c]; TUNEL-positivec[c]	Mn-SOD↑[c]; GSH↑[c]	115, 24
MELAS	Myoblasts	8-OHdG↑[c]; 4-HNE↑[c]	Mn-SOD (1.5-fold↑)[a]; Catalase (2.2-fold↑)[a]	113, 104
MELAS	Brain	8-OHdG↑[c]	Mn-SOD and Cu,Zn-SOD (↓)[c]	84
MELAS	Cybrids	H_2O_2(1.8-fold↑)	Mn-SOD (1.7-fold↑)[a]; CAT (2.0-fold↑)[a] Cu,Zn-SOD (1.6-fold↑)[a]	121
CPEO	Muscle	8-OHdG of m^a†DNA (40-fold↑)	Mn-SOD↑[c]; GSH↑[c]	115, 85
CPEO	Muscle	8-OHdG↑[c]; 4-HNE↑[c];Bcl-2?↓[c]; Caspase 3[c]↑	Mn-SOD↑[c];Cu,Zn-SOD (−)[c]	137
CPEO	Muscle fibroblasts	8-OHdG (8.7-fold↑); H_2O_2(1.9-fold↑); O_2–(1.6-fold↑)	Mn-SOD(2.2-fold↑)[a, b];CAT and GPx (−)[a]	88
KSS	Muscle	NA	Mn-SOD↑[c];CAT↑[c]; GPx↑[c]	138, 113
LHON	Skin fibroblasts	4-HNE (2.5-fold↑);MDA(2.4-fold↑)	Mn-SOD (2.9-fold↑)[a];Cu,Zn-SOD (−)[a]	116
LHON	Blood	Leukocyte 8-OHdG (4.3-fold↑)	NA	83
LHON	Cybrids	NA	GPx (50%↓)[a]; GR (34%↓)[a];Mn-SOD (50%↓)[a]; Cu,Zn-SOD (38%↓)[a]	118
NARP	Lymphoblasts	H_2O_2(1.5-fold↓)	Mn-SOD (2.1-fold↓)[a]; CAT (−)[a];Cu, Zn-SOD (2.8-fold↓)[a]	139
NARP	Cybrids	H_2O_2(3.0-fold↑); MDA(2.3-fold↑); 4-HNE (2.0-fold↑)	Mn-SOD (2.8-fold↑)[a]; Cu,Zn-SOD (−)[a]	140
Leigh syndrome	Skin fibroblasts	H_2O_2 (1.6-fold↑)	Mn-SOD(34%↓)[b]	86

Patients with mitochondrial diseases were aged between 30 and 60 years. "↑" indicates % of increase and "↓" indicates % of decrease in each of the parameters. "−" indicates no change in the indicated parameter. Superscripts a, b, and c indicate changes in the activity, protein expression, and immunohistochemistry results. Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; F_2-isoprostane, non-cyclooxygenase-derived prostanoids; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; NA, not available.

importance of these prosthetic groups in mitochondrial dysfunction of patients with mitochondrial diseases. It has been documented that deficiency in Fe-S clusters such as aconitase and subunits of complexes I and II are involved in mitochondrial encephalomyopathies and myopathies [100, 101]. In addition, a recent study demonstrated that mitochondrial dysfunction in myelodysplastic syndromes could be linked to the impairment of iron metabolism and heme biosynthesis in mitochondria [102]. Recently, heme deficiency has been considered as a vital factor in the mitochondrial decay, which is involved in the aging process and the pathogenesis of neurodegenerative diseases such as Friedreich ataxia (FRDA), AD, and PD [103]. To maintain cellular iron (Fe^{2+}) homeostasis, iron regulatory proteins (IRP1 and IRP2) regulate the expression of some iron-containing proteins required for mitochondrial function (e.g., aconitase and SDH) or cytosolic Fe^{2+} metabolism that includes transferrin receptor (TfR1) and ferritin through binding to the iron-responsive element (IRE) on the target mRNAs [104]. Mitochondrial aconitase (m-aconitase) functions in the TCA cycle to facilitate the dehydration-hydration reaction that reversibly converts citrate to isocitrate. Another isozyme is the bifunctional IRP1 that has cytosolic aconitase (c-aconitase) and IRE binding activities[105]. Therefore, the iron-dependent regulation of aconitase via IRP1 and the dependence of mitochondria on iron metabolism provide regulatory links between Fe^{2+} homeostasis and energy metabolism. It is rationalized that a disturbance in Fe^{2+} homeostasis can lead to mitochondrial dysfunction accompanied by the increase in the production of hydroxyl radicals via the Fenton reaction [24, 106].

Recently, we demonstrated [107] that the activities of both m- and c-aconitases were decreased, especially m-aconitase, in the primary culture of skin fibroblasts from MERRF patients with the A8344G mutation of mtDNA. In addition, we found that m-aconitase in the MERRF cybrids was more sensitive to H_2O_2 than that of the wild-type cybrids. It was shown that the loss of m-aconitase activity upon treatment of the mutant cybrids with H_2O_2 could be partially prevented by *N*-acetylcysteine (NAC), which is a precursor of glutathione that acts as the first-line intracellular antioxidant [108]. Moreover, we found a compensatory increase of m-aconitase expression but dramatic decrease of TfR1 expression in MERRF skin fibroblasts. The decrease of TfR1 may be a consequence of Fe^{2+} overload in response to elevated oxidative stress in mutant cells. It has been suggested that the oxidant-mediated Fe^{2+} overload can initiate and accelerate a vicious cycle that ultimately results in cellular damage and tissue degeneration [109]. Interestingly, we found that cytochrome *c* was decreased concurrently with the deficiency of heme *c*, suggesting that a functional abnormality of cytochrome *c* was induced in MERRF skin fibroblasts. The above-mentioned findings suggest that oxidative modification and deterioration of some proteins and enzymes that contain Fe-S clusters, such as aconitase, may also play an important role in the pathophysiology of mitochondrial diseases.

3.5 ALTERATION OF ANTIOXIDANT DEFENSE SYSTEM IN MITOCHONDRIAL DISEASES

In affected tissue cells with mitochondrial dysfunction-elicited oxidative stress, the induction of free radical scavengers is essential for efficient removal of ROS and some of the biological molecules with oxidative damage. Human cells have developed a ubiquitous antioxidant defense system during the evolutionary process to cope with the production of ROS by aerobic metabolism [110]. The antioxidant enzymes include SOD, CAT, GPx, and glutathione reductase (GR) together with a large number of low-molecular-weight antioxidants such as ascorbic acid, α-tocopherol, retinal, folic acid, lipoic acid, and glutathione (GSH), which can dispose of ROS and RNS to minimize their damaging effects. However, a decrease in low-molecular-weight antioxidants has been found in the serum of some patients with mitochondrial encephalomyopathies [111, 112]. In Table 3.2, we have compiled findings from previous studies on the changes of antioxidants status and the alterations in the expression or activity levels of antioxidant enzymes in mtDNA mutation-related mitochondrial diseases.

3.5.1 Differential Induction of Antioxidant Enzymes in Mitochondrial Diseases

Alteration in the expression of the antioxidant enzymes was first demonstrated by immunohistochemistry on skeletal muscle fibers from patients with mitochondrial encephalomyopathies including MELAS, CPEO, and KSS [113]. These findings indicate that the expression levels of Mn-SOD and, to a lesser extent, Cu,Zn-SOD are increased in RRFs with negative expression of cytochrome *c* oxidase [85, 114, 115]. It is noteworthy that the severity of cytochrome *c* oxidase deficiency was correlated with the increase of production of $O_2^{\bullet -}$ and induction of the expression of Mn-SOD [116]. Accordingly, it has been suggested that the dramatic induction of Mn-SOD in affected tissues and cells can be considered a biomarker for the onset of mitochondrial dysfunction in patients with a majority of mitochondrial diseases [115, 117]. On the other hand, a reduction in the protein expression and activity levels of Mn-SOD was observed in patients with LHON syndrome [118]. The disruption of Mn-SOD gene in the rat resulted in optic neuropathy, which was similar to the major symptom of LHON patients [119, 120]. On the other hand, the overproduction of ROS in cybrids harboring the A3243G or A8344G mutation of mtDNA led to an increase in the activities of antioxidant enzymes including SOD, CAT, and GPx [121]. Selenium (Se)-dependent and -independent GPx activities were also increased in response to the deficiency of respiratory enzymes in human myeloid leukemia U937 cells that had been exposed to mitochondrial stress such as chloramphenicol, an inhibitor of mitochondrial translation, or ethidium bromide, which depletes mtDNA [122].

In a previous study, we observed that the protein expression and activity levels of Mn-SOD, but not those of CuZn-SOD, CAT, and GPx, were increased in the primary culture of skin fibroblasts from CPEO patients compared with those of normal control subjects [88]. It is noteworthy that this imbalanced expression of antioxidant enzymes was much more pronounced in the fibroblasts cultured from muscle biopsies of patients with CPEO syndrome [123]. This might be due to higher oxidative stress in muscle fibroblasts, because the protein level of catalase of muscle fibroblasts was just half of that of skin fibroblasts. In addition, we also discovered that the upregulation of Mn-SOD in skin fibroblasts of patients with mitochondrial encephalomyopathies was associated with a dramatic increase in the expression of several matrix metalloproteinases (MMPs) due to increase of the intracellular level of H_2O_2 [124]. One of the most conspicuous features of the mitochondrial diseases in the affected cells was the striking derangement of the distribution and network of mitochondria,

which serve as a vehicle for efficient transmission of energy through the dynamic structural change of cytoskeleton in normal cells. Therefore, we suggest that the gross changes in morphology and network of mitochondria in the affected tissue cells from patients with mitochondrial disease is a result of activation of MMPs induced by enhanced oxidative stress [125, 126].

3.5.2 Regulation of Antioxidant Enzymes

A delicate balance exists between the expressions of both types of SOD and CAT plus GPx or thioredoxin reductase (TR) to confer cells with the ability to cope with oxidative stress. However, the imbalanced expression of antioxidant enzymes has repeatedly been observed in affected tissues and cultured cells from patients with mitochondrial diseases [127–129]. A low activity level of SOD relative to GPx or CAT could lead to the accumulation of ROS such as superoxide anions, while a high activity level of SOD relative to GPx or CAT could lead to an increased production of H_2O_2. It was demonstrated that the generation of ROS was elevated in skeletal muscle of transgenic mice with either overexpression or knockout of Mn-SOD [130]. In addition, several research groups demonstrated that cells with overexpression of CuZn-SOD alone or Mn-SOD alone were all much more susceptible to DNA strand breaks and growth retardation and more easily killed by an extracellular burst of $O_2^{\bullet-}$ and H_2O_2 [131–134]. On the other hand, the elevated oxidative stress and oxidative damage in single transfected SOD clones can be diminished after double transfection of CAT or GPx into the cells [135]. Therefore, coordination in the expression of antioxidant enzymes in mammalian cells to efficiently cope with oxidative stress is essential for cells to survive. Recently, it was found that the expression of Mn-SOD is also upregulated by PGC-1α, which is a transcription coactivator that controls mitochondrial biogenesis in response to increased energy demand such as exercise or cold exposure [34]. The research related to the regulation of antioxidant enzymes could provide useful information for the development of novel therapies to treat mitochondrial diseases.

3.6 REDOX THERAPY OF MITOCHONDRIAL DISEASES

Oxidative stress plays an important role in the pathophysiology of mitochondrial diseases, and thus reducing the deleterious effects of ROS by treatment with antioxidants can have therapeutic effects against a variety of ROS-mediated mitochondrial disorders. Indeed, coenzyme Q_{10} (CoQ_{10}) has been used as a remedy to treat mitochondrial diseases, even at dosages as high as 2000 mg daily [141]. It has been well documented that CoQ_{10} has dual roles as a component of the respiratory chain and as a potent ROS scavenger [142]. CoQ_{10} can not only restore the mitochondrial OXPHOS system but also reduce the ROS-induced DNA damage and the proportion of apoptotic markers in affected biopsies of patients with mitochondrial diseases. In addition, a number of studies reported that pretreatment with CoQ_{10} in human cells harboring mtDNA point mutations or large-scale deletion can reduce the increase of ROS and ROS-mediated cell apoptosis, which is induced by exogenous oxidative stress such as UV irradiation and H_2O_2 [143–145]. Clinically, the administration of CoQ_{10} in patients with sporadic KSS or CPEO syndrome and patients with RRF in muscles associated with seizures, ataxia, or mental retardation has been demonstrated to be extremely beneficial [146, 147]. In addition, in a trial with AD and PD, CoQ_{10} was found to restore the mitochondrial function and slow down the progression of the diseases [148]. Furthermore, in a study of lymphocytes from 12 patients with severe mitochondrial respiratory chain defects before and after 12 months of supplementation with a cocktail mixture including CoQ_{10} (350 mg daily), L-carnitine, vitamin B complex, vitamin C, and vitamin K_1, it was found that there was a significant increase in the capacity for ATP synthesis in the lymphocytes after the treatment [149].

In addition to CoQ_{10}, there are ongoing developments of other antioxidants for the therapy of mitochondrial diseases. Pyruvate is one of the antioxidants that not only reduce the intracellular ROS level but also boost mitochondrial function through activation of the pyruvate dehydrogenase complex (PDHC) by inhibiting the PDH kinase (PDK). Recently, long-term administration of sodium pyruvate to patients with Leigh syndrome was found to improve effectively the exercise intolerance and restore the mitochondrial function in affected tissues [150]. On the other hand, ascorbic acid is another important antioxidant used for the treatment of patients with mitochondrial diseases because it can directly enter mitochondria in its oxidized form via glucose transporter 1 (Glut1) and thus protects mitochondria from oxidative injury [151]. Dr. Peterson reported that treatment of patients with MERRF or MELAS syndrome with ascorbic acid (1 g twice per day) could improve medical complications and the patients survived longer with less functional disability [152]. Recently, a so-called "vitamin cocktail" containing ascorbic acid and CoQ_{10} has been developed and used for clinical treatment of mitochondrial diseases [153]. Furthermore, antioxidants including vitamin E, α-lipoic acid, glutathione, and NAC have also been shown to be effective in various animal models of mitochondrial

diseases and in cultured cells from some patients, but their clinical benefits need to be substantiated by well-designed clinical trials [154].

3.6.1 Strategy for Targeting Antioxidants to Mitochondria

The specific delivery of drugs or antioxidants into mitochondria has been proven to be a potential therapeutic approach. Recently, Murphy and Smith [155] developed one specific approach to selectively target antioxidants to mitochondria by conjugating them to lipophilic cations such as triphenylphosphonium (TPP). Lipophilic cations can pass easily through lipid bilayers because they carry a positive charge that is colocalized over a large surface area, and the electrochemical gradient drives their accumulation in mitochondria because of the large membrane potential across the mitochondrial inner membrane (150–180 mV). Therefore, the newly developed antioxidant $MitoQ_{10}$, a CoQ_{10} molecule attached to the TPP^+ ion, has been used in a wide range of mitochondrial disease models, and the results showed protection against oxidative damage [156, 157]. In fact, $MitoQ_{10}$ is more potent and effective than CoQ_{10} in preventing oxidative stress-induced apoptosis in skin fibroblasts from patients with mitochondrial diseases [158]. In addition, in vivo studies revealed that $MitoQ_{10}$ administered to mice and rats by intravenous injection was rapidly cleared from the plasma and accumulated in the heart, brain, liver, kidney, and skeletal muscle [159] and can selectively protect mitochondria from ischemia-reperfusion injury of the heart [160]. $MitoQ_{10}$ is now under clinical development (in phase II human clinical trials), and orally administered $MitoQ_{10}$ can be applied to a wide spectrum of human pathologies that involve mitochondrial oxidative damage such as mtDNA mutation-elicited mitochondrial diseases. Similarly, the Szeto–Schiller (SS) tetrapeptides have also recently been developed as small cell-permeant antioxidants in a mitochondrial membrane potential-independent manner [161]. The structure motif of SS peptides centers on alternating aromatic (phenylalanine, tyrosine, dimethyltyrosine) and basic (arginine, lysine) amino acid residues, in which tyrosine and dimethyltyrosine residues likely act as free radical scavengers. The SS peptides have been demonstrated to reduce ROS in mitochondria and clearly showed its potential in the treatment of mitochondrial disorders or oxidative stress-related diseases such as myocardial infarction and ischemic brain injury [162, 163].

3.6.2 Antioxidant Enzymes Targeted to Mitochondria

In light of the observation of the imbalanced expression of antioxidant enzymes found in most affected tissues of patients with mitochondrial diseases, it has been thought that increasing the expression of SOD, CAT, or GPx in affected individuals is another strategy for the therapy of mitochondrial diseases. For example, LHON cybrids with complex I deficiency were associated with decreased expression of Mn-SOD, and thus, by infection of the cells with recombinant adeno-associated virus (rAAV) containing the human Mn-SOD gene, the survival of LHON cells was increased up to 90% [108, 109]. On the other hand, the administration of SOD-CAT mimetics including manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin (MnTMPyP) and EUK-418 (developed by Eukarion Inc., USA) to boost the activities of Mn-SOD and CAT could protect cells from oxidative injuries in animal models of neurodegenerative diseases including PD, AD, and ALS [164]. In addition, supplementation with Se to activate GPx and TR in cybrids harboring 98% T8993G mutated mtDNA could decrease oxidative stress and oxidative damage [165]. Recently, with the redox approach of overexpression of CAT targeted to the mitochondria (mCAT) in mitochondrial mutator mice with DNA polymerase gamma (POLG) deficiency, mice were better protected from ROS-induced mitochondrial damage and had an extension of maximum life span [166, 167]. Other studies also revealed that overexpression of antioxidant enzymes such as peroxiredoxin [168], thioredoxin [169], and GPx [170] targeted to mitochondria could effectively protect cells from exogenous stress-induced oxidative damage and cell death. Although there are few reports on the therapeutic manipulation of antioxidant enzyme in animal models, the reduction of mitochondrial ROS by overexpression of antioxidant enzymes targeted to mitochondria can be considered a novel strategy to develop effective therapies for treatment of mitochondrial diseases in the future.

3.7 GENETICS-BASED GENE THERAPY FOR MITOCHONDRIAL DISEASES

Currently, there is no cure for mitochondrial diseases, and only treatments to relieve symptoms such as antioxidant therapy can be offered. This has led researchers to consider mitochondrial gene therapy, which can be carried out either by the expression of an engineered gene product to rescue defects caused by an nDNA mutation or by import of normal copies or relevant sections of mtDNA into mitochondria [171, 172]. Although gene therapy in patients with mtDNA mutations is a challenge because of the heteroplasmy of mtDNA, this idea has been tested and proved successful in yeast from Nagley's group in 1988 [173]. They demonstrated that yeast carrying an mtDNA mutation at *MTATP8* gene could be rescued when normal copies

of the gene were introduced into the nucleus, resulting in cytosolic translation of the gene product, the wild-type protein of ATPase 8. Recently, a successful targeting of *ATPase 6* gene in a cell model was shown to partially rescue a respiratory chain-deficient phenotype caused by a pathogenic mutation in the *MTATP6* gene [174]. However, it was pointed out that the assembly of complex V was not efficient to integrate correctly with the imported protein to make a mature and functional ATP synthase. The question is how to efficiently translocate engineered polypeptides to mitochondria, although the engineered polypeptides could be targeted with the mitochondria-targeting sequence in their N-terminals [175]. Recently, Corral-Debrinski and colleagues [176] attempted to optimize the expression of highly hydrophobic mitochondrial proteins by targeting the known protein (such as Mn-SOD) that is localized to the mitochondria. The results showed that facilitated cotranslational translocation of the gene product could improve mitochondrial import of the allotopically expressed proteins, preventing the formation and accumulation of cytosolic aggregates of the overexpressed proteins.

On the other hand, import of normal tRNA is another potential therapy for treatment of patients with mtDNA mutation in a tRNA gene [177]. Bhattacharyya and colleagues [178] first isolated a large tRNA import complex (RIC) from the inner mitochondrial membrane of *Leishmania tropica* and reconstituted it into phospholipid vesicles. With the application of RIC, Mahata and co-workers [179] suggested that the RIC can enter human cells through a caveolin-1-dependent pathway, and thereby induce import of endogenous cytosolic tRNAs, including $tRNA^{Lys}$, and restore mitochondrial function in the cybrids harboring a mutant $tRNA^{Lys}$ gene of mtDNA. The use of tRNA genes to rescue mitochondrial function may be beneficial for the management of such genetic disorders. Mitochondrial gene therapy has been established and tested in both cultured cells and animal models, and the experimental results have suggested that it holds some promise in clinical application for treatment of patients with mtDNA mutations in the future.

3.8 CONCLUSION

Mitochondrial diseases are caused by mutations in mtDNA and/or nDNA, but the molecular mechanisms underlying the pathogenesis of mitochondrial diseases are still poorly understood. It still remains a mystery as to how and why mutations in different genes lead to similar clinical symptoms and the same mtDNA mutations can manifest different clinical features. However, in light of the experimental data from our own and other laboratories, we believe that bioenergetic dysfunction and accumulation of deleterious metabolic intermediates in affected tissues or cells may be involved in the onset and progression of this prominent group of metabolic diseases (Table 3.1). In addition, defective mitochondria also generate more ROS and free radicals via electron leak from the respiratory chain. As a consequence, significant increases in oxidative stress and oxidative damage are often observed in the affected tissues and peripheral blood cells of most patients with mitochondrial diseases (Table 3.2). Therefore, it has been generally established that oxidative stress and oxidative damage are involved in the pathophysiology of mitochondrial diseases [12, 180]. Once the damage persists too long or is too serious to be repaired, the mitochondria would sense and integrate the extramitochondrial stress and signal to drive the cell into an irreversible death process (apoptotic mechanism) [34, 181]. This scenario may explain the clinically well-documented age-dependent progression and worsening of disease in the majority of mitochondrial diseases. On the other hand, with the development of cDNA microarray and proteomic techniques, altered expression of several clusters of genes and protein modifications have been observed in affected tissues and cultured cells of patients. Compensatory upregulation of OXPHOS genes and oxidative stress-responsive genes has been widely investigated in affected individuals with mitochondrial dysfunction [182, 183]. Specifically, the dramatic induction of Mn-SOD observed in affected tissues can be considered as an early sign of OXPHOS deficiency in the majority of mitochondrial diseases, and such manifestations are often correlated with the mtDNA mutation load and the proportion of abnormal mitochondria [115, 117].

To counteract the effects of oxidative stress, antioxidants have been utilized in the treatment of some mitochondrial diseases. Clinical trials of CoQ_{10} with other antioxidants such as vitamins C, E, and K as a form of "antioxidant cocktail" have been conducted to treat patients with mitochondrial diseases and neurodegenerative diseases [172]. In addition, reduction of mitochondrial ROS has been shown to be beneficial to patients with specific mitochondrial disorders. Thus the development of antioxidants targeting mitochondria such as $MitoQ_{10}$ has been a productive endeavor. Notably, there has been much research and development in the design of Mito-vitamin E, Mito-TEMPOL, and Mito-NAC for the treatment of diseases caused by mitochondrial disorders [184]. Therefore, targeting mitochondria with organelle-specific agents is proven to be an effective therapeutic strategy to specifically reduce the mitochondrial ROS in tissue cells of patients with a mitochondrial defect [185]. Although there have been

some designs of gene therapy to correct mitochondrial defects caused by a pathogenic mtDNA mutation, their clinical applications face many challenges. With consideration of the hurdles and potential risk of mitochondrial gene therapy, including the choice of appropriate viral or nonviral vectors, the high efficiency of the delivery of antioxidants to the affected tissues, and the low adverse (e.g., immunological) responses, redox therapy by antioxidants is a promising treatment for mitochondrial diseases and other diseases caused by mitochondrial dysfunction.

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