Mitochondrial Disorders Therapy: The Utility of Melatonin

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Abstract: Mitochondria play a central role in the cell physiology. It is now recognized that, besides their classic function of energy metabolism, mitochondria are enrolled in multiple cell functions including energy distribution through the cell, energy/heat modulation, reactive oxygen species (ROS) regulation, calcium homeostasis, and apoptosis control. Recently, evidence is accumulating for a direct participation of mitochondria in stem cell proliferation and/or differentiation. All these functions suggest that mutations in either nuclear or mitochondrial DNA may induce serious cell impairments, and there is now evidence of more than 200 mtDNA mutations responsible for human pathologies. Moreover, mitochondria are, simultaneously, the main producer and target of ROS and, thus, multiple mitochondrial diseases are related to ROS-induced mitochondrial injuries. Among these, neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), inflammatory diseases such as sepsis, and aging itself, are caused or accompanied by ROS-induced mitochondrial dysfunctions. With regard to its action spectrum as an antioxidant, melatonin may be regarded as a first-choice agent for preventing and/or reducing the excess of ROS, thereby maintaining mitochondrial homeostasis. Multiple *in vitro* and *in vivo* experiments have shown the protective role of melatonin on mitochondrial physiology, yielding a significant improvement in those diseases in which energy supply to the cell had been compromised. New lines of evidence suggest the participation of mitochondria in stem cell proliferation and differentiation, and preliminary data support the role of melatonin in these processes. This review accounts for the multiple functions of mitochondria and the mechanisms involved in the numerous beneficial effects of melatonin to maintain mitochondrial homeostasis.

Keywords: Oxidative stress, aging, mitodhondrial dysfunction, neurodegenerative diseases, stem cell differentiation, melatonin therapy.

MITOCHONDRIAL FUNCTION

Mitochondria are organelles found almost ubiquitously in eukaryotes. Their small size contradicts their fundamental magnitude to the life and metabolic economy of the cell, as they are the location of a number of vitally important metabolic pathways including Krebs cycle, fatty acid oxidation, and lipid and cholesterol synthesis. Most of these are "housekeeping" functions that are required for the maintenance of the cell's well-being. Foremost among these is formation of ATP, a versatile carrier of energy, in the respiratory chain/oxidative phosphorylation system. This aspect of mitochondrial function is unique insofar as the transformation of chemically bound energy is based on genes encoded in both mitochondrial and nuclear DNA. By virtue of possessing their own genome – mitochondrial DNA (mtDNA) – mitochondria are distinctive mammalian organelles. Human mtDNA is a 16,569 base pair, double stranded, circular molecule, which is maternally inherited [1]. MtDNA is continuously replicating in both dividing and non-dividing cells. Several nDNA-encoded proteins and other factors are involved in regulating human mtDNA replication and transcription [2]. The two strands of mtDNA have different densities determined by their G + T content and are described as heavy (H) and light (L) strands. Most of the information is encoded on the heavy (H) strand, with genes for two ribosomal RNAs (rRNAs), 14 transfer RNAs (tRNAs), and 12 polypeptides. The light (L) strand codes for 8 tRNA, and a single polypeptide. All thirteen polypeptides are subunits of the respiratory chain/oxidative phosphorylation system: seven belong to Complex I (C-I) or NADH-CoQ oxidoreductase), one to Complex III (C-III) or CoQ-cytochrome *c* oxidoreductase, three to Complex IV (C-IV) or cytochrome *c* oxidase (COX) and two to Complex V (C-V) or ATP synthase. These subunits are synthesized within the mitochondrion, where they assemble with a larger number of subunits encoded by nuclear DNA (nDNA) that are synthesized in the cytoplasm and are transported into mitochondrion [3]. The five mitochondrial complexes together with two electron carriers, coenzyme Q10 (CoQ_{10}) and cytochrome c, are embebed in the inner mitochondrial membrane, which is rich in cardiolipin, an unusual phospholipid directly involved in the mitochondrial function [4].

$NADH+H^+$ and $FADH_2$ produced by glycolysis, Krebs cycle, and β-oxidation of fatty acids are oxidized by the respiratory chain transferring electrons from these precursors

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to O_2. Incomplete reduction of oxygen occurs by electron leakage from especially C-I and C-III, perhaps also from C-IV. The resulting superoxide anion, $O_2\bullet^-$, is the source of other reactive intermediates, H_2O_2 and HO•. These three molecules, which represent endogenous oxidotoxins, are collectively denoted as reactive oxygen species (ROS). They can be detoxified by the antioxidant defense system that includes superoxide dismutase (SOD), glutathione redox cycle, catalase and melatonin [5]. According to the chemiosmotic hypothesis [6], the free energy made available in the course of sequential electron transfer is used by C-I, C-III and C-IV to pump protons from the matrix to the intermembrane space. This results in a proton gradient ($\Delta\mu_H^+$) over the mitochondrial inner membrane. This proton gradient is a source of free energy that is dissipated when protons enter the inner mitochondrial membrane via the ATP synthase. During this process, ADP is phosphorylated to ATP, a high energy molecule consumed by many enzymes and numerous cellular processes [6]. Dissipation of energy as heat to maintain body temperature at a level higher than in the environment is another important function of mitochondria. The mechanism is called thermoregulating uncoupling of respiration and phosphorylation, and this function seems to depend on the so-called uncoupling proteins (UCPs) [7]. UCPs are proton transporters across the inner mitochondrial membrane, driven only by the membrane potential [8]. Five types of UCPs have been identified in mammals, UCP1, UCP2, UCP3, UCP4 and UCP5, and they constitute a subfamily within the gene family of mitochondrial anion carriers [9]. While UCP1 is known to play an important role in regulating heat production during cold exposure in some mammals including human babies, possible roles for other UCPs are still controversial and they may include the control of ROS production by mitochondria [10, 11]. These UCPs probably do not transport protons except in the presence of specific activators [12]. Fatty acids, ROS and free-radical-derived alkenals are activators of proton transport through UCPs, whereas purine nucleotides are inhibitory (10;11). CoQ has been also proposed as a regulatory factor of UCPs but the results are still controversial [13-16]. A failure to control ROS damage can induce the collapse of multiple vital functions, including mitochondrial energy conservation, which culminates in loss of membrane integrity and cell death by necrosis and/or apoptosis [7]. The UCP-dependent uncoupling leads to suction of electrons and, consequently, reduces electron overflow and, thus, superoxide formation [17]. Thus, uncoupling can reduce the probability of mitochondrial permeability transition pore (mtPTP) opening and should, therefore, prevent the proapoptotic cascade.

Mitochondria play also an important role in the signaling for apoptotic cell death. Apoptosis may be initiated in the course homeostatic regulation, aging, mitochondrial dysfunction and to eliminate potentially tumorigenic cells. Mitochondria promote the release of proapoptotic factors including cytochrome *c* in the intermembrane space [18], activating the apoptotic cascade [19]. The PTP may be regulated by the ROS leaking from the ETC. A shift from a low to a high-conductance state is promoted by the oxidation of NADPH by oxidative stress. This impairs the antioxidant function of glutathione (GSH) [20]. The participation of ROS in the opening PTP is supported by the fact that this pore remains closed in the absence of molecular oxygen [20]. The PTP possesses at least two redox-sensitive sites which increase the probability of opening after oxidation: the S-site, a dithiol in apparent redox equilibrium with matrix GSH, and the P-site, in apparent redox equilibrium with the pyridine nucleotides [21]. Oxidized glutathione (GSSG) is probably the immediate oxidant of the S-site and many inducers of pore opening such as hydrogen peroxide (H_2O_2) appear to affect the pore through changes at the level of GSH rather than the direct oxidation of the S-site. In turn, oxidation of the P-site by oxidized pyridine nucleotides can induce PTP opening under conditions where the GSH pool is maintained in a fully reduced state. Under conditions of oxidative stress, the mitochondrial levels of GSH and reduced pyridine nucleotides are connected through energy-linked transhydrogenase and glutathione reductase (GRd) and thus it is difficult for these compound to independently modulate the S- and the P-site *in vivo* [21].

PRIMARY MITOCHONDRIAL DISORDERS

Primary respiratory chain defects are caused by mutations in mtDNA or nDNA genes that encode subunits of the mitochondrial respiratory chain. About 200 mtDNA point mutations have now been associated with human diseases, whereas only a small but increasing number of nDNA gene mutations have been identified [22].

Mutations in mtDNA protein–coding genes include mutations in ATP6 causing neuropathy, ataxia, retinitis pigmentosa (NARP) or maternally inherited Leigh syndrome (MILS) [22, 23]; mutation in ATP8 causing hypertrophic cardiomyopathy and nephropathy [24]; mutations in ND1, ND4 and ND6 causing Leber hereditary optic neuropathy (LHON); and mutations in ND1, ND4, cyt b, COXI, COXII or COXIII causing myopathy, encephalopathies, multisystemic diseases or another phenotypes [22, 23]. mtDNA deletions are frequently associated with chronic progressive external ophthalmoplegia (CPEO) or with Kearns-Sayre syndrome. mtDNA deletions and duplications are also associated with diabetes and myopathy [22, 23].

The most common mtDNA tRNAs mutations are the 3243 A>G $tRNA^{Leu}$ causing mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS); and the 8344 A>G $tRNA^{Lys}$ causing myoclonic epilepsy with ragged red fibers (MERRF). In addition, a large number of tRNAs mutations have been associated with different phenotypes including myopathy, cardiomyopathy, encephalopathy, Leigh syndrome, deafness and diabetes (22;23). Mutations in 12S and 16S rRNAs have been associated with deafness and/or cardiomyopathy [22, 23].

Mutations in nDNA genes encoding mitochondrial respiratory chain subunits have been identified in a few cases, relative to the pertinent numerous mtDNA mutations known. Consequently, it has been suggested that most deleterious mutations in these nDNA genes are incompatible with life. The reported cases are mutations in C-I subunits (NDUFA1; NDUFS1,2,3,4,6,7,8; and NDUFV1,2) causing Leigh syndrome and leukodystrophy [22, 23]; mutations in SDH subunits (SDHA,B,C,D) yielding Leigh syndrome and ataxia [22, 23]; mutation in the UQCRB subunit of C-III producing Leigh syndrome [25]; and mutation in the COX6B1subunit of CIV causing severe encephalopathy [26].

In mtDNA-related mitochondrial encephalomyopathies, cells die because the lack of an adequate energy supply and the decrease in $\Delta\psi_m$ that triggers PTP opening and apoptosis. However, a relatively small number of non-mutated mitochondrial chromosomes seems to be sufficient for protecting cells from respiratory chain defects. In neurons, the incapacity to maintain adequate ATP levels would lead to a partial neuronal depolarization and excitotoxicity, and muscle cells seem to die mainly by apoptosis [22]. In addition, oxidative stress can also participate to a variable extent in the pathology of primary mitochondrial disorders. Transmitochondrial cybrids harbouring homoplasmic mutations in mitochondrial tRNA genes (A3243G in tRNA LeuUUR and A8344G in tRNA Lys) showed an increase of ROS production and an increase in the antioxidant enzyme activities when they were grown under glucose-rich medium [27]. In contrast, homoplasmic COX mutant cybrids (carrying the stop-codon mutation G6930A in the COXI gene) grown under similar conditions did not show any sign of oxidative stress [27]. By contrast, cybrids harbouring homoplasmic mutations (Leber's hereditary optic neuropathy mutations) in mtDNA encoding subunits of complex I, ND1, ND4 and ND6, showed alterations of the antioxidant defense when the cells were grown in galactose medium (which forces ATP production through oxidative phosphorylation [28], but not under glucose-rich medium [29]. Likewise, mutations in ATP6 (T8993G and T8993C) have shown variable grade of oxidative stress depending on the severity of the mutation, degree of heteroplasmy or/and the culture conditions [30]. Therefore, the involvement of oxidative stress in the pathology of primary mitochondrial disorders seems to depend of the severity of the mutation, the heteroplasmy level and the energy requirement of the cell.

SECONDARY MITOCHONDRIAL DISORDERS

Secondary respiratory chain defects may encompass all the etiologies other than primary gene mutations of respiratory chain subunits. It can include defects of nuclear-encoded mitochondrial proteins responsible for the synthesis of components of the mitochondrial respiratory chain, the assembly of respiratory chain complexes, mtDNA integrity, replication and transcription, transport of nuclear-encoded proteins, regulation of the composition of mitochondrial membranes, mitochondria fusion and fission, proteins involved in critical mitochondrial biochemical pathways, and general intracellular biochemical derangements that have an impact on mitochondrial respiratory chain function [22, 23].

The biosynthesis of CoQ_{10} is carried out in the mitochondrial inner membrane and at least 8 enzymes participate in this process [31]. Mutations in the genes that encode these enzymes are responsible of primary CoQ_{10} deficiency, an autosomal recessive syndrome with a clinical spectrum that encompasses five major phenotypes [31]. Although it was already described 30 years ago, molecular defects were identified only recently. Specifically, the first mutations in two genes encoding the initial two CoQ_{10} biosynthetic enzymes (*PDSS2* subunit of COQ1, and *COQ2*) were identified in infants or children with encephalomyopathy or Leigh syndrome and nephrotic syndrome [32, 33]. Later, mutations in *PDSS1* and others in *COQ2* were indentified in infants or children with encephalomyopathy and/or nephrotic syndrome [34, 35]. Recently, a mutation in *COQ9*, a CoQ_{10} biosynthetic gene with unknown function, was identified in a child with intractable seizures, global developmental delay, hypertrophic cardiomyopathy and renal tubular dysfunction [36]. Nevertheless, the consequences of primary CoQ_{10} deficiency include a severe bioenergetic defect or a combination of mild bioenergetic defect and oxidative stress, which seems to be dependent on CoQ_{10} levels [37]. Other syndromes have also been associated with a secondary CoQ_{10} deficiency. These include autosomal recessive cerebellar ataxia of unknown etiology in children and is caused by mutations in *ADCK3* in adults [31, 38], the syndrome of ataxia and oculomotor apraxia (AOA1) caused by mutations in the aprataxin gene (*APTX*) [39], and a predominantly myopathic form of glutaric aciduria type II (GAII) caused by mutations in the electron transfer flavo-protein dehydrogenase gene (*ETFDH*) [40]. The usual treatment of CoQ_{10} deficient patients is the oral supplemen-tation with CoQ_{10}. However, the cause of the lack of positive response in some patients requires investigation [31, 32, 35, 36, 38].

The most frequent mutation in assembly factor genes have been identified in patients with COX deficiency associated with Leigh syndrome, myopathy and encephalopathy. These are the cases of mutations in COX10, COX15, LRPPRC, SCO1, SCO2 and SURF1 [22, 23]. Mutations in assembly factors for other mitochondrial complexes include the NDUFA12L gene resulting in defective assembly of C-I [22, 23]; SDHAF1 producing defective assembly of C-II [41]; BSC1L gene resulting in defective assembly of C-III [22, 23]; and ATPAF2 gene producing defective assembly of C-V [22, 23].

mtDNA integrity, replication and transcription depend on several nDNA-encoded proteins. Then, mutations in these nDNA genes yield quantitative or qualitative abnormalities of mtDNA. A quantitative mtDNA alteration is a partial or severe mtDNA depletion which frequently causes myopathy or hepatopathy. A particular disease in this category is MNGIE (mitochondrial neurogastrointestinal encephalomyopathy) since a combination of mtDNA depletion, multiple mtDNA deletions and point mutations are found in many organs of MNGIE patients [42-44]. The primary cause of the disease is a mutation in *TYMP* gene [42], which encodes thymidine phosphorylase (TP), an enzyme that catalyzes the phosphorolysis of thymidine and deoxyuridine. A defect in the function of TP causes an accumulation of thymidine and deoxyuridine provoking an unbalance in the mitochondrial deoxynucleotides pool, which in turns causes mtDNA alterations [45].

The vast majority (99%) of the mitochondrial proteins are encoded by nDNA genes, so they have to be imported from the cytoplasm into mitochondria. Mitochondrial proteins synthesized in the cytoplasm possess mitochondrial targeting signals that direct them to the appropriate compartment within the organelle. Transport across outer and inner membranes requires a well coordinated machinery that include docking proteins, chaperonins, and proteases, and it involves unfolding and refolding of the protein to be translocated. Thus, mutations in the mitochondrial targeting signals and defects in the import machinery cause fails in the nuclear-coded protein transport into mitochondria [22].

The mitochondrial respiratory chain complexes are embedded in the lipid milieu of the inner mitochondrial membrane. Therefore, alterations or damage in the inner mitochondrial membrane may affect mitochondrial function. The inner mitochondrial membrane is rich in cardiolipin, an unusual phospholipid directly involved in the mitochondrial function. Alteration in the concentration and composition of cardiolipin leads to altered mitochondrial architecture and function. These changes are representative of Barth syndrome, an X-linked recessive disorder characterized by mitochondrial myopathy, cardiomyopathy, and growth retardation, and caused by mutations in the gene encoding a phospholipid acyltransferase called tafazzin (*TAZ*) [22].

Mitochondria are dynamic organelles and form tubular networks that may favor the delivery of ATP to areas of high energy demands. Then, defects in proteins responsible of mitochondrial motility-fusion-fission cause another group of mitochondrial disorders. At least mutations in 13 different genes, i.e. *OPA1*, *MFN2* and *GDAP1*, have been associated with defects in mitochondrial dynamics [22].

Finally, secondary respiratory chain defects also include secondary consequence of other factors such as the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) and the subsequent widespread deleterious effects (oxidation and/or nitrosylation of DNA, lipids and proteins) of these intermediates. The generation of toxic reactive intermediates has been implicated in aging and a number of relatively common disorders, including neurodegenerative diseases, sepsis, diabetes and cancer.

PATHOLOGIES ASSOCIATED WITH MITOCHONDRIAL IMPAIRMENT

Sepsis is a systemic inflammation characterized by the activation of a number of intracellular signaling pathways; some of these signals lead to the expression of inducible nitric oxide synthase (iNOS). The iNOS produces nitric oxide (NO•), a crucial molecule in the inflammatory response because of its vasodilator properties and bactericide action [46, 47]. However, the excessive and prolonged production of NO• has toxic effects in the organism [46, 47]. In mitochondria, NO• can inhibit mitochondrial respiratory chain complexes and compete with oxygen for its binding site to complex IV [48, 49]. In experimental models of sepsis, it has demonstrated that huge amounts of NO• produced by iNOS are responsible of mitochondrial dysfunction and increased oxidative damage in different tissues [50-54]. The result is a bioenergetic crisis that contributes to the mortality of septic patients [55, 56].

Neurodegenerative diseases of different etiologies may share mitochondrial dysfunction, oxidative/nitrosative stress and apoptosis in particular brain areas as a final common pathway. Mitochondrial involvement in Parkinson's disease (PD) is revealed by deficiency of mitochondrial complex I (C-I) in *substantia nigra* [57], with a parallel reduction in GSH levels, suggesting the existence of oxidative stress. In platelets of PD patients C-I is also decreased, and in some cases, this is accompanied by C-II, C-III and C-IV deficiencies. Studies with cybrids have shown that alterations in C-I is due to a defect in the mtDNA [57]. Mitochondrial involvement in the pathology of PD has been genetically supported by the finding of *POLG* mutations in early-onset Parkinsonism in different families [58, 59]. However, there is currently no convincing proof for a primary role of mtDNA mutations in this neurodegenerative disorder [60]. However, a series of nuclear genes (*PARK2*, *PARK7*, *PINK1*, *SNCA*, *LRRK2* and *HTRA*2) has been found to be associated with the familial form of PD [22]. Moreover, some environmental toxins seem to interact with the products of these genes, which provokes oxidative damage, mitochondrial dysfunction and cell death [61]. These toxins including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone and paraquat, cause mitochondrial dysfunctions comparable to those in PD, stimulate nNOS activity and NO• production [62], and decrease the content of mtDNA [63].

Mitochondria are also involved in the pathology of Alzheimer's disease (AD), including reduction in brain energy metabolism [64], defects of mitochondrial metabolic enzymes [65, 66], and deficiencies of respiratory chain complexes [67, 68]. In a recent study, female triple transgenic Alzheimer's mice (3xTg-AD) showed an early mitochondrial bioenergetic deficit and increased oxidative stress in brain which were exacerbated in the reproductive senescence [69]. However, thorough analyses found little evidence in support for a role of mtDNA mutations in the development of AD [60]. Furthermore, it has been shown that β-amyloid peptide generates ROS in a metal-catalyzed reaction, which induces neuronal cell death in a ROS-mediated process resulting in damage to neuronal membrane lipids, proteins and nucleic acids.

Aging has been also related to the increased oxidative damage and mitochondrial dysfunction [70, 71]. mtDNA point mutations and duplications in tRNA, protein-coding genes and D-loops have been found to accumulate in some post-mitotic tissues during human aging [72-74]. However, it seems that the proportion of mutant mtDNAs is too low to cause a significant impact on mitochondrial function in aging tissues. On the other hand, the distribution of the mutant mtDNA in the cell and tissue is still unknown and the answer to this matter could resolve important questions regarding the importance of mtDNA mutation in aging [75]. Additionally, mitochondrial polymerase γ (POLG) deficient mice accumulate high levels of mtDNA mutations resulting in a premature aging phenotype without increase of ROS generation and oxidative damage [76, 77]. These results would support the idea of a direct involvement of mtDNA mutations in aging but would cast doubt on the vicious cycle theories of aging and oxidative stress [78]. However, some explanations have been proposed to account the lack of oxidative stress in POLG deficient mice: aging is the result of alterations in many pathways; alterations in POLG may be downstream from mechanisms that generate ROS; and extensive mtDNA mutations could prevent the generation of ROS [78]. Contrary to the POLG mutator mice, skin fibroblasts harboring an mtDNA point mutation associated with aging show an alteration in the expression profile of antioxidant enzymes [79]. Age-related mtDNA deletions have been also detected in humans with a dissimilar pattern in different tissues [80], and the correlation of the increased of mtDNA deletions and mitochondrial respiratory chain malfunction during aging has been amply reported [81-83]. The mechanisms of mtDNA deletions during aging are still

controversial but oxidative damage to DNA associated with single- or double-strand breaks has been proposed. This idea has been supported by some studies: the relative amount of mtDNA deletions correlates with the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) [84]; and treatment of human skin fibroblasts with sub-lethal dose of oxidants and environmental insults inducers of ROS results in the formation and accumulation of the 4977 bp deletion in mtDNA [85, 86]. Oxidative damage in aging is also reflected by oxidation of mitochondrial proteins in a variety of arganisms [75]. Proteins containing Fe-S clusters seem to be the most susceptible to oxidation [75]. Several reports have revealed that oxidation of aconitase, adenine nucleotide translocase and mitochondrial respiratory chain complexes increase during aging and, consequently, the activities of these enzymes may diminish [87-89]. Oxidative injury is not limited to mtDNA or proteins but is also detected in mitochondrial membranes. This may lead to a progressive lipid peroxidation (LPO) and cross-linking damage, with simultaneous changes in the respiration rate, ATP synthesis, membrane fluidity and permeability, Ca^{2+} homeostasis and apoptosis [90]. Alterations in the expression and activities of the antioxidant enzymes in response to the oxidative environment in the aging cells has been found in human blood [91-93] and muscle [94, 95], and in a variety of tissues from rats and mice, including skeletal muscles, brain and heart, which are tissues with high energy demand. Additional information about the physiological changes in mammalian aging has been obtained in studies performed in the senescence-accelerated mouse (SAM) [96]. SAM includes two strains, one prone to accelerated senescence (SAMP) and one resistant to accelerated senescence (SAMR). SAMP8, a sub-strain of SAMP, shows relatively strain specific age-associated phenotypic pathologies such as a shortened life span and early manifestation of senescence (including loss of activity, alopecia, lack of hair glossiness, skin coarseness, periophthalmic lesions, increased lordokyphosis and systemic senile amyloidosis), similar to several geriatric disorders observed in humans [96, 97]. SAMP8 mice show a general hyperoxidative status manifested by increased mitochondrial electron leakage and ROS production, increased LPO and protein carbonyl content, changes in the antioxidant enzymes activities and increase of GSSG: GSH ratio [98-104]. The results are a decrease in the mitochondrial respiratory chain activity, ATP synthesis and energy status of the organism, suggesting that the mechanism of senescence acceleration in SAMP8 mice is related to free radical damage in a variety of tissues [105-107]. SAMP8 mice also show an age-dependent increase in IFN-γ and TNF-α, a reduction in IL-2 levels and an increase on nitric oxide (NO•) levels [108], suggesting the existence of an inflammatory process during aging. The increase in NO• levels is particularly relevant since this radical can react with (O_2^{-}•) in mitochondria yielding peroxynitrite [109], which irreversibly impairs the mitochondrial respiratory chain and decreases the efficiency of the oxidative phosphorylation, leading to energy depletion and cell death [49, 110].

MITOCHONDRIA AND STEM CELLS

Stem cells are classified depending on their capacity of differentiation to mature cells, processes which are regulated by many factors. In the last years, some studies have lead to propose a mitochondrial involvement in the regulation of pluripotency and differentiation of stem cells. Differentiation of mouse and human embryonic stem cells (ESC) results in changes in mitochondrial structure, morphology and pattern of cytoplasmic localization. Mitochondria in stem cells tend to localize perinuclearly [111]. Moreover, ESC have relatively few mitochondria with poorly developed cristae [112, 113], and restricted oxidative capacity. As cells are allowed to differentiate, the mtDNA copies are amplified and the differentiated cells contain larger numbers of mitochondria with distinct cristae, dense matrices and high membrane potentials. These features suggest the initiation of metabolic activity through OXPHOS [114]. Because ESC display low oxygen consumption and thus, poor OXPHOS, an elevation in ATP content per cell may therefore reflect a loss of stemness and the subsequent onset of differentiation [111, 113]. Remarkably, transmitochondrial embryonic stem cells harboring pathogenic mtDNA mutations have shown to be compromised in neuronal differentiation when the mitochondrial respiratory chain function is severely affected [115]. Therefore, preservation of immature mitochondria with a perinuclear arrangement, reduced expression of OXPHOS enzymes and low metabolic activity in ESC has led to the suggestion that these mitochondrial properties might be important for the maintenance of pluripotency and should be considered as another ESC marker.

The increase in mitochondrial mass is accompanied by elevated ATP production and, thus, by a greater generation of ROS. Undoubtedly, the intracellular levels of ROS are higher in differentiated than in undifferentiated cells, due to the increase in OXPHOS metabolism in the former [116]. An increase in ROS levels might have a role in cell signaling and regulation of proliferation and differentiation. Exposure to low levels of ROS has been reported to enhance ESC differentiation whereas continuous exposure to high levels of ROS results in inhibition of differentiation [116]. Therefore, differentiating cells probably activate effective antioxidant systems, including catalase, glutathione peroxidase (GPx) and others. However, a recent study has revealed high levels of ROS in *Drosophila* multipotent haematopoietic progenitor cells. The ROS levels are decreased when the progenitor cells undergo differentiation. And interestingly, this differentiation can be modulated by scavenging or increasing the ROS [117]. In summary, successful differentiation of embryonic cells *in vivo* or ESC *in vitro* involves initiation of mtDNA transcription and replication, an increase in the number of mitochondria, and regulation of the enzymes required for aerobic metabolism in order to fulfill the elevated ATP requirements of fully differentiated cells. Additionally, ROS and antioxidant defenses may also be involved in the regulation of the differentiation.

THE ANTIOXIDANT CAPACITY OF MELATONIN AND ITS ACTIONS ON MITOCHONDRIA-RELATED DISORDERS

Melatonin is an ancient indoleamine derived from tryptophan, present from unicellular organisms to mammals [118, 119]. In mammals, melatonin is synthesized by the pineal gland in a circadian manner and it is released to the

blood, where it can attain up to 0.5 nM [120]. Melatonin is also produced in most of the tissues and organs of the body and this extrapineal biosynthesis of melatonin is, in total, much higher than that formed by the pineal [121, 122]. The different sources of melatonin are related to the different actions of the indoleamine: the pineal melatonin is mainly a mediator of the circadian cycles and circannual reproductive rhythms [123, 124]; the extrapineal melatonin participates in the immune system modulation, anti-inflammatory mechanisms and antioxidant actions [5, 125]. Interestingly, melatonin is concentrated by subcellular compartments including nucleus and mitochondria, the latter showing 100-200 times more melatonin than cytosol [126, 127]. Therefore, melatonin is available in the mitochondrion, the cell organelle in which free radicals are being maximally generated, and can counteract a potential damage [128, 129].

The earliest evidences of the antioxidant capacities of melatonin were reported in the 90´s. First, it was shown that one molecule of melatonin scavenges two molecules of HO•, producing in that reaction cyclic 3-hydroxymelatonin [130, 131]. This molecule can be detected in the urine of human and rats under oxidative stress conditions and treated with melatonin [131]. Moreover, several oxidative pathways of melatonin yield N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) [132]. Although it was proposed that AFMK is a byproduct of the interaction of melatonin with H_2O_2 [133, 134], it was reported that melatonin does not directly scavenge H_2O_2 *in vitro* [135]. Fowler *et al.* [135] claim that the direct interaction of melatonin with H_2O_2 *in vitro* occurs only when traces of transition metal ions are present in the reaction medium, Thus, *in vivo*, in conditions under tissue injury, the presence of transition metal ions may favor the reaction between melatonin and H_2O_2. In addition, the two metabolites, cyclic 3-hydroxymelatonin and AFMK, may also function as scavengers of toxic reactants [136-138]. However, other melatonin metabolites, *N*-acetyl-5-methoxykynuramine (AMK) and 6-hydroxymelatonin, are also capable of neutralizing some ROS. A cascade of scavenging reaction seems to be initiated by melatonin and thus could explain the high efficacy of melatonin in reducing oxidative damage [138]. Other ROS have been reported to be scavenged by melatonin [138]. Interestingly, melatonin can also react with peroxynitrite producing several metabolites including *N*-nitrosomelatonin [138-141].

Besides its ability to directly scavenge ROS and RNS, melatonin has a number of indirect actions by which it may reduce oxidative/nitrosative stress. It has been reported that melatonin can increase the SOD and catalase activities and induces the expression and activity of GPx and GRd, regulating the redox cycle of glutathione [142-147]. The regulation of glutathione system by melatonin is also reflected by the stimulation of the rate limiting enzyme in glutathione synthesis, γ-glutamylcysteine synthase [148]. Moreover, melatonin can reduce the expression and activity of iNOS and, thereby, decrease the levels of NO and peroxynitrite [149, 150]. Therefore, the actions of melatonin can be classified according to receptor- and non-receptor-mediated processes [151] depending of its direct and indirect action. Receptor-mediated effects of melatonin involve both membrane and nuclear receptors [152-155].

Based on all these properties of melatonin, the indolamine has been used in a wide variety of experimental conditions and cellular and animal models of diseases. Especially relevant are the studies in which mitochondrial function is compromised by the toxic effects of ROS and RNS. The ability of melatonin to influence mitochondrial homeostasis was initially tested *in vivo*. In this study it was shown that melatonin administered to normal rats significantly increased the activities of the C-I and C-IV of the mitochondrial respiratory chain measured in isolated mitochondria obtained from brain and liver, whereas C-II and C-III were unaffected [156]. Melatonin also counteracted ruthenium red-induced inhibition of C-I and C-IV in brain and liver mitochondria when melatonin was given simultaneously with ruthenium red [156].

To further test the antioxidant potency of melatonin against mitochondrial oxidative stress, *in vitro* experiments using isolated mitochondria prepared from rat brain and liver were performed. Oxidative stress was induced by incubation of these mitochondria with t-butyl hydroperoxide (t-BHP), which oxidizes pyridine nucleotides, depletes the mitochondrial GSH pool and inhibits both GPx and GRd activities [157]. In this situation, 100 nM melatonin counteracted these effects, by restoring basal levels of GSH and the normal activities of both GPx and GRd. N-acetyl cysteine (NAC) and vitamins E and C were unable to exert any significant effect on t-BHP-induced oxidative stress in mitochondria despite the high doses of these compounds used [158]. Interestingly, melatonin increased the activity of the C-I and C-IV in a dose-dependent manner, the effect being significant at 1 nM melatonin [158]. Melatonin was also able to counteract the cyanide-induced inhibition of C-IV, restoring the levels of cyt $a+a_3$. Moreover, melatonin increased the activity of C-I separated by blue native polyacrylamide gel electrophoresis (PAGE). The effects of melatonin are of likely physiological significance since the indoleamine increased the mitochondria respiratory chain activity coupled to OXPHOS, which was reflected in an elevated ATP synthesis, either in normal mitochondria or in mitochondria depleted of ATP by cyanide incubation [127, 159].

Recently, a study performed *in vitro* with normal mitochondria addressed some of the mechanisms involved in the actions of melatonin on this organelle [127]. Using high-resolution respirometry, rat liver mitochondria were analyzed for oxygen consumption, ROS generation, membrane potential, ROS generation, and activities of mitochondrial complexes. Melatonin decreased oxygen consumption in the presence of ADP in a concentration-dependent manner; it reduced the membrane potential and, consequently inhibited the production of O_2^{-}• and H_2O_2. At the same time, melatonin maintained the respiratory control ratio (RCR) and the efficiency of oxidative phosphorylation and ATP synthesis while increasing the activity of the respiratory complexes (mainly C-I, C-III, and C-IV). Kinetic experiments showed that mitochondria take up melatonin in a time- and concentration-dependent manner and thus, the effects of melatonin on this organelle were due to its presence within the mitochondria [127]. These data support the hypothesis that melatonin participates in the physiological regulation of mitochondrial homeostasis.

The effect of melatonin in the reduction of nitrosative stress and mitochondrial damage has been amply studied in animal models of sepsis. The administration of pharmacological doses of melatonin in rodents with sepsis induced by lipopolysacharide injection or cecal ligation and puncture (CLP) produced a decrease in the expression and activity of iNOS, and consequently nitrite levels, nitrosative/oxidative stress and mitochondrial function were normalized in muscle tissues and liver [51-54, 149, 160]. Likewise, the survival of CLP mice treated with melatonin increased significantly. Interestingly, the increase of iNOS expression was more pronounced in aged (18 m.o.) than young rats (3 m.o.), but melatonin was able to reduce the expression in both groups [51, 161]. Because of the efficacy of melatonin in the treatment of experimental sepsis, Gitto and co-workers tested this treatment for sepsis in human premature newborns [162]. Twenty newborns diagnosed with sepsis were randomly divided into 2 groups of 10 infants each; all were given conventional antibiotic therapy but 10 were also supplemented with 2 doses of 10 mg oral melatonin within the first 12 hours after after diagnosis, separated by a 1-hour interval. Melatonin improved the clinical outcome of the septic newborns as judged by measurement of sepsis-related serum parameters after 24 and 48 h. Three of 10 septic children who were not treated with melatonin died within 72 h after diagnosis of sepsis; none of the 10 septic newborns treated with melatonin died [162]. Therefore, melatonin may assist considerably in reducing the mortality of sepsis.

In a series of studies, we have demonstrated the efficacy of melatonin to prevent oxidative damage and mitochondrial dysfunction in aging. In SAMP8 mice, chronic melatonin administration in the drinking water for 9 months (10 mg/kg b.w.) completely prevented the mitochondrial impairment in heart, diaphragm, skeletal muscle, and brain, maintaining or even increasing ATP production [105-107, 163]. Likewise, melatonin prevented the increase of mitochondrial LPO and increased GPx and GRd activities normalizing the GSSG/ GSH ratio [105-107, 163]. Moreover, melatonin prevented the age-dependent increase in serum pro-inflammatory cytokines in SAM mice [108], and age-dependent nitrosative status in mitochondria was also prevented by melatonin administration [107, 108]. All these effects of melatonin treatment led to a significant increase of both half-life and life span of SAM mice [163]. Although melatonin treatment increased survival in SAMR1 mice, the main effect of the indoleamine was produced in SAMP8 mice, in which the survival was increased by 4 months, so that the animals reached the same age as SAMR1 animals [163]. This is an interesting finding, and support experimental data from other pathophysiological conditions, reporting that melatonin is more efficient in the regulation of mitochondrial function when it is impaired.

Since many of neurodegenerative diseases share a common pathway of mitochondrial dysfunction, the properties of melatonin are also of interest for the treatment of these disorders. Although it was recently suggested that PD could be an endocrine disorder of the endogenous melatonin [164], most of the experimental data supports the neuroprotective properties of the indoleamine on this disease. In mouse models of PD induced by MPTP melatonin administration normalized complex I activity and oxidative status in mitochondria from *substantia nigra* and striatum. Looking for the targets of melatonin action, it was recently shown that melatonin reduced the activity of the mitochondrial iNOS (i-mtNOS), thus decreasing mitochondrial NO• levels, preventing the respiratory inhibition produced by NO• at the level of complex IV [165]. Melatonin also protects against excitotoxicity by reducing the autoxidation of dopamine (DA) which occurs in PD [166]. These effects were demonstrated in MPTP-induced PD in mice [167, 168] and in PC12 cells incubated with 6-hydroxydopamine [169]. Melatonin also abrogated cell death induced by cysteamine pretreatment of the PC12 cells; cystamine treatment involves mitochondrial iron sequestration [170]. The age-associated accumulation of redox-active iron in subcortical astrocytes may facilitate the bioactivation of DA to neurotoxic free radical intermediates and thereby predispose the nervous system to PD and other neurodegenerative diseases. In rats injected with kainic acid to produce excitotoxicity-induced apoptotic cell death, melatonin significantly attenuated apoptosis, an effect linked to the reduction in oxidative damage and an increased GSH content [171]. In a spontaneous, age-induced model of apoptosis using cerebellar granule cells, it was shown that melatonin and Ca^{2+}-channel blockers such as amlodipine, inhibited spontaneous apoptosis [172]. This antagonism between melatonin and Ca^{2+}-channels was also demonstrated in electrophysiological and binding experiments [173]. Striatal neurons growing in low density culture in serum-free medium and in the absence of glia die within 3 days by apoptosis. The presence of melatonin rescues striatal neurons from impending cell death, which may have important consequences in neurodegenerative diseases involving nigrostriatal pathway as in PD [174].

In AD patients melatonin levels are decreased in blood and cerebrospinal fluid (CSF) and that reduction seems to parallel the progression of AD neuropathology [175, 176]. Moreover, CSF melatonin levels are already decreased in pre-clinical AD individuals [175]. The administration of melatonin has been tested in order to reduce the neurodegenerative manifestations in AD [177]. When neuroblastoma cells were incubated with Aβ, more than 80% of the neurons died due to apoptosis, but the presence of melatonin reduced cellular death and DNA damage in a dose-dependent manner [178]. In human platelets, melatonin also protected against Aβ-induced damage [179, 180]. Recently, melatonin treatment has been tested in the APP + presenilin-1 double transgenic (Tg) mouse, which is considered a mouse model with characteristics of the neuropathology of AD [181]. Melatonin administered in the drinking water (100 mg/L water) for four months was able to protect AD mice from cognitive impairment in a variety of tasks of working memory, spatial reference learning/ memory, and basic mnemonic function. Immunoreactive Aβ deposition was significantly reduced in hippocampus (43%) and entorhinal cortex (37%) of melatonin-treated AD mice. The levels of TNF-α was decreased in hippocampus of AD mice treated with melatonin, as well as the cortical mRNA expression of SOD-1, GPx and catalase. Taken the results together, the authors suggested that melatonin's cognitive benefits could involve its anti-Aβ aggregation, anti-inflammatory, and/or antioxidant properties [181]. In AD patients, melatonin has been able to stabilize cognitive function over a 2–3 year period [180, 182]. An additional retrospective study reported that individuals with mild cognitive impairment

given melatonin for sleep enhancement also showed significantly better cognitive performance in two widely utilized cognitive assessment tests [183].

MELATONIN, MITOCHONDRIA, AND NEURAL STEM CELLS

A role of mitochondria in stem cell proliferation and/or differentiation begins to have experimental support, but the role of melatonin remains unclear. One can presume that, in view of the specific and significant effects of melatonin on mitochondrial physiology, the indoleamine may also affect mitochondrial physiology in stem cells. It has been shown in a recent study that the differentiation of multipotent haematopoietic progenitor cells from *Drosophila* can be regulated by the levels of ROS in that cells [117]. Then, the antioxidant capacity of melatonin could modulates the proliferative and differentiation potential. Related to that, it was recently reported that melatonin modulates the proliferative and differentiative ability of the neural stem cells from fetal mouse brain, depending on concentration and exposure-timing [184]. When applied during the proliferation period, pharmacological concentrations of melatonin (1-100 μM) applied during the proliferation period, caused a decrease in cell division. Interestingly, neural differentiation of these cells increased without affecting astroglial differentiation. Other data point towards a net hippocampal neurogenesis in adult mice by melatonin [185]. The effects of melatonin on neural proliferation and differentiation might partly result from melatonin´s activity in mitochondria and thus, additional studies are required to uncover underlying melatonin´s actions on neural stem cells. Finally, if oxidative and nitrosative environment in the aged brain and its interaction with mitochondrial function are critical factors for the long-term survival and phenotypic stability of stem cell-derived neurons or glial cells newly generated or transplanted in some brain areas [186], melatonin could, thus, be of potential utility in order to increase the survival of these cells.

CONCLUDING REMARKS

Due to the multiple functions of the mitochondria in the cell, any alteration in this organelle might have a considerable impact on the functioning of the cell and, potentially, the entire organism. One of the main aspects here revised regarding mitochondrial dysfunction is the participation of ROS/RNS in mitochondrial pathologies. Although no convincingly effective treatment is to date available for most mitochondrial-dependent diseases, an interesting and, perhaps, promising therapeutic approach might consist in the use of free radical scavengers and/or antioxidants to prevent/counteract the damage induced by ROS/RNS. In this regard, melatonin treatment may become a first-line therapy because of its multiple actions on mitochondria. In fact, mitochondria take up melatonin in a concentration- and time-dependent manner and, once inside the organelle, melatonin exerts a series of actions with the consequence of maintaining their bioenergetic capacity. Melatonin increases the activity of the respiratory chain and the ATP production, reducing at the same time the O_2 consumption. Consequently, melatonin avoids an excess of ROS, preventing PTP opening and apoptosis. Additionally, melatonin also prevents changes in the $\Delta\psi_m$ that could account for bioenergetic alteration and PTP opening. Due to the lack of side effects of melatonin, the indoleamine is an excellent candidate for the co-treatment of many of the mitochondrial-related diseases. The recent findings on the role of mitochondria on stem cell differentiation open a promising line of research for melatonin.

ABBREVIATIONS

8-OHdG	=	8-hydroxy-2'-deoxyguanosine
Aβ	=	Amyloid β
AD	=	Alzheimer disease
AFMK	=	N^1-acetyl-N^2-formyl-5-methoxykynuramine
AMK	=	*N*-acetyl- 5-methoxykynuramine
C-I	=	Complex I
C-II	=	Complex III
C-III	=	Complex III
C-IV	=	Complex IV
C-V	=	Complex V
CLP	=	Cecal ligation and puncture
CoQ_{10}	=	Coenzyme Q10
COX	=	Cytochrome *c* oxidase
DA	=	Dopamine
ESC	=	Embryonic stem cells
GPx	=	Glutathione peroxidase
GRd	=	Glutathione reductase
GSH	=	Glutathione (reduced form)
GSSG	=	Glutathione (oxidized form)
iNOS	=	Inducible nitric oxide synthase
LPO	=	Lipid peroxidatios
MPTP	=	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	=	Mitochondrial DNA
nNOS	=	Neuronal nitric oxide synthase
OXPHOS	=	Oxidative phosphorylation
PD	=	Parkinson disease
POLG	=	Mitochondrial DNA polymerase γ
PTP	=	Permeability transition pore
RCR	=	Respiratory control ratio
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
SAMR	=	Senescence accelerated mouse - resistant
SAMP	=	Senescence accelerated mouse - prone

SOD = Superoxide dismutase

t-BHP = *t*-butyl hydroperoxide

TP = Thymidine phosphorylase

UCP = Uncoupling proteins

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