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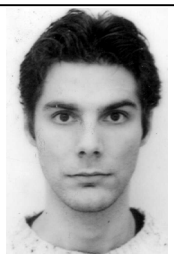
Anti-Oxidants in Parkinson's Disease Therapy: A Critical Point of View

Roberta Filograna, Mariano Beltramini, Luigi Bubacco and Marco Bisaglia*

Molecular Physiology and Biophysics Unit, Department of Biology, University of Padova, Via Ugo Bassi 58/B, 35121 Padova, Italy



M. Bisaglia

Abstract: Parkinson's disease (PD) is a degenerative neurological syndrome, which is characterized by the preferential death of dopaminergic (DAergic) neurons in the *Substantia Nigra*. The pathogenesis of this disorder remains poorly understood and PD is still incurable. Current drug treatments are aimed primarily for the treatment of symptoms to improve the quality of life. Therefore, there is a need to find out new therapeutic strategies that not only provide symptomatic relief but also halt or reverse the neuronal damage hampering PD progression. Oxidative stress has been identified as one of the major contributors for the nigral loss in both sporadic and genetic forms of PD. In this review we first evaluate the current literature that links oxidative stress and mitochondrial dysfunction to PD. We then consider the results obtained through the treatment of animal models or PD patients with molecules that prevent oxidative stress or reduce mitochondrial dysfunction.

Keywords: Antioxidants, iron chelators, mitochondria, oxidative stress, radical scavengers, reactive oxygen species.

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder characterized by the preferential death of dopaminergic (DAergic) neurons in the midbrain area known as *Substantia Nigra* (SN), resulting in a decrease of dopamine (DA) levels in its striatal projections. DA is pivotal for normal movement because it is the neuro-transmitter that allows information on movement to be transferred from the SN to the *striatum*, which then initiates and controls the ease and balance of movement [1]. Coherently with dopamine depletion, the main clinical feature of PD is the presence of several motor dysfunctions, which include resting tremor, muscular rigidity, bradykinesia and postural instability. However, this neurodegenerative disease also affects cholinergic, serotonergic and noradrenergic systems accounting for other clinical non-motor manifestations, which are present in most patients, such as cognitive impairment, olfactory deficits, sleep disturbance, depression and constipation [2].

Although the discovery of monogenic, heritable forms of the disease, which represent 5-10% of all cases, has been very important in helping to delineate the cellular pathways leading to this pathology, PD is generally an idiopathic neurological disease. The etiology of sporadic forms of PD is still poorly understood and aging is considered the most important risk factor. Strong evidence now exists indicating that oxidative stress and mitochondrial dysfunction play a central role in the progression of the disorder [3].

*Address correspondence to this author at the Marco Bisaglia, Molecular Physiology and Biophysics Unit, Department of Biology, University of Padova, Via Ugo Bassi 58B, 35121 Padova, Italy; Tel: +390498276329; Fax: +390498276300; E-mail: marco.bisaglia@unipd.it

OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTION IN PARKINSON'S DISEASE

Oxidative stress occurs when the ability of the endogenous antioxidant systems is overwhelmed by the generation of reactive oxygen species (ROS). ROS encompass both free radical species such as the superoxide anion ($\bullet O_2^-$) and the hydroxyl radical (•OH), as well as other non-radical molecules such as hydrogen peroxide (H_2O_2) [4]. Mitochondria are recognized as the main source of ROS [5]. Actually, ROS are by-products of aerobic respiration and in particular of oxidative phosphorylation that occurs at mitochondrial level. Specifically, the formation of mitochondrial superoxide radicals takes place primarily at two discrete sites in the electron transport chain, at level of complex I and complex III. Once the $\bullet O_2^-$ is generated, it can induce the production of other ROS, such as H_2O_2 and •OH. The latter can be also produced through the Fenton reaction in the presence of intracellular iron, or it can be formed *via* the Harber-Weiss reaction. Moreover, $\bullet O_2^-$ can combine spontaneously with intracellularly synthesized NO to form peroxynitrite ($ONOO^-$), which is a highly reactive nitrogen species. Neuronal cells are considered hypersensitive to ROS-induced damage because of their massive oxygen consumption associated with a high energy demand and of the low regenerative capacity due to their post-mitotic nature. These properties might explain the vulnerability of neuronal tissues to chronic and degenerative diseases, including PD. ROS are highly reactive molecules that can interact with several cellular components, such as nucleic acids, proteins and lipids, causing cell injury.

The association between oxidative stress and the degeneration of DAergic neurons has been highlighted through *post mortem* examinations of PD brains, in which a strong evidence of oxidative damage was found in the analyzed tissues. Lipid peroxidation is defined as the

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degradation of cell membrane components mediated by ROS. Unsaturated lipids are susceptible to oxidative modifications at the level of their carbon-carbon double bonds generating a radical chain reaction which leads to the production of 4-hydroxyl-2,3-nonenal and malondialdehyde. Both these markers have been observed in DAergic cells of PD brains [6, 7]. Additionally, patients present an increase at level of nigral neurons of 8-hydroxyguanosine, a characteristic product derived from nucleic acids oxidation [8]. Other targets of ROS-mediated oxidation are proteins. Here again, different investigations revealed the presence of a high content of protein carbonylation, a hallmark of oxidative protein damage induced by ROS, in brains of PD patients [9, 10]. Nevertheless, it is worth mentioning that it is impossible to discern from the aforementioned observations, whether oxidative stress plays a role in promoting PD or it is merely a consequence of neuronal cell loss.

Additional support to the role of oxidative stress in the loss of dopaminergic neurons arises from the observation that a significant iron accumulation occurs in the SN of patients with PD [11-13], where neuromelanin-granules with iron overload are present [14]. Moreover, besides an increase of 176% in the levels of total iron, a 225% increase of iron (III) was also found in the SN of the PD patients, indicating that changes in the ratio Fe(III):Fe(II) could be implicated in the pathology [13]. Iron can generate oxidative stress in several ways [15], the most prominent being its ability to take part to the Fenton reaction to produce hydroxyl radicals. Strictly related to the preferential neuronal degeneration, which characterizes PD, it has been also indicated that iron in the presence of DA and H_2O_2 can catalyze the synthesis of the endogenous neurotoxin 6-hydroxydopamine (6-OHDA) [16, 17]. Accordingly, in a work performed using mice fed with a high iron diet, significantly increased brain iron concentrations were found compared to controls, associated with an increased fraction of oxidized (GSSG) versus reduced (GSH) glutathione and enhanced levels of hydroxyl radicals in striatum and brainstem [18]. In the same model, the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a molecule able to induce a parkinsonian phenotype also in humans (see below), were strongly enhanced in iron-loaded mice, in comparison to controls.

To deal with the accumulation of free radical species, cells possess several antioxidant defense systems, which work synergistically to avoid oxidative stress production. Among them, GSH is very abundant in mammalian cells, attaining concentrations up to 12 mM [19]. It is worth mentioning that an age-dependent reduction in GSH levels has been described [20], and that the SN has the lower concentration of GSH in comparison to other brain regions [21]. Additionally, in agreement with the proposed link between oxidative stress and PD, a further reduction in the concentration of GSH has been described in the SN of early and advanced PD patients leading to the hypothesis that the drop of GSH concentration in the SN could be an early event for PD [22-24].

The direct association between mitochondrial dysfunction and PD was firstly proposed, based on *post-mortem* examinations, which indicated a loss of complex I activity in the SN of patients who had died from PD [25, 26]. Accordingly to the notion that complex I is one of the main mitochondrial sites of superoxide radical production [5], mitochondrial dysfunctions were observed in association with increased oxidative stress, emphasizing the interrelationship between these events [27, 28]. More recently, additional support emerged from the identification of genetic causes of familial PD. Specifically, mutations in four genes, encoding parkin, DJ-1 PINK1 and Fbxo7, are involved in recessive forms of parkinsonism [29-32]. The main cellular effects, which are common to all four genes, concern mitochondrial functioning and oxidative injure, indicating a potential overlap among the pathways that lead to recessive parkinsonism. Specifically, Parkin, PINK1 and Fbxo7 proteins have been described to exert a central role in the mitophagic process to selectively remove damaged mitochondria. Consequentially, dysfunction of these proteins affects mitochondrial morphology and integrity [33-36]. Although the precise biological function of DJ-1 is not known, a general consensus exists that it plays a role in the cellular responses to oxidative stress [37]. Interestingly, it has been suggested that DJ-1 could act in maintaining the mitochondrial functioning in the presence of oxidative stress, by working in parallel to the PINK1/ parkin pathway [38]. The impairment of mitochondrial function in idiopathic forms of PD is further highlighted by the finding that the exposure to environmental toxins, that affect mitochondrial functions, is a significant risk factor for PD. These same toxins produce parkinsonian phenotypes when used in animal models (see [39] for a review). In conclusion, strong experimental evidence implicates oxidative stress as being involved if not in the pathogenesis of PD, at least the propagation of cellular injury associated to the pathology. Oxidative stress is tightly linked with an integrated series of cellular phenomena, such as protein aggregation, mitochondrial dysfunction, protein clearance etc, which all seem to contribute to neuronal loss. The relation among these processes may not be necessarily a cascade, but the events can be correlated in a cyclic way, in which oxidative stress is a major component. Fig. **1** emphasizes how oxidative stress seems to be involved in the progression of PD.

In light of the aforementioned evidence of the central role played by oxidative stress and mitochondrial dysfunction in the progression of PD, antioxidant molecules have been considered attractive therapeutic drugs to cure PD. Several neurotoxin-based animal models, that played an important role in studying the mechanisms of PD pathogenesis, are often used to evaluate the efficacy of various therapeutic agents in PD. The degeneration of dopaminergic neurons observed in these animal models is associated to severe sensory and motor impairment similar to those seen in PD patients [40,41]. The most accepted model is probably attained by the administration of the neurotoxin MPTP. After administration, MPTP crosses the blood-brain barrier and is metabolized to 1-methyl-4-phenylpyridinium ion (MPP+), by monoamine oxidase. Through the action of the DA transporter, MPP+ is then specifically taken up by dopaminergic neurons. MPP+ toxicity results from the mitochondrial inhibition of complex I, which leads to oxidative stress [40]. Other helpful models are attained by injecting 6-OHDA into the *striatum* of rats or mice, or by the systemic or intracerebral administration of rotenone, or paraquat [40]. In

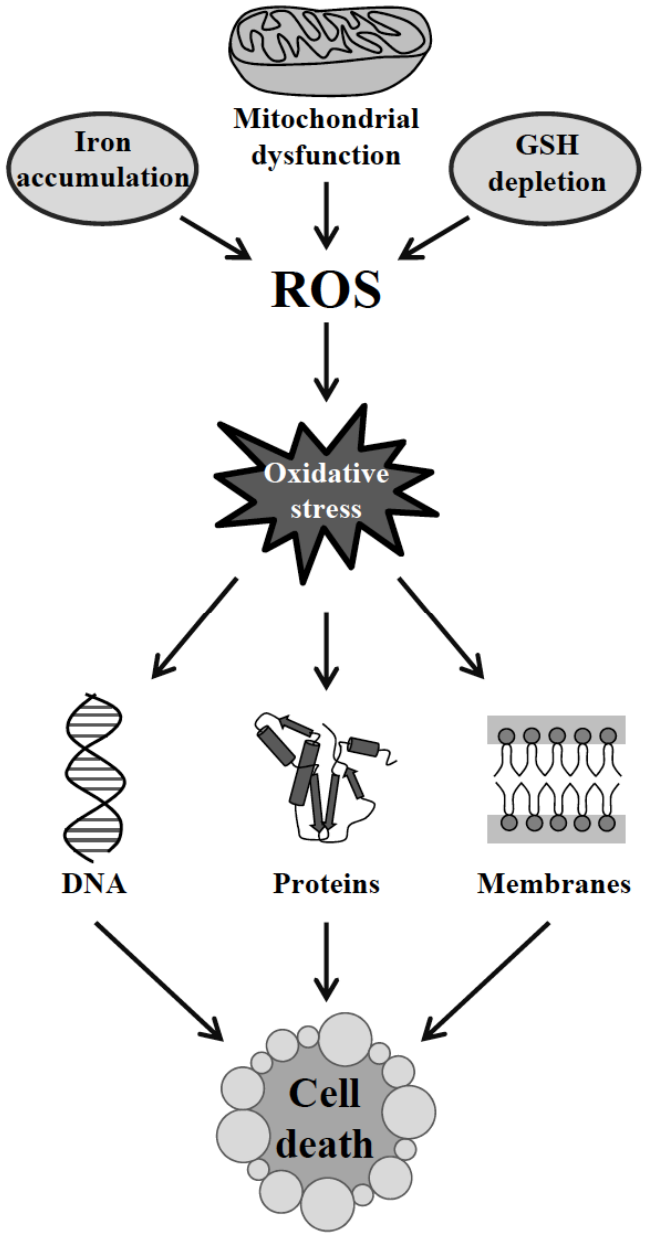

Fig. (1). Interrelationship between mitochondria and oxidative stress in PD progression. Mitochondria are the main source of ROS inside cells. *Post-mortem* analyses indicated complex I deficiency in the SN of patients, as well as iron accumulation and GSH deficiency. ROS are highly reactive molecules that can interact with several cellular components such as lipids, proteins and nucleic acids. Accordingly, lipid peroxidation, protein carbonylation and the presence of 8-hydroxyguanosine were observed in DAergic neurons of PD patients.

the following sections we will discuss some results obtained with the use of several antioxidants, whose chemical structure is represented in Fig. **2**.

VITAMIN E

Vitamin E is a lipid-soluble endogenous antioxidant, which acts as a scavenger of several ROS, including hydroxyl and peroxyl radicals, thus inhibiting lipid peroxidation. The results achieved by supplementing vitamin E to animal models of PD were often contradictory [42-45]. Nevertheless, clinical trials were carried out in the same way to evaluate the potential neuroprotective properties of this molecule providing again controversial results. In a first pilot open-labeled trial, a combination of ascorbate and α-tocopherol (a component of vitamin E) was administered to patients with early PD. The results suggested that the administration of these antioxidants may slow the progression of the disease [46]. Furthermore, a large cohort study demonstrated that dietary intake (from food only) of vitamin E diminishes risk of PD among both men and women. However, the same study indicated that the use of vitamin E supplements did not appear to reduce PD risk [47]. In contrast with these promising data, another study questioned the results on food intake of vitamin E and its efficiency in slow down PD progression. Specifically, a case-control study took into consideration the possible role against PD onset of long-term dietary antioxidant intake. The antioxidant intake, adjusted for age, education, smoking, rural living, and total energy intake, was not associated with a reduced PD risk [48]. Results of two double-blind, randomized controlled trials were also disappointing, as vitamin E did not show beneficial effects in PD. The first multicenter controlled clinical trial did not reveal any evidence of benefits of α-tocopherol (2000 IU per day) in either improving the clinical features of PD or delaying functional decline [49], while a second one demonstrated that α-tocopherol did not affect the duration of life in the early PD patients examined [50].

COENZYME Q_{10} (COQ_{10})

CoQ_{10} is a constituent of the mitochondrial electron transport chain and participates in ATP production. Three redox states of CoQ_{10} are known: a fully oxidized, often called ubiquinone, a semiquinone, and a fully reduced form, also called ubiquitol. The ability of CoQ_{10} to exist in a completely reduced or oxidized form enables it to work in the electron transport chain, and, at the same time, to act as an antioxidant. Actually, CoQ_{10} behaves as a free radical scavenger in the inner mitochondrial membrane [51]. Interestingly, levels of CoQ_{10} were found significantly lower in platelet mitochondria from PD patients [52].

Neuroprotective effects of CoQ_{10} have been shown in various *in vivo* models of PD. In mice, CoQ_{10} protected against MPTP-induced loss of DAergic neurons [53]. Short-term oral administration of CoQ_{10} also prevented DAergic cell degeneration after MPTP administration in monkeys [54]. Up to now several clinical trials with CoQ_{10} have been carried out on PD patients. In a first open label phase-I pilot study performed on 15 PD patients CoQ_{10} at doses of 400, 600 and 800 mg/day for 1 month was well tolerated and its plasma levels significantly increased in a dose-dependent manner. Nevertheless no significant benefit in Unified Parkinson's Disease Rating Scale (UPDRS) score was observed [55]. More recently a placebo-controlled, double-blind and dosage-ranging phase II clinical trial of CoQ_{10}, called QE2, has been conducted in 80 early PD patients by administering placebo, 300, 600 or 1200 mg/day of CoQ_{10}

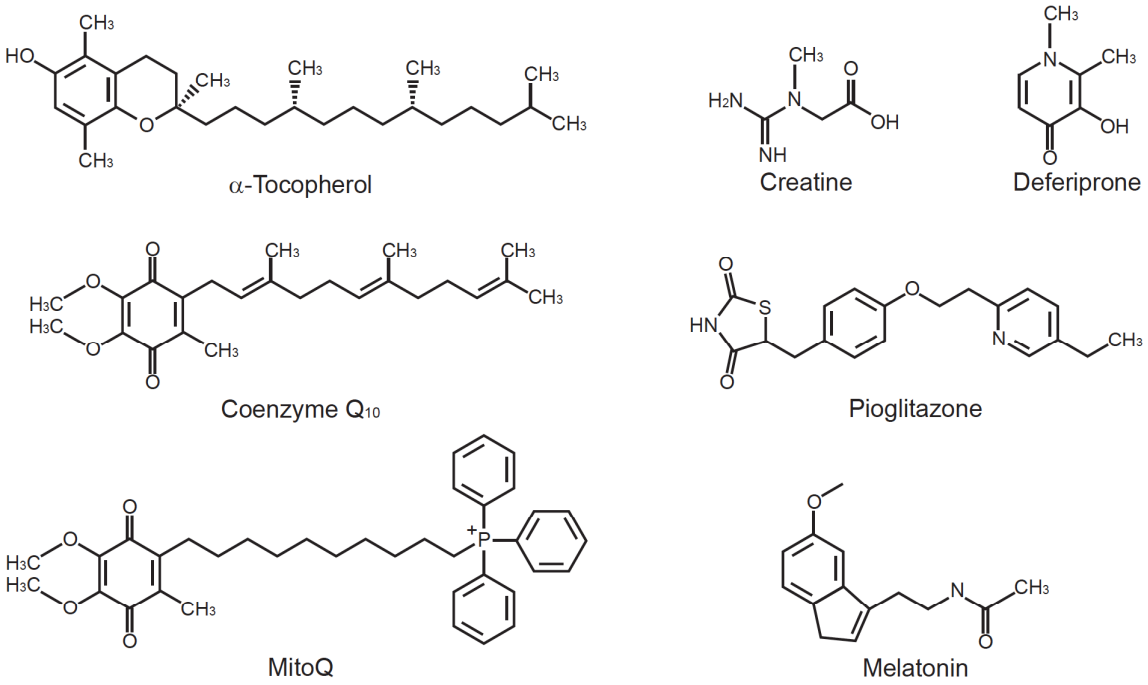

Fig. (2). Chemical structure of the antioxidant molecules described in the present paper.

for 16 months. In PD subjects who received CoQ_{10} a statistically significant dose-dependent reduction in UPDRS score was observed [56]. Two additional clinical trials contributed to raise the hope of a therapeutic use of CoQ_{10}. First, in a placebo controlled, double-blind trial conducted on 28 PD patients, CoQ_{10} supplementation (360 mg/day) for 4 weeks significantly improved the UPDRS score and the performance on color visual testing, with no benefit on motor symptoms [57]. Second, in another open pilot study, 12 patients with idiopathic PD were treated with 1000 mg/day of CoQ_{10} for a period of 3 months followed by a period of 3 months at 1500 mg/day. At the end of the study, although most motor variables tested did not improve significantly with CoQ_{10} therapy, the sum score showed a statistically significant beneficial effect of CoQ_{10} on motor performance, which was mainly due to the 1500-mg dose [58]. However, the therapeutic prospective of CoQ_{10} is bound to decline if one considers the results of a large clinical trial that appeared very recently [59]. QE3 a clinical trial that followed the QE2 study, was designed to evaluate whether high dosages of CoQ_{10} could slow functional decline in early PD. The QE3 study was well powered to detect substantially smaller therapeutic effects of CoQ_{10} than those seen in the QE2 study by recruiting a larger number of patients (600 participants), but overall the QE3 protocol closely matched the QE2 protocol. The QE3 study showed no evidence of a benefit from high dosages of CoQ_{10}, and it failed to confirm the results of the QE2 study. Moreover, the adverse trend in the primary outcome measure crossed the futility threshold, leading to the termination of the study [59].

MITOQUINONE (MitoQ)

The failure of CoQ_{10} as a therapeutic agent against PD may be rationalized by the scarce ability of the molecule to permeate into the brain at a sufficient dose to improve mitochondrial and neuronal function, even at a relatively early clinical stage of PD [60, 61]. To enhance its therapeutic potential, a mitochondria-targeted CoQ_{10} analog, called MitoQ, has been designed [62]. MitoQ is formed by covalent binding of the lipophilic triphenylphosphonium (TPP) cation and the ubiquinone, the active antioxidant moiety of CoQ_{10} [63]. Due to the TPP moiety, MitoQ is able to easily cross all biological membranes and the blood-brain barrier. Furthermore, driven by its positive charge and by the large membrane potential, it accumulates several-hundred fold within mitochondria [62, 63]. MitoQ resulted protective in many animal models not related to PD [64-66]. To test whether it could act as a disease-modifying agent, a clinical trial was conducted for 1 year on 130 participants with a daily dose of 40 or 80 mg. While the study demonstrated that MitoQ can be safely administered to patients for a year, it did not reduce the progression of the disease [60]. A potential explanation that has been suggested for the absence of clinical benefits observed after MitoQ intake is that, by the time PD is diagnosed, approximately 50% of dopaminergic neurons and 80% of striatal dopamine levels have already been lost [67] and the fate of the surviving neurons is already decided so that neuroprotection cannot avoid their degeneration.

CREATINE

Creatine is a nitrogenous guanidine molecule that occurs naturally in vertebrates and helps to supply energy to muscle and nerve cells. Strong evidence suggests that creatine also possesses antioxidant properties; it can reduce mitochondrial dysfunction and, in general, it shows neuroprotective properties in *in vivo* models of several neurodegenerative disorders, among which PD (see [68] for a review). For example, in the MPTP mouse model of PD, creatine

supplementation decreased dopaminergic neuron degeneration and dopamine depletion [69]. In addition, in rodent models of PD, the combination of creatine with CoQ_{10} generated additive neuroprotective effects [70]. Different clinical trials have been performed to evaluate the therapeutic potentiality of creatine in PD. A pilot 2-year placebo-controlled randomized clinical trial based on 60 PD patients found that creatine improved patient mood (a non-motor symptom of PD), but had no effect on the UPDRS scores [71]. In a parallel randomized, double-blind, phase II futility clinical study conducted on 200 patients who were within 5 years of a PD diagnosis, 10 g daily creatine administration showed reduced UPDRS scores and was not rejected as futile [72]. In addition, another double-blind study carried out on a small number of PD patients (20 persons) demonstrated that creatine supplementation (20 g/day) can enhance the benefits of resistance training [73]. In 2007, a phase III clinical trial was announced by the National Institute of Neurological Disorders and Stroke [74]. More than 1700 early-stage PD patients were considered in this large long-term study, which involved 51 medical centers in the United States and Canada. Creatine was administered with a dose of 10 g/day. Regrettably, the study was stopped early for futility based on results of a planned interim analysis of participants enrolled at least 5 years prior to the date of the analysis (n=955). Overall, the results indicate that the intake of creatine for at least 5 years, do not produce clinical benefits. In conclusion, these findings do not support the use of creatine in patients with PD [75].

IRON CHELATORS

The accumulation of iron found in the SN of PD patients has been the rationale for addressing metal depletion as a novel therapy concept. As a consequence, numerous iron chelators have been designed and successfully used in preclinical studies of PD (see [76] for a review). One of the most studied molecules, desferrioxamine (DFO) was firstly described already in the 60' [77]. In a chronic iron-loaded mice model, the effects of DFO on striatal iron content, GSSG/GSH ratio, •OH and dopamine levels were measured after treatment with MPTP. The results demonstrated that DFO inhibited the iron accumulation and thus hampered the increase in GSSG, GSSG/GSH ratios, hydroxyl radical and lipid peroxidation levels [44]. As one of the pathways through which 6-OHDA induces dopaminergic neuron degeneration relies on the metal-catalyzed free radical formation, the effects of DFO were also investigated on a 6-OHDA-based rat model. Specifically, intraventricular injections of DFO were able to partially prevent the decrease in striatal dopamine levels induced by 6-OHDA [78] and to restore almost normal behavioral responses, such as spontaneous movements in a novel environment and rearing, which resulted altered after 6-OHDA administration [79]. The main concern in relation to a possible therapeutic use of DFO derives from both its hydrophilic nature and its large molecular size, which restrict its gastrointestinal absorption and prevent it from crossing the blood brain barrier [80]. To overcome this issue, new non-toxic, hydrophobic, blood brain barrier-permeative iron-chelating molecules were developed [81]. Among them, the iron chelator VK28 has been successfully used in 6-OHDA-based model of PD [82]. These studies suggest that iron chelation could represent a valuable therapeutic approach against PD. Accordingly, a pilot, double-blind, delayed-start placebo-controlled randomized clinical study was recently set up to assess the safety and the efficacy of the iron chelator deferiprone (DFP) [83]. DFP seems to target regional iron accumulation, without significantly altering normal essential functions that rely on the physiological presence of iron. In the trial, 40 patients with early-stage PD randomly received either DFP for 12 months (the early-start, ES group) or placebo for 6 months followed by DFP for the following 6 months (the delayed-start, DS group). With the exclusion of three cases, no relevant side effects were reported throughout the trial. After DFP treatment, a significant decrease of the magnetic resonance signal associated to the iron in the SN of both ES and DS group was observed. Notably, patients in the ES group showed a significantly higher motor performance at both 6 and 12 months in comparison to patients in the DS group. These latter ones showed a significant motor performance impairment during the first 6 months and only during the next 6 months of treatment with DFP they showed a relative improvement [83]. The results of this clinical trial indicate that a moderate regimen of iron chelators, that avoids changes in systemic iron levels, could represent a novel therapeutic strategy against PD.

PPARγ COACTIVATOR-1α (PGC-1α)

Considering the central role that mitochondria play in cellular ROS production and the existing link between mitochondria dysfunction and PD, an enhancement of the number of mitochondria in neurons has been postulated to be able to compensate for bioenergetic defects observed in PD pathology. In this frame, the PGC-1α protein has emerged as a master regulator of mitochondrial biogenesis and respiration [84, 85] and for this reason it has been considered to be a potential therapeutic target for early intervention. Interestingly, PGC-1α has also been shown to take part to an homeostatic cycle that controls ROS accumulation [86]. Indeed, the induction of many antioxidant enzymes by an oxidative stressor involves PGC-1α, which, in turn, is powerfully induced by ROS. Importantly, the induction of antioxidant proteins by PGC-1α is not limited to mitochondria, but it also includes enzymes which are substantially found in the cytosol or peroxisomes, such as glutathione peroxidase 1, catalase, and SOD1 [86].

A genome-wide meta-analysis revealed that many genes controlling cellular bioenergetics, expressed under the control of PGC-1α, were underexpressed in PD patients [87]. Accordingly, while PGC-1α knockout mice resulted more susceptible to MPTP-induced neurodegeneration [86], transgenic mice overexpressing PGC-1α in dopaminergic neurons were more resistant against MPTP-induced cell loss [88]. PGC-1α expression is activated by specific compounds [89] and a large body of evidence suggests that PGC-1a agonists could improve some of the pathological features of PD. For example, the PGC-1α activator resveratrol, a natural polyphenolic compound found in a wide variety of plants, has been described to induce the expression of genes involved in mitochondrial biogenesis, oxidative phosphorylation and endogenous antioxidant defense [90-92]. Although the

effects of resveratrol in PD are uncertain, it seems to protect against different cytotoxic neurotoxins such as MPTP [88, 93-95] and 6-OHDA [96-98]. Another PGC-1α agonist, pioglitazone, has been demonstrated to protect against bradykinesia, depressive-like behavior, learning and memory impairment, and dopaminergic neuron loss in a MPTP-based rat model of PD [99]. Furthermore, pioglitazone has been shown to significantly reverse the reduction of locomotor activity in rats treated with rotenone [100]. It also significantly reversed the reduced striatal dopamine level [100]. Pioglitazone is a drug already used in the treatment of diabetes and considering its already tested safety, it could easily be the object of clinical trials to test its efficacy against PD progression. Actually, a trial of pioglitazone in early Parkinson's disease has been completed and results should appear in a short time (ClinicalTrials.gov identifier NCT01280123).

MELATONIN

While most of the clinical trials so far performed with antioxidants did not reach the expected results, with some exception, some new drugs have been described possessing very interesting properties that might be exploited in the context of PD therapy. Among them melatonin is probably the most promising molecule.

In mammals, melatonin is produced in the pineal gland, from where it is directly released into the cerebrospinal fluid of the brain's third ventricle. Then melatonin readily diffuses into the surrounding neural tissue [101]. Actually, because of its amphiphilicity, melatonin readily passes across all morphophysiological barriers (such as the blood-brain barrier) and diffuses to all cells compartment or body fluid. Interestingly, a decreased expression of melatonin receptors has been observed in the human amygdala and SN of patients with PD in comparison to normal subjects [102]. Melatonin has been described to possess relevant antioxidant properties, *in vivo*. In several MPTP-based mouse models of PD, melatonin administration was found to prevent neuronal degeneration in the nigrostriatal pathway [103] by counteracting MPTP-induced oxidative and nitrative stress, [104-106]. The neuroprotective action of melatonin has been also demonstrated in a 6-OHDA animal model of PD [107]. In addition, rats and mice treated with paraquat (or paraquat and maneb), known to promote free radicals production, showed a significant reduction in the oxidative damage when concomitantly treated with melatonin [108, 109]. Finally, the administration of melatonin attenuated the rotenone-induced GSH depletion and increased the activity of the antioxidant enzymes superoxide dismutase and catalase in the nigrostriatal pathway [110]. However, it is worth mentioning that contradictory results have been also reported. For instance, two studies indicated that the administration of melatonin was ineffective, in mice, in protecting nigral dopaminergic neurons from MPTP-induced toxicity [111, 112].

Although at high concentration a direct role of melatonin in radical scavenging has been shown under numerous experimental conditions, its relevance at physiological levels should be questioned for stoichiometry reasons. Nonetheless, its antioxidant activity is not limited to free radicals scavenging, but also involves other indirect mechanisms, including the stimulation of the expression of several antioxidant enzymes and the down-regulation of pro-oxidant enzymes. For example, melatonin has been indicated to induce the expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme of GSH synthesis [113], and of both glutathione reductase and glutathione peroxidase, which are involved in the recycling of GSH to maintain a high GSH/GSSG ratio [114]. It has also be shown that melatonin administration augmented the mRNA levels of both SOD1, SOD2 and catalase while it significantly decreases the mRNA of nitric oxide synthases [115, 116].

Because of its amphiphilic feature, differently from other free radical scavengers that are either hydrophilic or lipophilic, melatonin can limit oxidative damage in both the lipid and aqueous phases of cells. In fact, the neuroprotective activity of melatonin could be also related to its ability to accumulate at the mitochondrial level due to its lipophilicity. Consistent with this hypothesis, a several studies have indicated that melatonin participates in regulating mitochondrial homeostasis. Safeguarding of respiratory electron flux, reduction of oxidant formation by lowering electron leakage and inhibition of the opening of the mitochondrial permeability transition pore are among the most important effects of melatonin in mitochondria. (see [117] for a review).

In spite of the numerous studies carried out on animal models, only a few clinical trials have been performed to assess therapeutic potential of melatonin in PD patients and they were mostly focused on sleep disorders associated to PD pathology. In a multi-site double-blind placebo-controlled cross-over trial based on 40 PD patients melatonin was administered during a 2 week treatment, in doses ranging from 5 to 50 mg/day. Even though the treatment with the highest melatonin concentration significantly increased actigraphically measured total sleep time, the authors suggested that the small improvement observed may not be clinically significant. However, they also found a significant improvement in subjective sleep disturbance and suggested that these modest effects may be clinically relevant in this patient population. The study also found that 50 mg/day of melatonin were well tolerated [118]. In another randomized, double blind, placebo-controlled study, 18 patients were treated with 3 mg of melatonin, or placebo, for 4 weeks. Melatonin was found to significantly improve subjective quality of sleep, but no benefit was observed in motor performance [119]. A double-blind, placebo-controlled, crossover study performed on 22 patients suffering from tardive dyskinesia, a dysfunction where oxidative stress-induced toxicity in the nigrostriatal pathway seems to be implicated, showed the efficacy of 10 mg/day of melatonin administered for 6 weeks [120]. Two considerations can be emerging from the clinical trials mentioned above. First, melatonin is safe and well-tolerated in humans. In this respect, in a group of 31 patients with amyotrophic lateral sclerosis, chronic high-dose (300 mg/day) rectal melatonin was well tolerated during an observation period of up to 2 years [121]. Second, the small size of the clinical trials conducted until now does not allow to achieve any

conclusion on the therapeutic potential of melatonin, pointing out the need to design and perform a large scale study. It is worth mentioning here that this latter aspect is critical, because very often initially reported significant effects were not reproduced in large scale trials, so that high skepticism toward the therapeutic use of melatonin in PD would be retained.

CONCLUSION

From the data discussed in the present review, it appears that the effects described in clinical trials with antioxidants are often modest and many antioxidants, such as vitamin E, creatine, coenzyme Q_{10} and mitoquinone, which were effective in animal models of PD, did not show significant effects on the evolution of the disease in humans.

Several issues have to be considered in the attempt to rationalize these substantially negative results. First, even though a multiplicity of animal models exists, none of them fully recapitulate the clinical and behavioral phenotypes associated to PD. The MPTP-based model appears to produce an irreversible and severe parkinsonian syndrome, characterized by many of the cardinal features of PD, and it has been the most used in pre-clinical studies. Nevertheless, it is far from being a perfect model. For example, Lewy bodies, which represent one of the hallmarks of the disease, have not been convincingly observed in MPTP-induced models [40]. Moreover, all regimens of MPTP intoxication, even when attained through a number of low dose injections, induce a unique or recurrent acute insult, very different from the clinical progression of PD [40].

Another potential explanation, recently suggested [122] is that antioxidants are often uniformly dispersed throughout the entire body or cell, while the oxidative damage may be restricted to specific brain regions and cell types, such as dopaminergic neurons in the SN, or organelles, such as mitochondria. Therefore, the local concentration of antioxidants may be inadequate to cope with specific hotspots of oxidative damage. This is the reason why the therapeutic potential of antioxidant molecules specifically directed inside mitochondria was tested, with great expectations, but, once again, without positive results. In this case it has been suggested that mitochondrial oxidative damage could not be the primary cause of neurodegeneration, being most probably a consequence of other pathological processes [60].

Another issue related to the massive oxygen consumption of neuronal cells and to the corresponding huge amount of anti-oxidant systems, is the very pronounced generation of reducing equivalents in neurons when compared to other sites in the body. This implies that what could be a pharmacologically induced significant increase of the anti-oxidant activity in the body might result in a trivial enhancement in the brain. Moreover, an open issue related to the previous one, is the lack of a demonstrated "target engagement" (an effect that indicates that the tested molecule is having the expected pharmacology for its target). This derives both for the lack of a unique or robust target of ROS and, more so, for the lack of an accepted ROS-based neurodegenerative mechanism to monitor in order to quantify the effectiveness of the tested molecule.

An additional convincing hypothesis implies that most neurons have already been lost by the time PD is diagnosed and likely the fate of the remaining neurons is already determined and neuroprotection cannot prevent their death [67]. Another potential limitation to the development of antioxidants relies on the fact that, in spite of the deleterious effects of free radicals on cellular components, increasing evidence supports that they are also involved in maintaining homeostasis and cell signaling [123]. Actually, oxidants can act as an additional class of small molecules that function as cellular messenger regulating cell proliferation, cell death (either apoptosis or necrosis), gene expression and metabolic response [123]. Additionally, it has been observed that ROS signaling is required for the normal regulation of autophagy [124]. Consequently, the indiscriminate use of an antioxidant could do more harm than good by disrupting essential signaling processes. Accordingly, it has been demonstrated that many antioxidants inhibit basal and induced levels of autophagy [125], a process that seems to be protective in PD [126].

In spite of the aforementioned aspects that could explain the modest results attained by the use of antioxidants against PD, it is worth mentioning that some molecules showed very promising results. This is the case of the iron chelator DFP. This suggests that molecules directed toward a specific pathway may offer a better protection in comparison to non selective systemic antioxidant molecules. Moreover, the results of some clinical trials involving antioxidants, such as pioglitazone, are still waited before a conclusion could be made. Finally, other molecules, such as melatonin, showed encouraging properties and, in light of their already demonstrated safety, they should be considered in clinical trials.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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