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REVIEW ARTICLE

Melatonin as a natural ally against oxidative stress: a physicochemical examination

Abstract: Oxidative stress has been proven to be related to the onset of a large number of health disorders. This chemical stress is triggered by an excess of free radicals, which are generated in cells because of a wide variety of exogenous and endogenous processes. Therefore, finding strategies for efficiently detoxifying free radicals has become a subject of a great interest, from both an academic and practical points of view. Melatonin is a ubiquitous and versatile molecule that exhibits most of the desirable characteristics of a good antioxidant. The amount of data gathered so far regarding the protective action of melatonin against oxidative stress is overwhelming. However, rather little is known concerning the chemical mechanisms involved in this activity. This review summarizes the current progress in understanding the physicochemical insights related to the free radical–scavenging activity of melatonin. Thus far, there is a general agreement that electron transfer and hydrogen transfer are the main mechanisms involved in the reactions of melatonin with free radicals. However, the relative importance of other mechanisms is also analyzed. The chemical nature of the reacting free radical also has an influence on the relative importance of the different mechanisms of these reactions. Therefore, this point has also been discussed in detail in the current review. Based on the available data, it is concluded that melatonin efficiently protects against oxidative stress by a variety of mechanisms. Moreover, it is proposed that even though it has been referred to as the chemical expression of darkness, perhaps it could also be referred to as the chemical light of health.

Annia Galano[1], Dun Xian Tan[2] and Russel J. Reiter[2]

[1]Departamento de Química. Universidad Autónoma Metropolitana-Iztapalapa. Col. Vicentina. Iztapalapa. México D. F. México; [2]Department of Cellular and Structural Biology, UT health Science Center, San Antonio, TX, USA

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Address reprint requests to Annia Galano, Departamento de Química. Universidad Autónoma Metropolitana-Iztapalapa. San Rafael Atlixco 186, Col. Vicentina. Iztapalapa. 09340 México D. F. México.
E-mail: agal@xanum.uam.mx

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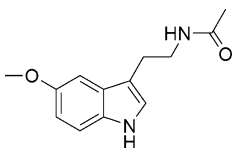
Introduction

Melatonin (N-acetyl-5-methoxytryptamine, Scheme 1) was first isolated in 1956 from the pineal gland and its structure was identified shortly after [1]. Even though melatonin is mainly produced by the pineal gland, it is also found in several extra-pineal organs including the retina, cerebellum, skin, ovary, liver, pancreas, kidneys, etc. [2–7]. The regulatory role of melatonin in circadian and seasonal rhythms is well established [8–10], and it is known as the chemical expression of darkness [11]. However, there is increasing evidence showing its involvement in many other functions [12]. For example, it is now accepted that it has immune-enhancing [13] and anti-inflammatory [14, 15] properties, that it exerts homeostatic roles in the mitochondrion [16–18], and that it inhibits cancer progression [19–21].



Scheme 1. Xxxxxx.

Accordingly, it is known that melatonin is a versatile and ubiquitous molecule, and it is not surprising that it has drawn the scientific community's attention since its discovery. In fact, according to the Scopus database [22], melatonin as a topic appears in more than 22,800 publications in the last 50 years (Fig. 1). Moreover, there is an increasing trend in the number of melatonin-related publications in recent decades.

So far, a very important function of melatonin has not been mentioned, i.e., its antioxidant, or free radical–scavenging activity [23–25]. This was first recognized almost 20 years ago [26], and it is the focus of this review. About 3700 scientific investigations on this particular and very important function of melatonin have been published since 1993, and the number per year is still increasing (Fig. 1). This interest is justified as there is still much to investigate and understand regarding the mechanisms of action of melatonin when fighting free radicals.

Free radicals and oxidative stress

From a chemical point of view, free radicals are species containing one or more unpaired electrons. This characteristic makes them highly reactive species that trigger chain

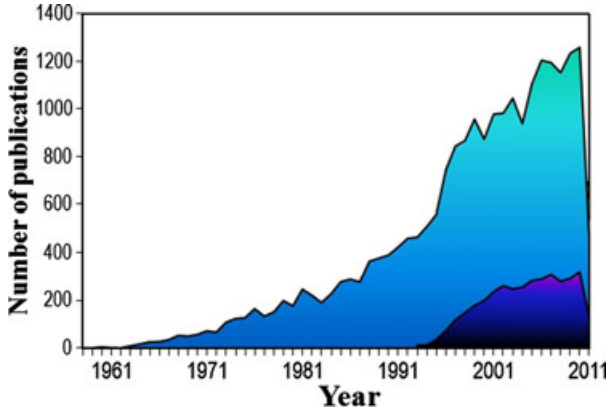


Fig. 1. Number of papers with *melatonin* as topic, according to Scopus database (Consulted June 1st, 2011). The darker region corresponds to the subset dealing with the antioxidant or free radical–scavenging activity of melatonin.

reaction mechanisms, which may involve molecules of high biological importance such as DNA and proteins.

Most radicals that occur in vivo are, or arise from, reactive oxygen species (ROS) or reactive nitrogen species (RNS). ROS include oxygen-based free radicals, including the superoxide radical anion ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), and hydroperoxyl ($HOO^{\bullet}$). RNS include peroxynitrite (ONOO−), nitric oxide ($NO^{\bullet}$), and nitrogen dioxide ($NO_2^{\bullet}$). ROS and RNS can be either harmful or beneficial to living systems [27], depending on their concentration. In healthy organisms, there is a delicate balance between the production and the removal of free radicals, which guarantees that they remain in low/moderate concentrations. Under such conditions, free radicals have beneficial effects. They are necessary for the maturation process of cellular structures [28], and induce mitogenic responses [29–32]. Free radicals are also involved in the defense system; phagocytes release them to destroy pathogenic microbes [33, 34]. They also participate in cellular signaling systems [29, 31, 32, 35] and seem to play a role in the regulation of insulin receptor kinase activity [33] and in the apoptosis of defective cells [36, 37]. In summary, free radicals at low/moderate levels are essential for optimal human health.

In contrast, at high concentrations, free radicals can be harmful to living organisms. Such high concentrations are caused by an imbalance between the production and the consumption of free radicals, which is commonly referred to as oxidative stress. More than 50 years ago, Gerschman and coworkers [38] discovered that free radicals are the toxic intermediates associated with oxygen poisoning and ionizing radiation. Surprisingly, despite its implications, not enough attention was paid to this discovery at the time. Currently, the role of free radicals in the development of a large variety of chronic and degenerative diseases is well documented. It has been established that oxidative damage to DNA is responsible for cancer development [27, 31, 32, 35, 39–43]. Oxidative stress is involved in several neurological disorders such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, memory loss, and depression [44–51]. There are also evidence supporting the role of oxidative stress in several cardiovascular diseases including atherosclerosis, cardiac hypertrophy, ischemia, hypertension, cardiomyopathy, and congestive heart failure [33, 52–60]. In addition, oxidative stress has been associated with ocular [61, 62], renal [33, 63], and pulmonary [64–67] diseases; rheumatoid arthritis [68], fetal growth restriction, and pre-eclampsia in prenatal medicine [69–73]. Based on such overwhelming data, it is evident that an excess of free radicals can be dangerous to human health.

These species are not only produced by endogenous sources but also by exogenous ones. Endogenous free radicals are generated from immune cell activation, inflammation, mental or physical stress, excessive exercise, ischemia, infection, cancer, and aging. Exogenous causes of free radicals come from environmental pollution, cigarette smoke, alcohol, heavy or transition metals, certain drugs, and radiation (Fig. 2) [28–35, 43, 74–79]. Therefore, efficiently detoxifying free radicals to keep them at healthy low/moderate concentrations is a difficult task in the modern world.

Chemical nature and reactivity of free radicals

The hydroxyl radical can be formed intracellularly by the Fenton-type reaction, by Haber-Weiss recombination, or by other radicals created from enzyme reactions [80–84]. It can also be produced by ultraviolet and ionizing radiations [85]. $^{\bullet}OH$ is the most electrophilic [86] and reactive of the oxygen-centered radicals, with a half-life of $\sim 10^{-9}$ s [87]. It can react although a wide variety of mechanisms [88] and its reactions with a large variety of chemical compounds take place at, or close to, diffusion-controlled rates [89, 90].It has been estimated that the $^{\bullet}OH$ is responsible for 60–70% of the tissue damage caused by ionizing radiations [91]. $^{\bullet}OH$ are so reactive that they react immediately at the site of their formation with little selectivity toward the various possible sites of attack [92]. Moreover, it has been proposed that it can react with almost any molecule in the vicinity of where it is generated [93] and to damage DNA [94–96]. Therefore, this radical is probably the most dangerous one, but the fact that a particular substance readily reacts with

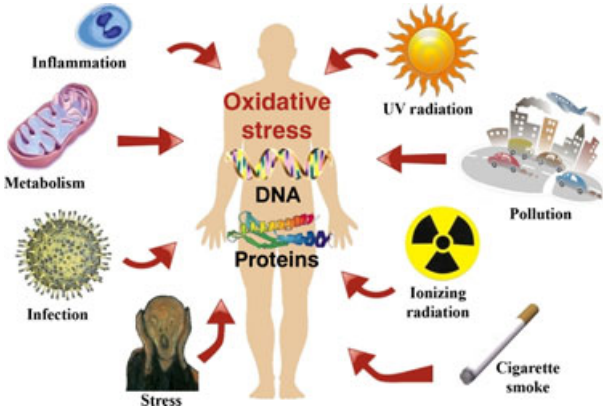


Fig. 2. Some sources of free radicals.

the $^{\bullet}OH$ does not necessarily mean that it is a good antioxidant.

Compared with $^{\bullet}OH$, peroxyl radicals are less-reactive species capable of diffusing to remote cellular locations [97]. Their half-lives are of the order of seconds [98], and their electrophilicity is significantly lower than that of $^{\bullet}OH$ [86]. However, they can also react with other chemical species through different mechanisms [99].

The simplest of the peroxyl radicals is the hydroperoxyl radical. It is the conjugated acid of the superoxide radical anion, and according to its pKa (4.8) [100], only ~0.3% of any superoxide present in the cytosol of a typical cell is in its protonated form [101]. However, it has also been demonstrated that $HOO^{\bullet}$ initiates fatty acid peroxidation [102]. Moreover, it has been proposed that the biological and biomedical importance of $HOO^{\bullet}$ exceeds by far the attention that it has received and that a more widespread appreciation of its possible role in biological systems would be of considerable benefit to biomedical research [101]. The reactions of $HOO^{\bullet}/O_2^{\bullet-}$ with bioorganic compounds are generally quite slow [90, 100, 103]; for example, unsaturated fatty acids react with $HOO^{\bullet}$ at rate constants $\sim 10^3$/M/s and with $O_2^{\bullet-}$ at rate constants about five orders of magnitude slower [99]. The negative charge of this species has an effect on its reactivity; for example, it has been demonstrated when reacting via electron transfer, while for most radicals the transfer takes place from the antioxidant to the radical, in the case of $O_2^{\bullet-}$ it occurs in the opposite direction [104].

Organic peroxyl radicals are also very important in living organisms. Hydrogen transfer processes from organic compounds or the addition of a radical to a double bond yield carbon-centered radicals, which in the presence of O_2 produce peroxyl radicals [99]. In general, they are less reactive than $HOO^{\bullet}$ when R is an alkyl or an alkenyl group [105, 106]. However, if R is a more electron-accepting group, for example R = $-CCl_3$, the ROO radicals significantly increase their reactivity toward organic molecules [105]. In particular, the rate constants for the electron-transfer reactions of $ROO^{\bullet}$ strongly depend on the chemical nature of R, increasing to a great extent as the electron-withdrawing character of the substituents increases [107]. As this particular mechanism involves the formation of charged species, it is also strongly influenced by the solvent. It has also been reported that the reactions of halogenated peroxyl radicals with alkenes are much faster than the corresponding reactions of alkylperoxyl radicals [108–110].

Alkoxyl radicals are formed from the reduction of peroxides and are significantly more reactive than ROO radicals, provided that R is the same in both species [106, 111–114]. On the other hand, they are less reactive than $^{\bullet}OH$ [106, 111–114]. This intermediate activity makes them ideal candidates to test the efficiency of antioxidants, as well as the relative site reactivity of any species reacting with oxygenated free radicals [106, 115, 116].

Regarding RNS, the chemical reactivity of $NO^{\bullet}$ is rather low, and therefore its direct toxicity is actually minor [117–119]. However, it reacts with $O_2^{\bullet-}$ yielding peroxynitrite [120], which is a potent oxidant. $ONOO^-$ itself is a very damaging species able to react with proteins, lipids, and DNA [121–123]. Therefore, the reaction between two rather innocuous free radicals produces a much more reactive one [124, 125]. Moreover, this reaction has one of the largest rate constants known among the NO• reactions [126–129], and is believed to occur in vivo [124, 130]. Nitrogen dioxide is a moderate oxidant, and its reactivity is somewhere between those of $NO^{\bullet}$ and $ONOO^-$ [99]. NO_2 reacts with organic molecules at rates that range from $\sim 10^4$ to 10^6/M/s, depending on the pH [131, 132].

Desirable properties of free radical scavengers

A series of properties that would characterize an ideal free-radical scavenger has been identified [93]. Owing to their high reactivity, most free radicals have short half-lives in biological systems. This means that they would react with molecules in the vicinity of their place of formation. Therefore, an efficient antioxidant should be ubiquitous and should also be present in adequate amounts in cells. As mentioned earlier, melatonin is widespread within cells. Its concentrations in human serum and cerebrospinal fluid varies widely [133] with time of day of collection. Likewise, in the aqueous humor of the eye, the concentration changes over the light: dark cycle [134]. Unfortunately, rather little is known concerning the intracellular concentrations of melatonin. Melatonin's concentration in plasma varies during the day and from childhood to adulthood. In adults, melatonin levels in the blood are usually below 10 pg/mL at night [135, 136]. Melatonin concentration has also been estimated to be as high as 112 pg/mL in the ovarian follicular fluid [137], 5–15 pg/mL in saliva [138], and ~100 (day) to ~400 (night) pg/mg in the pineal gland [139]. In hepato-gastrointestinal tissues, its concentration has been reported to be 80, 176, 163-214, and 214 pg/100 mg wet weight in liver, duodenum, colon, and rectum, respectively [140]. While these are measurements from specific reports, the values estimated by others can vary widely [141, 142]. The point is made, however, that melatonin has a very wide distribution.

A good antioxidant should be versatile, i.e., it should easily react with a wide variety of free radicals. As mentioned before, the free radicals present in living organism are of diverse nature, and their reactivity varies accordingly. Melatonin has been proven to scavenge hydroxyl [26, 143–151], alkoxyl [152, 153], and peroxyl [150, 152, 154–157] radicals, albeit its scavenging activity against $ROO^{\bullet}$ is highly influenced by the chemical nature of R [105]. Melatonin has also been found to scavenge NO [129, 158, 159] and $^1O_2$ [160, 161]. Therefore, it can be stated that melatonin is a broad-spectrum antioxidant [162].

Another desirable property for a good antioxidant is the ability to cross physiologic barriers and to be quickly transported into the cells [149, 158, 163–165]. Thus, it must be available to all cells [93]. As melatonin is highly soluble in lipids and partially soluble in water, it has such ability [166–169]. Moreover, its intermediate size is optimum for transportation across cellular membranes [93].

It is also important for an antioxidant to be available. To be accessible when needed, antioxidants should be acquired in the diet, i.e., obtained from exogenous sources; or be produced *in situ*, i.e., by endogenous sources. Melatonin is endogenously produced, and it is ingested in the food as it is widely available in fruits, vegetables, nuts, etc. [170–174].

Hence, melatonin is produced internally and is also ingested in the diet.

Antioxidants undergo regeneration [93]. The reaction between an antioxidant and a free radical yields an oxidized form of the antioxidant. This new species has, by definition, less scavenging activity than the original compound. Therefore, many antioxidants have physiologically reducing mechanisms or its oxidized forms can still efficiently react with new free radicals. There is some evidence that melatonin can be regenerated after radical quenching through different process [175–177]. It can also assist in regenerating other antioxidants including glutathione [178] and is reportedly regenerated by other species such as ascorbate and urate [175]. Moreover, melatonin's metabolites arising from its reaction with ROS still possess free radical–scavenging activities [179–189].

Rose and Bode [93] have also proposed that an ideal antioxidant should be suitable for reabsorption after being filtered by the kidneys. Otherwise, large urinary losses would occur and the half-life will be short (< 1 hr). Only small amounts of melatonin are excreted into urine in its unchanged form [190]. This has two explanations, which are as follows: it is a lipophilic compound that easily diffuses through biological barriers to enter cells, and it is rapidly metabolized, mainly in the liver [191] to 6-sulfatoxymelatonin. This compound is a recognized major urinary metabolite of melatonin [192]. Therefore, the short half-life of melatonin should not be associated with urinary loss but with its rapid uptake into cells. The half-life of melatonin in serum has been estimated to be 41 min [193], 52 min [194], 30–47 min [191], and 32–40 min [195].

An important aspect to consider for evaluating the suitability of a compound as an antioxidant is its toxicity. It should be nontoxic prior to and after the free radical–scavenging process takes place. Arendt [196] found that the only significant short-term side effect after oral ingestion of less than or equal to 5 mg of melatonin by normal healthy adults is sleepiness. Numerous in vivo studies on animals involving massive doses of melatonin have shown that acute and chronic toxicity of melatonin is extremely low [197–200]. Moreover, oral doses (up to 1 g daily) taken by human volunteers resulted in no negative side effects [201]. There is widespread agreement that melatonin has minimal toxicity over a very wide dose range.

After donating an electron to $^{\bullet}OH$, melatonin becomes a free radical itself, the indoyl radical cation [26, 105, 202]. However, its reactivity is very low, and therefore, it is not toxic to cells [203]. Melatonin in the process of scavenging two $^{\bullet}OH$ radicals forms cyclic-3-hydroxymelatonin [204]. In fact, this compound is considered a foot print molecule, excreted in small amounts in the urine, and evidence of the in vivo scavenging activity of melatonin.

Based on the properties discussed earlier, melatonin exhibits most of the features of an ideal antioxidant.

Melatonin reducing oxidative stress

The role of melatonin regarding inhibition of lipid peroxidation has been well established [205–209]. The amount of data gathered so far regarding the protective action of melatonin against oxidative stress is overwhelming. Moreover, based on the ability of melatonin to scavenge a wide variety of ROS and RNS, it has been hypothesized that its primary function in living organisms is to protect them from oxidative stress [182, 210].

The brain is particularly sensitive to free radical damage. This is because of its high utilization of O_2, its relatively poorly developed antioxidant defense, and the fact that it contains large amounts of easily oxidizable fatty acids [6]. As melatonin is generated in situ in the brain, and is released into the cerebrospinal fluid [141], its antioxidant activity may acquire special relevance to protect against neurodegenerative disorders. There are numerous studies documenting the protective effects of melatonin against oxidative damage–related disorders affecting the brain [211]; for example, it has been demonstrated that melatonin limits ischemia/reperfusion-induced damage in the brain [212–215] and in other organs [216]. This has been related to the melatonin's ability to scavenge the free radicals produced during such events [217, 218].

Experimental models of Alzheimer's disease have been used to evaluate the effect of melatonin, and it has been found that it reduces degenerative changes [219–227]. Moreover, melatonin administration to humans has been shown to significantly slow the progression of this disease [228, 229]. There are also numerous studies showing the neuroprotective effect of melatonin in Parkinson's disease [230]. This effect has been related to the regulatory role of melatonin in the circadian rhythm of dopamine [231] and to the protective action of melatonin against autoxidation of dopamine [232]. Moreover, the efficiency of melatonin for the latter has been described to be higher than those of vitamins E and C, and that of Ldeprenyl [233].

Whereas the central nervous system is extensively destroyed by free radicals, not only is the brain susceptible to oxidative stress, but all organs suffer from these devastating processes. It has been shown that melatonin protects the mucosa of the stomach [234–236], the liver [237], and the heart [238, 239] from the damaging effects of free radicals generated during ischemia–reperfusion.

Mechanism of action

Despite the numerous reports on the antioxidant activity of melatonin and its metabolites, the information on the mechanism, or mechanisms, determining this activity is not yet well defined. Based on what is known of other antioxidants [240–246], it is presumed that the mechanisms are as follows:

$$\text{Radical adduct formation (RAF)}: \quad melatonin + {}^{\bullet}R \rightarrow melatonin - R^{\bullet}$$

$$\text{Hydrogen atom transfer (HAT)}: \quad melatonin + {}^{\bullet}R \rightarrow melatonin(-H)^{\bullet} + HR$$

$$\text{Single electron transfer (SET)}: \quad melatonin + {}^{\bullet}R \rightarrow melatonin^{\bullet+} + R^{-}$$

Additionally, SET can occur rapidly followed by, or simultaneously with, proton transfer, which are known as

sequential electron proton transfer (SEPT) and proton-coupled electron transfer (PCET) mechanisms, respectively:

$$\text{Sequential electron proton transfer (SEPT)}:$$
$$(a)\ MLT + {}^{\bullet}R \rightarrow MLT^{\bullet+} + R^{-} \rightarrow MLT(-H)^{\bullet} + HR$$
$$(b)\ MLT + {}^{\bullet}R \rightarrow MLT(-H^{+})^{-} + R^{\bullet} \rightarrow MLT(-H)^{\bullet} + HR$$
$$\text{Proton} - \text{coupled electron transfer (PCET)}:$$
$$MLT + {}^{\bullet}R \rightarrow MLT(-H)^{\bullet} + HR$$

It should be noted that in the particular case of SEPT, the sequential transfer can take place in two different ways: (*a*) a SET process followed by deprotonation of the formed radical cation, or (*b*) a deprotonation followed by a SET process from the formed anion.

Even though SEPT and PCET yield the same products as HAT, the influence of the solvent and of the nature of the reacting radical on their feasibility is expected to be different. While SET and SEPT are likely to be favored by polar environments that promote solvation of the intermediate ionic species, the PCET mechanism might be also viable in nonpolar media because the transfer of the proton and the electron occurs simultaneously in this case and, therefore, no charged intermediaries are formed.

To elucidate the predominant chemical mechanism of action of any antioxidant is a huge challenge. Such action takes place in a very complex environment, which means that there are large numbers of compounds present and involved in competing chemical reactions. This implies many chemical processes that can occur simultaneously, depending on the relative reactivity of the reacting species. Moreover, because of the chemical nature of the free radicals, a chain reaction mechanism may also be involved. Therefore, the first oxidation step can be rapidly followed by subsequent chemical processes. Also, different radicals can react in different ways, and the polarity of the environment can also affect the relative importance of the diverse mechanisms.

The colossal task of identifying the main mechanism, or mechanisms, of action can be attained both experimentally and theoretically. A good experimental strategy is to perform detailed product analyses. However, this approach also involves a large degree of inference because the processes are usually very fast and composed by several steps. Therefore, the detectable products are frequently those formed after various elementary reactions. Moreover, as mentioned earlier, different mechanisms might lead to the formation of the same products. Computational strategies also present a series of difficulties, mainly related to the frequent necessity of using simplified models and to the availability of strategies to properly include solvent effects. Accordingly, combined experimental–theoretical efforts are probably the best way to address this important part of the antioxidant activity–related investigations.

Applying chemical intuition

Sometimes the available physicochemical data helps narrow the number of possible mechanisms involved in the free radical–scavenging activity of chemical compounds; for example, based on its pKa values, the neutral form of melatonin predominates under physiological conditions [105, 175, 247, 248]. Therefore, any mechanism of action starting with the deprotonation of melatonin can be ruled out. Consequently, the SEPT (b) route is not expected to contribute to the antioxidant activity of this compound.

Based on structural considerations, it has been proposed that because melatonin does not have phenolic hydrogens, it should react more easily by electron transfer than by hydrogen atom transfer [249]. However, not all free radicals have sufficient electron-acceptor character to be involved in such mechanisms. Therefore, the nature of the reacting free radical also has a role in its viability; for example, haloperoxyl radicals have been proposed to be highly electron-deficient and therefore capable of reacting via electron transfer [250]. It should be kept in mind, however, that while such a mechanism is viable in aqueous solution, it is highly improbable in the lipid phase.

Melatonin's metabolite, N-acetyl-N-formyl 5-methoxykynuramine (AFMK), is formed in the rat brain by an enzyme that requires $O_2^{\bullet-}$ as substrate [251], and it has been identified in a system generating $O_2^{\bullet-}$ by means of xanthine oxidase in the presence of iron-EDTA [252], or hemin [253], as catalysts. Based on this evidence, Hardeland et al. [254] proposed that melatonin might trap $O_2^{\bullet-}$. Moreover, they found AFMK among the products from the reaction of melatonin with •OH [254]. This led them to suggest that the $O_2^{\bullet-}$ scavenging activity of melatonin takes place through a mechanism involving a SET process to a strong oxidant yielding the indolyl cation radical, which then reacts with $O_2^{\bullet-}$ to produce AFMK [254]. This intuitive leap was later confirmed spectrophotometrically [152, 255, 256].

Learning from the experiments

SET and HAT have been identified, from experimental data, as the main mechanisms for the reactions of other indole derivatives (tryptophan and N-methylindole) with different free radicals [$N_3^{\bullet}$, $Br_2^{\bullet}$, and $(SCN)_2^{\bullet}$] [249]. Accordingly, based on the structure similarities, these mechanisms are also expected to contribute to the overall free radical–scavenging activity of melatonin. In fact, melatonin was found to scavenge one-electron oxidants ($N_3^{\bullet}$ and $Br_2^{\bullet}$) in aqueous solution. When the reaction is conducted at pH = 7, distinctive absorption bands at 335 and 500 nm appear [175], which resemble the transient spectra of indolyl radical species [247]. At pH = 3, the protonated form of the transient is form [175], which has a strong absorption band at 450 nm [247] in addition to the typical ones [247, 257]. For the reactions of melatonin with $ONOO^{-}$ and alkoxyl radicals, similar spectra were observed [152, 256]. This evidence supports the importance of SET and HAT mechanisms for the antioxidant activity of melatonin.

For the reaction of melatonin with $^{\bullet}OH$, it was observed that the spectra is also coherent with those of the indolyl-like radicals [175, 255]. However, in this case, there was also evidence of the formation of another structure, which was suggested to arise from RAF processes, in particular from $^{\bullet}OH$ additions to the carbon sites in the indole ring [255]. This is not surprising as because of the high reactivity of this radical, it is expected to be less selective and form a

wider variety of products. It is also in agreement with an early study in which, using different techniques for measurements, other products of melatonin + $^{\bullet}OH$ reaction were identified, and related to OH addition at the C3 site of the indole moiety [204].

For the reaction of melatonin with $ONOO^-$, there is evidence for the formation of cyclic 2-hydroxy melatonin, cyclic 3-hydroxymelatonin, and 6-hydroxymelatonin [256, 258]. This led the authors to suggest that the initial step of the melatonin + $ONOO^-$ reaction involves one electron transfer from melatonin to $ONOO^-$ [256] and, alternatively, that nitrated species are intermediates in the oxidation process [258]. However, the 6-hydroxymelatonin is not formed when the reaction takes place in the presence of CO_2, suggesting that its formation involved an activated form of peroxynitrite that can only exist in the absence of bicarbonate [125, 259].

Learning from the theory

The rapid development of the computer capabilities available to scientists in the last few decades has made possible the application of computational chemistry to a wide variety of chemical problems. The size of the systems that can be studied at a reliable level of theory is nowadays large enough and allows securing valuable information on the antioxidant activity of a wide variety of substances. In fact, it has been used to predict the scavenging activity of systems as large as carbon nanotubes [114, 116, 260–263]. Therefore, computational techniques have much to offer to the study of the chemical process related to the oxidative stress.

The first two theoretical studies on the free radical–scavenging activity of melatonin appeared in 1998 [264, 265]. Migliavacca et al. [264] investigated the viability of the SET mechanism. For that purpose, they located and characterized six conformers of the radical cation of melatonin. Their calculated adiabatic ionization energies (IE), which by definition are directly related to the facility for electron donation, were found to be small. Based on this finding, they proposed that the antioxidant properties of melatonin are because of the stability of its radical cation. These authors also investigated the viability of the HAT mechanism from the N-atom of the indole moiety. They found that this channel of reaction is endothermic, and therefore unlikely to occur. They also studied the hepatic metabolite of melatonin, 6-hydroxymelatonin, and proposed that the additional electron-donating group in the aromatic moiety should contribute to a better stabilization of the radical cation. They also proposed that for this metabolite, the HAT mechanism is plausible. Based on these findings, they supported previous experimental results, which had suggested that the antioxidant activity of 6-hydroxymelatonin is higher than that of melatonin itself.

Turjanski et al. [265] studied two channels of the melatonin + $^{\bullet}OH$ reaction: one involving the abstraction of an indolic hydrogen (HAT) and yielding an indolyl neutral radical; and another involving the $^{\bullet}OH$ addition to the indolic moiety (RAF) to form 2-hydroxymelatonin. The computed ΔG values were found to be −30 and −31 kcal/mol, respectively. Based on these thermochemical results, they proposed that melatonin can directly scavenge hydroxyl radicals both in vacuum and in aqueous solution. However, they ruled out the SET mechanism because the ΔG values predicted by the AM1 method for this route were positive: 161 and 8 kcal/mol in vacuum and aqueous solution, respectively. They also modeled different pathways for the conversion of melatonin into its metabolite AFMK (N1-acetyl-N2-formyl-5-methoxykynuramine) and found this process is highly exergonic in both vacuum and aqueous solution. Based on this finding, they proposed that besides its interaction with the endogenous antioxidant defense system [265], melatonin itself may exert direct antioxidant effects. In addition, these authors investigated the structure–antioxidant activity relationship and found that the 5-methoxy and the N-acetyl group of melatonin do not seem to significantly influence its thermodynamic capacity for scavenging •OH.

Velkov et al. [148] performed an investigation on the antioxidant activity of melatonin, using a combined approach that involves both theory and experiments. They used the theoretical approach to calculate several descriptors (first ionization energies, bond dissociation energies, and spin densities) and tested their suitability for evaluating the antioxidant activity. Moreover, they proposed to associate these descriptors with different mechanisms of reaction. The experimental data were found to support the usefulness of the computed scavenging parameters of melatonin.

Stasica et al. [266] studied five RAF channels of the reaction of melatonin with $^{\bullet}OH$, using AM1 semi-empirical calculations, and proposed that all of them should occur easily and with low selectivity. Based on the particularly reactive nature of $^{\bullet}OH$, they call attention to the possibility that other reaction paths could also be feasible.

In a recent study, all the reaction paths involved in the interaction of melatonin with hydroxyl and a series of peroxyl radicals have been studied using the density functional theory (DFT) [105]. In this research, five different mechanisms of reaction were considered: RAF, HAT, SET, SEPT, and PCET. In addition, as melatonin is highly soluble in lipids and partially soluble in water, the influence of the environment's polarity was also analyzed. Melatonin was found to react with hydroxyl radicals in a diffusion-limited way, regardless of the polarity of the environment, which is in line with previous findings indicating that this molecule is an excellent $^{\bullet}OH$ scavenger. Melatonin was also predicted to be a very good $^{\bullet}OOCCl_3$ scavenger, both in aqueous and in lipid media. However, it was also found that melatonin is rather ineffective for scavenging less-reactive peroxyl radicals, such as alkenyl peroxyl radicals and $^{\bullet}OOH$. Therefore, it was concluded that the protective effect of melatonin against lipid peroxidation does not take place by directly trapping peroxyl radicals, but rather by scavenging more reactive radicals, such as $^{\bullet}OH$, which initiate the degradation process.

An estimation of the relative importance of the different mechanisms and reaction paths was also performed in the same work [105]. Different criteria were used to differentiate between HAT and PCET mechanisms. Based on these, it was proposed that the H transfers from the N2 atom in

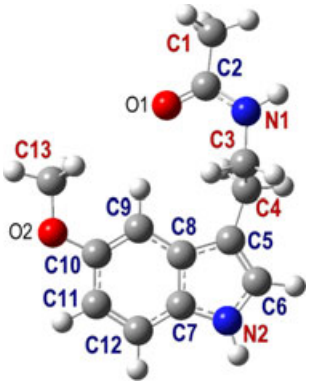


Fig. 3. Site numbers: blue labels represent radical adduct formation sites of reaction, and red labels, HAT or proton-coupled electron transfer sites of reaction.

melatonin, to $^{\bullet}OH$ and $^{\bullet}OOCCl_3$ radicals take place by PCET, while H atoms in the alkyl sites are transferred by HAT (the numbers of the reaction sites are shown in Fig. 3). In aqueous solution, SET was predicted to be the most important mechanism for the reaction of melatonin with $^{\bullet}OH$, accounting for the 44.1% of the overall reactivity. However, in aqueous solution, at physiological pH, the formed radical cation is expected to immediately deprotonate from site N2 [175]. Therefore, such a process more corresponds to the SEPT mechanism, which was accordingly identified as the main mechanism in this case. RAF and H transfer were proposed to contribute by 17.8% and 38.1%, respectively, to the overall $^{\bullet}OH$ scavenging activity of melatonin, in aqueous solution. For the reaction of melatonin with $^{\bullet}OOCCl_3$, RAF was proposed to account for 57.7% and H transfer for 42.3%, also in aqueous solution. In nonpolar environments, the RAF mechanism was predicted to be the one contributing the most to the overall reaction with $^{\bullet}OOCCl_3$ (92.9%), while H transfer was identified as a minor path (7.1%). On the other hand, for the reaction with $^{\bullet}OH$, both mechanisms were proposed to be similarly important (RAF 54% and H transfer 46%) in nonpolar media.

Regarding the relative reactivity of the different sites of reaction, a wide product distribution was proposed for the melatonin reaction with $^{\bullet}OH$ [105]. In nonpolar environments, significant populations were estimated for products formed by HAT at sites C1, C3, C4, C13 and N2, and by RAF at sites C5 to C12 (Fig. 3). In aqueous media, the main product was predicted to be that formed from the SEPT mechanism, with the deprotonation taking place at N2. The other products that were predicted to be formed to a significant extent are those from HAT at C4, C13, and N2 and from RAF at C5, C6, C9, C10, C11, and C12. For the reaction with $^{\bullet}OOCCl_3$ in nonpolar environments, only two products were predicted to be observed: those formed from RAF on C6 site (major product) and from H transfer from N2 (minor). In aqueous solution, these two products were also predicted to be the main ones, but they were predicted to be formed in a similar proportion in this case. For the reactions of melatonin with peroxyl radicals $^{\bullet}OO–CH = CH_2$ and R4 = $^{\bullet}OO–CH_2–CH=CH_2$, only the HAT product from C4 was predicted to be generated.

Table 1. Rate constants (k) of melatonin's reactions with different radicals

Radical	k (per M/s)	Approach	Main solvent	Ref.
$^{\bullet}OH$	2.7×10^{10}	Experimental	Water	[149]
$^{\bullet}OH$	6×10^{10}	Experimental	Water	[267]
$^{\bullet}OH$	4×10^{10}	Experimental	Water	[268]
$^{\bullet}OH$	1.3×10^{10}	Experimental	Water	[150]
$^{\bullet}OH$	1.2×10^{10}	Experimental	Water	[255]
$^{\bullet}OH$	1.85×10^{10}	Theoretical	Water	[105]
$^{\bullet}OH$	1.25×10^{10}	Experimental	Mix	[175]
$^{\bullet}OH$	2.23×10^{10}	Theoretical	Benzene	[105]
$^{\bullet}OOCCl_3$	6×10^{8}	Experimental	Mix	[175]
$^{\bullet}OOCCl_3$	2.7×10^{8}	Experimental	Mix	[272]
$^{\bullet}OOCCl_3$	1.40×10^{9}	Theoretical	Water	[105]
$^{\bullet}OOCCl_3$	4.40×10^{8}	Theoretical	Benzene	[105]
$^{\bullet}OOL$	$\sim 10^{-2}$	Theoretical	Water	[105]
$^{\bullet}OOL$	~2–5	Theoretical	Benzene	[105]
t-But$O^{\bullet}$	3.4×10^{7}	Experimental	Acetonitrile	[152]
t-But$O^{\bullet}$	2.8×10^{9}	Experimental	Mix	[175]
di-t-Cum$O^{\bullet}$	6.7×10^{7}	Experimental	Acetonitrile	[152]
$O_2^{\bullet-}$	$< 1.0 \times 10^{4}$	Experimental	Water	[150]
$^{\bullet}N_3$	7.5×10^{9}	Experimental	Mix	[175]
$^{\bullet}N_3$	9.8×10^{9}	Experimental	Water	[150]
$^{\bullet}NO$	3×10^{7}	Experimental	Mix	[175]
$^{\bullet}NO_2$	3.7×10^{6}	Experimental	Mix	[175]

For the reaction with $^{\bullet}OOH$, on the contrary, it was proposed that in addition to the product formed by HAT from C4, the product yield from RAF at site C6 would also be formed. Moreover, in this particular case the later was identified as the main product of the reaction, regardless of the polarity of the environment.

Kinetics

There are several reports on the kinetics of the reactions of melatonin with free radicals, mainly from experiments but also from theoretical calculations (Table 1). The most abundant data reported so far involve melatonin's reaction with the $^{\bullet}OH$. They have been estimated from different experimental techniques and by DFT calculations. There is an excellent agreement among all the reported values, which range from 1.2 to 6×10^{10}/M/s [105, 149, 150, 175, 255, 267, 268]. Accordingly, it seems that there is little doubt that melatonin scavenges this very reactive and dangerous radical at diffusion-controlled limits, regardless of the polarity of the environment.

The reaction of melatonin with trichloromethylperoxyl radicals is slightly slower than that with $^{\bullet}OH$, but still very fast. Even though the solvents used in the different estimations of the rate constant of the melatonin + $^{\bullet}OOCCl_3$ reaction are different, the values are all coherent and indicate, beyond any doubt, that melatonin is a very efficient scavenger of this radical. In addition, the experimental and theoretical results are in very good agreement.

The most controversial results regarding the kinetics of the reactions of melatonin with free radicals relate to the reactions involving lipoperoxyl radicals. Pieri et al. [269] carried out a fluorescent assay, using dihydrochloride (AAPH) as the source of peroxyl radicals, and concluded that melatonin was twice as effective as vitamin E. On the

contrary, other researchers have reported that melatonin is only a limited lipoperoxyl radical scavenger [105, 156, 207, 270]. Livrea et al. [156] studied the interaction of melatonin with lipoperoxyl radicals from soybean phosphatidylcholine (PC) liposomes, using AAPH as a radical initiator. Based on fluorescence measurements, they found that the antioxidant activity of melatonin in soybean PC liposomes is much lower than that of α-tocopherol, under comparable assay conditions. Longoni et al. [207] investigated the lipoperoxyl radical–scavenging activity of melatonin using assays that included peroxidation of linoleic acid micelles by either AAPH, or Fe^{2+}–EDTA, and of dilinoleoyl phosphatidylcholine multilamellar liposomes for which peroxidation was induced by Fe^{2+}–EDTA. From comparisons of the results obtained from the three variations, they concluded that melatonin was poorly effective at scavenging peroxyl radicals. Antunes et al. [270] designed several experiments to evaluate the kinetics of the lipoperoxyl radical scavenging activity of melatonin and concluded that it is not a peroxyl radical trapping antioxidant. In addition, it was estimated, from a DFT study [105] that the rate constants of the reactions of melatonin with two model lipoperoxyl radicals are several orders of magnitude (seven to nine) slower than that of the melatonin + $^{\bullet}OOCCl_3$ reaction. Accordingly, it seems to be a general agreement on the poor effectiveness of melatonin as lipoperoxyl radical scavenger.

Regarding the alkoxyl radical scavenging activity of melatonin, the data gathered so far support the efficiency of melatonin to trap these species. Scaiano [152] studied the reactions of melatonin with tert-butoxyl radicals (t-ButO$^{\bullet}$) and di-tert-cumyloxyl (di-t-CumO$^{\bullet}$) radicals and found that the rate constants are 3.4 and 6.7×10^7/M/s, respectively. Mahal et al. [175] also studied the reaction of melatonin with t-ButO$^{\bullet}$ and estimated a rate constant of 2.8×10^9/M/s, i.e., 82 times faster than the previous estimation. Despite this difference, both values indicate that melatonin is capable of efficiently scavenging alkoxyl radicals. On the contrary, Barsacchi et al. [271] reported that melatonin does not quench the ESR signals in a system in which galvinoxyl radicals in CH_2Cl_2 were used as the hydrogen abstractor. This supports the importance of the chemical nature of the reacting radical on the total scavenging process. It also demonstrates the necessity of further studies on these complex processes.

Regarding the reaction of melatonin with superoxide radical anions, Marshall et al. [272] reported that melatonin is not able to scavenge $O_2^{\bullet-}$, in agreement with the findings of Chan and Tang [273]. On the other hand, Zang et al.[274] found that melatonin may react with this ROS, but only with a modest effectiveness. In line with this finding, Roberts et al. [150] estimated an upper limit for the melatonin + $O_2^{\bullet-}$ reaction of 1.0×10^4/M/s. Compared with the rate constant for the scavenging of this species by superoxide dismutase (2×10^9/M/s, at pH = 7), they concluded that melatonin cannot be considered an efficient quencher of $O_2^{\bullet-}$; which seems to be the current general agreement.

With respect to the reactions of melatonin with RNS, there are several estimations of rate constants. For its reaction with $^{\bullet}N_3$, the two available values [150, 175] (Table 1) are in very good agreement and support the efficiency of melatonin for trapping this radical. Melatonin is also a good $^{\bullet}NO$ and $^{\bullet}NO_2$ scavenger, even though its reactions with these species are slower than that with $^{\bullet}N_3$ [175]. It has been proposed that melatonin is also reactive toward ONOOH and its anion [258]. The rate constant for the reaction with ONOOH was found to be second-order and equal to 1.59×10^2/M/s. Accordingly, melatonin is much less efficient for quenching this species than its for quenching $^{\bullet}N_3$, $^{\bullet}NO$ and $^{\bullet}NO_2$.

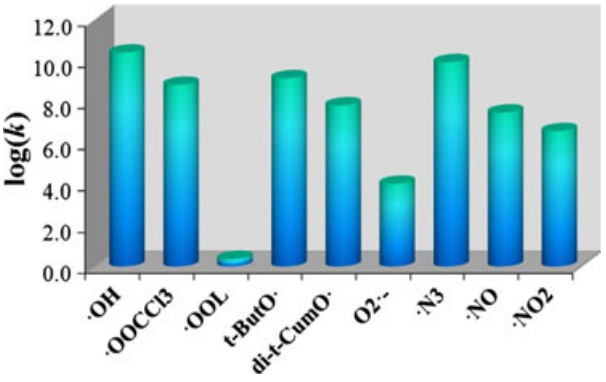


Fig. 4. Comparative efficiency of melatonin for scavenging free radicals of different nature.

The efficiency of melatonin as free radical scavenger depending on the reacting radical has been plotted in Fig. 4. In this plot, the rate constant (k) has been considered as the ultimate indicator of such reactivity. For every considered free radical, we have calculated the average value of k, including all the values reported in Table 1. As this plot shows, melatonin is a versatile free radical scavenger. It is excellent for trapping $^{\bullet}OH$, and $^{\bullet}N_3$, very good for $^{\bullet}OOCCl_3$ and alkoxyl radicals, and good for $^{\bullet}NO$ and $^{\bullet}NO_2$. However, it is rather ineffective for scavenging $O_2^{\bullet-}$ and lipoperoxyl radicals. This reinforces previous reports on the role of the chemical nature of the free radical involved in the quenching process on the scavenging activity of antioxidants.

Concluding remarks

Melatonin has most of the desirable characteristics of good free radical scavengers: (i) it is widely distributed in the body, and is present in adequate concentrations; (ii) it is a broad spectrum antioxidant; (iii) it easily transported across cellular membranes; (iv) it can be regenerated, after radical quenching, and its metabolites still present antioxidant activity. Finally, (v) it has minimal toxicity.

Elucidating the chemical mechanisms determining the antioxidant activity of any substance is a huge task, because of the high complexity of the systems. It can be addressed by both experimental and theoretical methodologies, but it is proposed that the best approach is to combine both of these. Hydrogen transfer and electron transfer has been identified as the main mechanisms determining the free radical–scavenging activity of melatonin. However, there are other mechanisms, such as the radical adduct (RAF)

formation, which have non-negligible contributions to the overall free radical–scavenging activity of melatonin.

The chemical nature of the reacting free radical also has an influence on the relative importance of the different mechanisms of reaction. It has been shown that melatonin reacts with a wide variety of radicals at high rates. Some of them are $^{\bullet}OH$, $^{\bullet}OOCCl_3$, $RO^{\bullet}$, and $^{\bullet}N_3$. On the contrary, there seems to be a general agreement on the modest efficiency of melatonin as antioxidant for detoxifying other radicals such as $LOO^{\bullet}$, $ROO^{\bullet}$ and $O_2^{\bullet-}$. In any case, there is no doubt that melatonin efficiently inhibits lipid peroxidation. Most likely such action does not take place by directly trapping peroxyl radicals, but rather by scavenging more reactive species, such as $^{\bullet}OH$, which initiate the degradation process.

Based on all the gathered data it can be concluded, without hesitation, that melatonin efficiently protects against oxidative stress, by a variety of mechanisms. Moreover, even though it has been referred to as the chemical expression of darkness, perhaps it could also be referred to as the chemical light of health.

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