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Melatonin: Action as antioxidant and potential applications in human disease and aging

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ABSTRACT

This review aims at describing the beneficial properties of melatonin related to its antioxidant effects. Oxidative stress, i.e., an imbalance between the production of reactive oxygen species and antioxidant defences, is involved in several pathological conditions such as cardiovascular or neurological disease, and in aging. Therefore, research for antioxidants has developed. However, classical antioxidants often failed to exhibit beneficial effects, especially in metabolic diseases. Melatonin has been shown as a specific antioxidant due to its amphiphilic feature that allows it to cross physiological barriers, thereby reducing oxidative damage in both lipid and aqueous cell environments. Studies on the antioxidant action of melatonin are reported, with a special mention to water gamma radiolysis as a method to produce oxygen-derived free radicals, and on structure–activity relationships of melatonin derivatives. Mass spectrometry-based techniques have been developed to identify melatonin oxidation products. Besides its ability to scavenge several radical species, melatonin regulates the activity of antioxidant enzymes (indirect antioxidant properties). Efficient detection methods confirmed the presence of melatonin in several plant products. Therapeutic potential of melatonin relies either on increasing melatonin dietary intake or on supplementation with supraphysiological dosages. Clinical trials showed that melatonin could be efficient in preventing cell damage, as well under acute (sepsis, asphyxia in newborns) as under chronic (metabolic and neurodegenerative diseases, cancer, inflammation, aging). Its global action on oxidative stress, together with its rhythmicity that plays a role in several metabolic functions, lead melatonin to be of great interest for future clinical research in order to improve public health.

1. Oxidative stress involvement in human disease and aging

Reactive oxygen species (ROS) are continuously produced under aerobic conditions; they are involved in several processes, i.e., transformation, regulation or degradation (Gardès-Albert et al., 2003; Favier, 2003). Nevertheless, ROS concentration is strictly controlled by endogenous antioxidants, thereby protecting human beings against the potentially deleterious effects of ROS (Fig. 1). ROS include superoxide anion radical ($O_2^{\bullet -}$) -especially produced in cytosol, mitochondria and endoplasmic reticulum-, hydrogen peroxide (H_2O_2) produced in peroxysomes, the highly reactive hydroxyl radical ($^{\bullet}OH$) and singlet oxygen ($^1O_2$). Reactive nitrogen species (RNS) can also be involved in cell damage, such as nitric oxide ($^{\bullet}NO$) that is produced by the NO-synthases, and that reacts with $O_2^{\bullet -}$ to form peroxynitrite ($ONOO^-$), an efficient oxidative and nitrosative agent (Halliwell and Gutteridge, 1999). An oxidative imbalance, i.e., a disturbance in the balance between the production of ROS (especially free radicals) and antioxidant defenses, has been described as an oxidative stress status (Halliwell and Gutteridge, 1999). This can result in several kinds of cell damage, leading to a loss of function and integrity. Oxygen radical-mediated tissue damage (Betteridge, 2000) has been involved in a large number of pathological conditions (Dalle-Donne et al., 2006; Fisher-Wellman et al., 2009) including cardiovascular diseases (Bolli et al., 1989; Charniot et al., 2007; Sawyer et al., 2002), neurological disorders (Kontush, 2001; Dexter et al., 1998), cancer (Jung-Hynes et al., 2010) and aging process (Calabrese et al., 2010). As an example, the oxidative stress theory of aging proposed by Harman suggested that ROS formed as by-products from metabolic processes can play a key role in aging (Harman, 1956). Mitochondria are specifically involved in these

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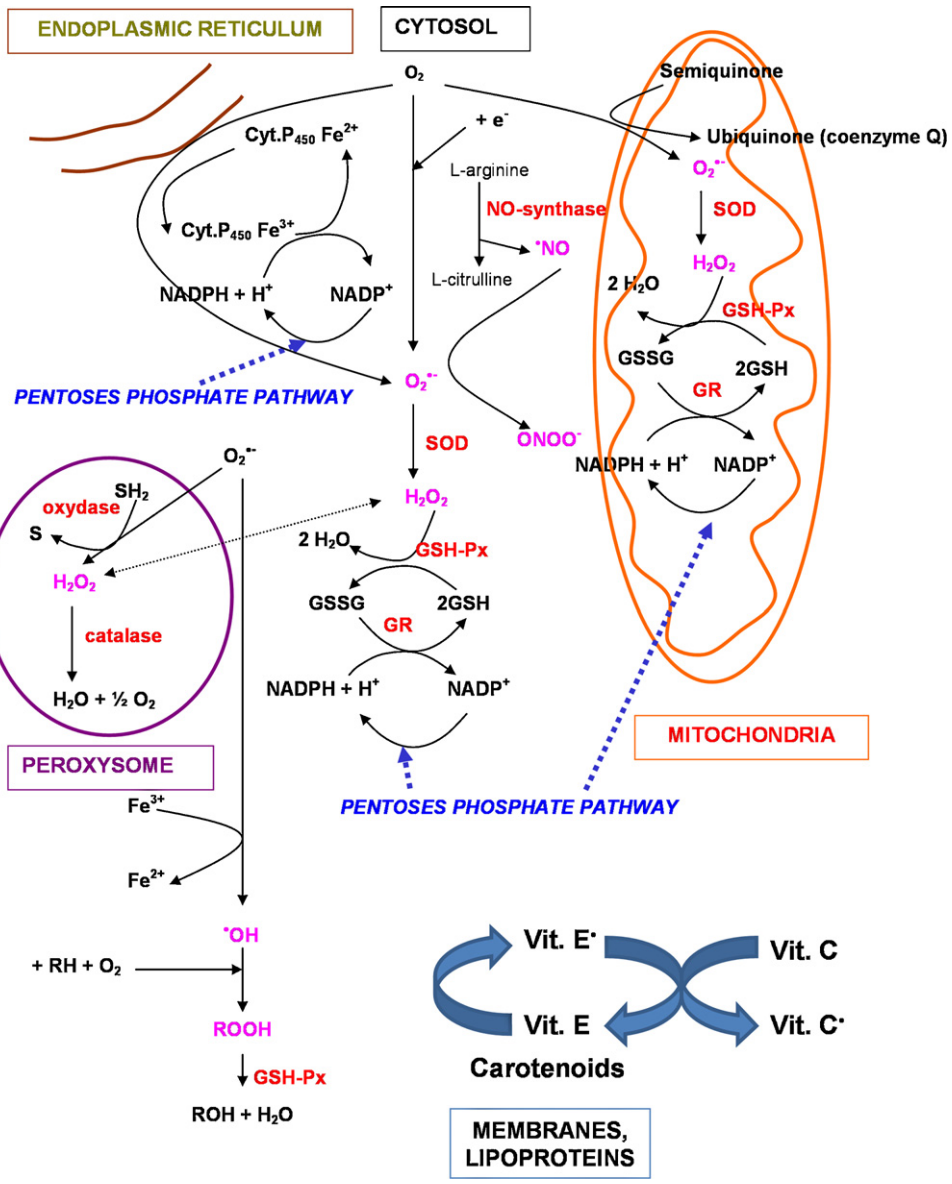

Fig. 1. The main cellular sources of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and the interrelationships between the antioxidant defenses. RH: polyunsaturated fatty acid; ROOH: lipid hydroperoxide; SH_2: reductive substrate; S: oxidized substrate; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; GR: glutathione reductase; NADH + H^+: reduced form of the nicotinamide adenine dinucleotide phosphate; NAD^+: oxidized form of the nicotinamide adenine dinucleotide phosphate; Vit. E: vitamin E; Vit. C: vitamin C.

processes (Harman, 1972) and in related diseases (Salmon et al., 2010).

ROS play a physiological role by acting at low concentrations as second messengers able to regulate apoptosis processes (Curtin et al., 2002), to activate transcription factors (NF-kB, p38-MAP kinase, ...) responsible for the activation of genes involved in immune response (Owuor and Kong, 2002), or genes coding antioxidant enzymes (Holgrem, 2003). The role of oxidative stress is crucial in the modulation of cellular functions, notably for neurons astrocytes and microglia, such as apoptosis and excitotoxicity, both involved in neuronal death. Mitochondrial dysfunction, i.e. cell energy impairment, apoptosis and overproduction of ROS, is a final common pathogenic mechanism in aging and in neurodegenerative disease such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis. Nitric oxide is also a highly diffusible radical and biological messenger, that plays a prominent role in the physiology of the central nervous system (Yun et al., 1996). Three isoforms account for •NO production and include neuronal NO synthase (nNOS; type I), inducible NO synthase (iNOS; type II) which is produced in very large amounts by activated microglia (macrophages), and endothelial NO synthase (eNOS; type III), In the central nervous system, nNOS, whose expression is regulated by both physiological and pathophysiological stimuli, accounts for most •NO production, and allows a further formation of the highly reactive peroxynitrite, via the rapid reaction of $O_2^{•-}$ with •NO (Emerit et al., 2004).

Antioxidant defense systems help the organism to fight against a ROS excess (Fig. 1). These systems comprise non enzymatic proteins (transferrin, ferritin, ceruleoplasmin), enzymes (superoxide dismutases (Cu, Zn-SOD and Mn-SOD), catalase, glutathione peroxidase, ...), oxidizable molecules (glutathione, vitamins E, A, C, carotenoids, flavonoids...) and trace elements (copper, zinc, selenium). Some of them are endogenous whereas others are provided by alimentation. These antioxidants constitute three lines of defense: a first line of defense limits an overproduction of ROS by inactivating endogenous cations such as Fe^{2+} or Cu^+; this line con-

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tains enzymes (ferroxidase; Epsztejn et al., 1999) and iron chelating proteins (e.g., transferrin) or copper chelating proteins (e.g., albumin) (Luza and Speisky, 1996). In plasma, albumin is the main thiol-containing protein and can thus be considered as an important extracellular antioxidant, especially due to its high plasma concentration (about 0.5 mM) (Halliwell and Gutteridge, 1990). The second defense line is mainly constituted by three enzymes that react synergistically, i.e., superoxide dismutases (SOD) (reaction 1), catalase (reaction 2) and glutathione peroxidase (GSH-Px) (reaction 3). Cu,Zn-SOD (SOD1) is the major cytoplasmic superoxide scavenger and is also located in the mitochondrial intermembrane space (Fridovich, 1999; Okado-Matsumoto and Fridovich, 2001); Mn-SOD (SOD2) is the main scavenger of superoxide in the mitochondrial matrix (Fridovich, 1999); catalase is an ubiquitously expressed antioxidant enzyme primarily located in the peroxisomes that catalyses the decomposition of hydrogen peroxide into oxygen and water (Halliwell and Gutteridge, 1999); GSH-Px 1 is the most abundant isoform of the mammalian GSH-Px and is responsible for much of the detoxification of H_2O_2 within the cytoplasm (Halliwell and Gutteridge, 1999). GSH-Px are more generally enzymes able to reduce hydroperoxides (ROOH) into alcohols (ROH) with the concomitant oxidation of reduced glutathione (GSH) in its oxidized form (GSSG). All these antioxidant enzymes exhibit a complementary action of the radical cascade finally leading to the formation of water and molecular oxygen

$$O_2^{\bullet -} + O_2^{\bullet -} \xrightarrow{2H^+} H_2O_2 + O_2 \quad (1)$$

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2 \quad (2)$$

$$2GSH + H_2O_2 \rightarrow 2H_2O + GSSG \quad (3)$$

The third line of defense is constituted by molecules able to scavenge ROS, such as vitamin E tocopherols and tocotrienols, pro-vitamin A carotenoids, ascorbate (vitamin C), glutathione. The resulting protection thus depends on the feature of the molecule (lipophilic or hydrophilic).

These different kinds of defense systems limit the *in vivo* ROS-induced damage. Nevertheless, these systems could be overwhelmed under pathological conditions so that research for new antioxidant molecules able to inhibit oxidative stress by restoring the balance between ROS and antioxidants is always in progress (Favier, 2003). However, clinical studies using classical antioxidants have been often disappointing, especially in cardiovascular disease (Vivekananthan et al., 2003). As an example, the oxidative theory of atherosclerosis (Steinberg et al., 1989) claims that lipoproteins, especially low density lipoproteins (LDL) oxidized within the arterial wall are able to activate or damage endothelial cells, attract monocytes within the intima and participate to their transformation into foam cells that constitute the first step in the formation of fatty streaks. According to this theory, the use of antioxidants would be beneficial in human CVD (Steinberg and Lewis, 1997). However, against prediction, the antioxidant supplements did not improve the clinical course of human atherosclerosis during several placebo-controlled trials, and this addresses the issue of the usefulness of these antioxidants (Kuller, 2001; Stocker and Keaney, 2004; Zureik et al., 2004). In all pathologies where oxidative stress is involved, the development of new antioxidant management, especially with melatonin (N-acetyl-5-methoxytryptamine) appears of great interest, as will be described below. Indeed, experimental studies have shown that melatonin turned out to be more effective than classical antioxidants for protection against oxidative and nitrosative stress-induced damage (Baydas et al., 2002; Gitto et al., 2001a; Martinez-Cruz et al., 2002).

2. Melatonin as an antioxidant defense system

Melatonin is a pineal hormone produced according to a circadian rhythm, with a maximal secretion at night. Optimum melatonin production is only achieved in complete darkness. Melatonin concentration in blood is thus highly variable, from 10 to 60 pg mL^{-1} (43–258 pmol L^{-1}), during day and night, respectively (Brzezinski, 1997), and remains high during sleepness. Moreover, it is worth noting that melatonin concentration in other human fluids can be higher than that measured in blood (Reiter and Tan, 2003), so that melatonin could exhibit its antioxidant properties, i.e., bile (Tan et al., 1999a; Koppisetti et al., 2008), gut (Bubenik, 2002), cerebrospinal fluid from the third ventricle (Skinner and Malpaux, 1999; Tricoire et al., 2002; Longatti et al., 2007; Leston et al., 2010) or some cells (from skin, Slominski et al., 2002; retina, Faillace et al., 1995; bone marrow, Tan et al., 1999b). Another advantage of melatonin is its amphiphilic feature: in contrast to other antioxidants that are either hydrophilic or lipophilic, melatonin can cross physiological barriers, thereby reducing oxidative damage in both the lipid and aqueous environments of cells (Reiter et al., 2004).

Both direct and indirect antioxidant properties of melatonin have been reported (Tan et al., 2002; Hardeland, 2005). Melatonin exhibits indirect antioxidant role by supporting superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities (Reiter et al., 2002; Rodriguez et al., 2004; Reiter et al., 2005b), possibly via epigenetic mechanisms (Korkmaz and Reiter, 2008), which constitutes a specific behaviour for an antioxidant. It also stimulates glutathione production in cells (Winiarska et al., 2006); melatonin stimulates γ-glutamylcysteine synthase thereby increasing glutathione level, and it promotes the activity of glutathione reductase which converts oxidized glutathione (GSSG) back to its reduced form (GSH) (Reiter et al., 2002).

Direct antioxidant properties of melatonin have been shown both *in vitro* (Vijayalaxmi et al., 1995; Gulcin et al., 2002) and *in vivo* (Vijayalaxmi et al., 1996; Kaya et al., 1999; El-Missiry et al., 2007), towards lipid peroxidation and DNA degradation. The latter action could be due to a direct scavenging of free radicals and/or the activation of DNA reparation enzymes (Vijayalaxmi et al., 1998). The protection of DNA against adduct formation induced by the carcinogen safrole has also been reported, partly due to its hydroxyl radical scavenging capacity (Tan et al., 1994). As mentioned above, melatonin is known to be both lipophilic (Roberts et al., 1998) and hydrophilic (Shida et al., 1994), which allows it to cross physiological barriers and enter cells. This property confers to melatonin an advantage as compared to other antioxidants whose action is limited due to their solubility limiting their partitioning between intra- and extra-cellular compartments. The efficiency of radical scavenging by melatonin is therefore highly dependent on its partitioning between the lipidic and aqueous phases. In order to better assess the location of melatonin in lipid systems, Mekhloufi et al. (2007) used model assemblies such as linoleate micelles, phosphatidylcholine liposomes or LDL. The proportion of melatonin in the aqueous and lipid phases of each system has been determined (concentrations of the antioxidants ranging between 3×10^{-5} and 10^{-4} M) by assaying melatonin by HPLC/UV detection in liposomes and LDL, or by fluorescence in micelles. The results showed that melatonin was preferentially located in the aqueous phase of micelles (68.4%) whereas only 30.5% were found in the lipid phase. By contrast, melatonin was essentially present in the lipid phase (73.5%, vs. 25.9% in the aqueous phase) of phosphatidylcholine liposomes. In the case of LDL, 99.9% of the melatonin added was found in the methanol/water extracting phase containing phospholipids, unesterified cholesterol and apolipoprotein B100. The partitioning of melatonin in linoleate micelles allowed a more accurate determination of the lower limit values of the reaction rate constants of melatonin with

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lipid peroxyl radicals than previously done (Mekhloufi et al., 2005), i.e. $k(LOO^{\bullet} + \text{melatonin}) \geq 9.0 \times 10^4\ M^{-1}\ s^{-1}$. The partitioning data obtained in these lipid systems could thus help explain the ability of melatonin to scavenge free radicals according to their production site, and thereby to protect lipids and/or proteins against the oxidation process.

Several studies demonstrated the ability of melatonin to scavenge oxygen-derived free radicals, such as hydroxyl (Poeggeler et al., 2002; Roberts et al., 1998; Matuszak et al., 1997) and peroxyl (Livrea et al., 1997; Pieri et al., 1994) radicals. By contrast, melatonin seems poorly able to react with superoxide radicals (Zang et al., 1998; Walters-Laporte et al., 1998). Moreover, protective effects of melatonin have been reported in the case of the *in vitro* oxidation of LDL by copper or by macrophages (Bonnefont-Rousselot et al., 2002, 2003; Walters-Laporte et al., 1998; Duell et al., 1998; Livrea et al., 1997; Abuja et al., 1997). This protection has been explained by the ability of melatonin to directly scavenge the free radicals involved in these oxidation processes (Allegra et al., 2003). Similarly, models of melatonin-leaded nanoparticles have shown their efficiency to protect liposomes or microsomes against lipid peroxidation (Schaffazick et al., 2005). Melatonin is also able to neutralize singlet oxygen, peroxynitrite anion, and nitric oxide (Allegra et al., 2003; Reiter et al., 2004; Gilad et al., 1997; Ucar et al., 2007; Topal et al., 2005). Although some metabolites have been identified *in vivo* (e.g., 3-cyclohydroxymelatonin, Tan et al., 1998), the mechanisms by which melatonin displays its direct antioxidant properties have not totally been unravelled. As previously specified, melatonin is known to exhibit antioxidant properties towards a wide range of biological systems, as for example linoleate model system (Mekhloufi et al., 2005) or LDL (Pieri et al., 1996; Kelly and Loo, 1997; Bonnefont-Rousselot et al., 2002). In the latter model, melatonin exhibited a relatively low activity. This could be explained in term of lipophilicity since replacing acetyl by nonanoyl group dramatically increases the antioxidant activity (Gozzo et al., 1999). Other functional groups play an important role in the beneficial effect of melatonin in the prevention of oxidation. If the amide function is not an absolute requirement for the antioxidant activity of melatonin, it was suggested that the presence of the acyl terminal group could prevent a possible prooxidant behaviour (Tan et al., 1993). This was confirmed when 5-methoxytryptamine was found to shorten the lag phase duration during oxidation of the LDL model (Gozzo et al., 1999). However, the acyl terminal group had

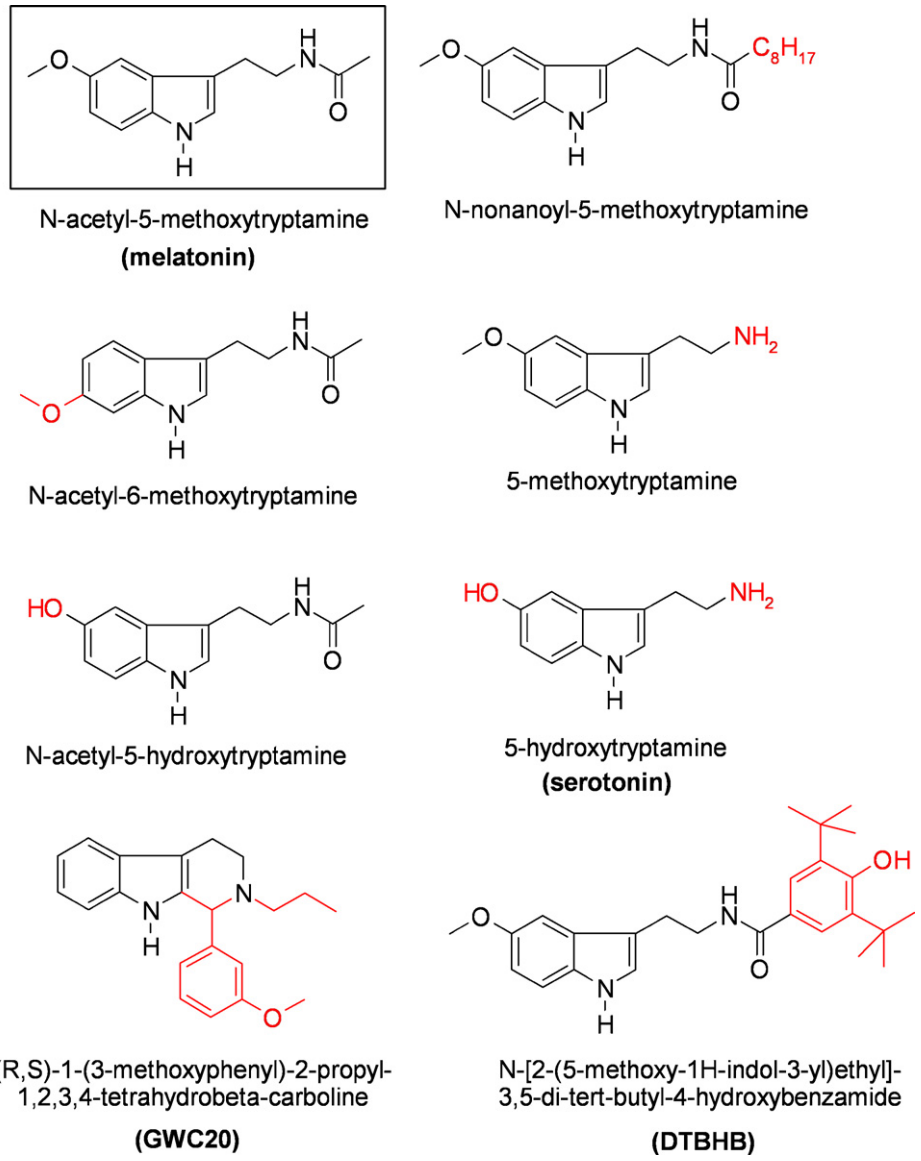

Fig. 2. Chemical structures of melatonin and some derivatives.

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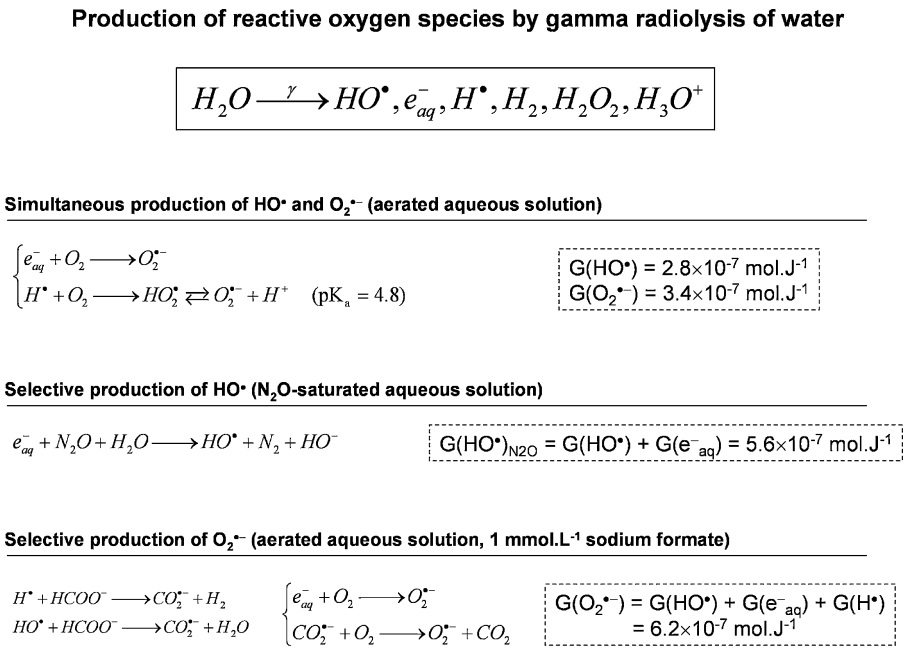

Fig. 3. Selective production of •OH/$O_2^{\bullet-}$, •OH, $O_2^{\bullet-}$/$HO_2^\bullet$ radical species upon water gamma radiolysis.

no influence on the scavenging capacity of hydroxyl radicals *in vitro* since rate constants for the reaction of hydroxyl radicals with melatonin and 5-methoxytryptamine were similar (respectively 2.7 and 2.3×10^{10} M^{-1} s^{-1}; Matuszak et al., 1997).

The methoxy group has also a beneficial effect in antioxidant activity of melatonin, whatever its position on the indole ring (5- or 6-methoxy). However, the methoxyl function is not an absolute requirement since phenolic derivatives of melatonin are very active. In particular, N-acetyl-5-hydroxytryptamine activity is increased by one order of magnitude comparing to melatonin (Gozzo et al., 1999) (Fig. 2). This latter compound seems to have beneficial effects in preventing both lipid and protein oxidation in synaptosomal membranes (Millán-Plano et al., 2010). Thus, methoxy and acyl groups are essential substituents for the antioxidant properties of melatonin. The compound lacking both these groups (namely 5-hydroxytryptamine) was found to efficiently promote the generation of •OH radicals in the presence of ferric iron and hydrogen peroxide (Matuszak et al., 1997) (Fig. 2).

3. Gamma radiolysis as a tool to study molecular antioxidant properties of melatonin

In order to improve the knowledge of melatonin antioxidant mode of action, a study of the direct antioxidant properties of melatonin has been performed *in vitro* against different oxygen-derived free radical species generated in aqueous solution by gamma radiolysis. Gamma radiolysis of water is a well known method, that has many advantages, such as the homogeneous production of defined concentrations of free radicals (as superoxide anion $O_2^{\bullet-}$ or hydroxyl radical •OH), as well as the possibility to selectively produce one specific radical to be studied at a time (Bonnefont-Rousselot, 2005). Free radicals thus generated have been used to initiate one-electron oxidation reaction(s) on melatonin dissolved in water.

Radiolysis can be defined as the chemical transformations of a solvent due to the absorption of ionizing radiations (Spinks and Woods, 1990). These radiations can be of several kinds (electrons, photons, neutrons, heavy charged particles, but the more frequently used is the γ radiation emitted by ^{60}Co (1.17 and 1.33 MeV photons) or by ^{137}Cs (≈600 keV photons). Those photons ionize the aqueous (or ethanolic) solvent, leading to the very rapid production (within a few nanoseconds) of a homogeneous solution of radical species. Radiolytically generated free radicals are independent of the nature and the concentration of the dissolved compound as long as its concentration remains lower or equal to 10^{-2} mol L^{-1} (Spinks and Woods, 1990). Under these conditions, the compound dissolved into the irradiated solvent is not directly subjected to the ionizing radiations, but to the action of the free radicals produced by the solvent radiolysis.

Briefly, water γ-radiolysis leads within a few nanoseconds to the production of a homogeneous solution of radicals and molecular species whose nature depends on the experimental conditions (Fig. 3). The mechanisms of formation of the primary species (•OH, e^-_{aq}, •H, H_2, H_2O_2) are well known (Spinks and Woods, 1990). The latter are produced with the following radiolytic yields (G) expressed as the number of molecules or radicals produced per unit of energy absorbed, i.e. per Joule (Spinks and Woods, 1990; Draganic and Draganic, 1971).

This method has been used to evaluate antioxidant properties of melatonin, firstly when melatonin was included in lipid systems, then with a molecular approach with analysis of melatonin oxidation products by mass spectrometry.

Regarding lipid systems, low density lipoproteins (LDL) are of special interest since oxidized LDL are involved in the pathogenesis of atherosclerosis (Rizzo et al., 2009). In a comparative study, LDL oxidation was initiated *in vitro* either by defined free radicals [i.e., superoxide anion ($O_2^{\bullet-}$) and ethanol-derived peroxyl radicals ($RO_2^\bullet$)] produced by gamma radiolysis or by copper ions. The antioxidant effect of melatonin was evaluated at 100 μM. Under these experimental conditions, melatonin did not protect endogenous LDL alpha-tocopherol from oxidation by $RO_2^\bullet$/$O_2^{\bullet-}$ free radicals. By contrast, it protected β-carotene from the attack of those radicals and partially inhibited the formation of products derived from lipid peroxidation (conjugated dienes and thiobarbituric acid-reactive substances or TBARS) (Bonnefont-Rousselot et al., 2002, 2003). As previously reported (Walters-Laporte et al., 1998), melatonin inhibited copper-induced LDL oxidation by increasing 1.60-fold the lag phase duration of conjugated diene formation over the 8 h of the experimental procedure. This study also showed a stronger antioxidant activity of some

melatonin derivatives (Fig. 2), i.e., N-[2-(5-methoxy-1H-indol-3-yl)ethyl]-3,5-di-tert-butyl-4-hydroxybenzamide (DTBHB) and (R,S)-1-(3-methoxyphenyl)-2-propyl-1,2,3,4-tetrahydro-beta-carboline (GWC20) which is a pinoline derivative, so that it seemed of interest to test *in vivo* whether DTBHB and GWC20, which exhibited a strong capacity to inhibit *in vitro* LDL oxidation, would reduce atherosclerosis in animals susceptible to this pathology. Nevertheless, it is worth remembering that an *in vitro* protective effect of molecules is not always related to a prediction of their capacity to inhibit *in vivo* atherosclerosis development (Tailleux et al., 2005).

4. Melatonin analysis by mass spectrometry-based techniques

Mass spectrometry (MS)-based techniques are among the most efficient and versatile analytical techniques that are used increasingly to determine, both quantitatively and qualitatively, endogenous substances in biological samples. Melatonin determination by mass spectrometry makes identity confirmation possible, thus increasing the validity of the results. In addition, when coupled to liquid chromatography, mass spectrometry allows working directly on complex samples such as saliva or serum without sample preparation step. This is made possible by the use of the common discriminative working modes of mass spectrometry (selected ion monitoring (SIM), selected reaction monitoring (SRM) and multiple reaction monitoring (MRM)) that enhance the specificity, and thus the sensitivity, of the analytical method. Motoyama et al. (2004) have determined endogenous melatonin in saliva by using a simple quadrupole mass spectrometer coupled to reverse-phase liquid chromatography and working in the SIM mode with in-source fragmentation focused on the ion at *m/z* 174. This latter is usually one of the most intense fragments of melatonin, generated by the fragmentation of the aliphatic chain of melatonin (formal loss of CH_3CONH_2, −59 Da). It has been used both in SIM and SRM mode for the quantitative determination of melatonin by electrospray ionisation (ESI) triple quadrupole mass spectrometry in complex plant samples (Cao et al., 2006) or human serum (Yang et al., 2002).

Separative methods are widely used for qualitative and quantitative analysis of melatonin in complex samples prior to mass spectrometric detection. Among them, enzyme immunoaffinity chromatography (IAC) is successfully applied to determine melatonin in biological sample such as human serum, plasma and saliva. As tandem MS techniques separate specifically single ion that can be assigned to single analyte, IAC is able to separate one specific analyte among myriads of compounds, with recovery varying from 92 to 98% for amounts of melatonin ranging from 50 to 400 fmol (Rolcik et al., 2002). Enzyme immunoassay is also employed to determine melatonin in food samples such as olive oil or grape skin (De La Puerta et al., 2007; Iriti et al., 2006). Other more innovative methods are tentatively developed for melatonin separation from complex samples. One method, based on the use of copolymers of N-isopropylacrylamide (PNIPAM), N-*tert*-butylacrylamide and acrylic acid, exhibiting both pH- and temperature-dependent properties, was proposed for the qualitative and quantitative analysis of melatonin (Ayano et al., 2007). The copolymers were modified with cross-linked hydrogel applied onto aminopropyl silica beds and packed as an HPLC column. The lower critical solution temperature of PNIPAM is 32 °C: below, the polymer hydrates and has hydrophilic properties, while above, it dehydrates to get a compact hydrophobic conformation. This property was successfully used to separate melatonin from L-tryptophan and serotonin by working isocratically with aqueous mobile phase and by varying the temperature.

As melatonin possesses several reaction sites for oxidation by $HO^{\bullet}$ radicals, such as the indole ring, one pair of electrons of nitrogen atoms or the hydrogen on the aliphatic chain, the prediction of structures for oxidation products remain difficult. Further information on the molecular mechanisms of the *in vitro* radical-induced oxidation of melatonin and the identification of the resulting products is often given by mass spectrometry analysis. In an investigation on the possible role of melatonin in melanogenesis, melatonin was submitted to the attack of $HO^{\bullet}$, generated by the decomposition of hydrogen peroxide under UV irradiation (Rizzi et al., 2006). Oxidation of melatonin was found to lead to several products, including 2-hydroxymelatonin, N^1-acetyl-N^2-formyl-5-methoxykynurenin (AFMK) and cyclic 3-hydroxymelatonin (c3HO-MLT), identified by Matrix Assisted Laser Desorption Ionization—Time-Of-Flight (MALDI-TOF) mass spectrometry, fluorescence and UV–visible spectrophotometry. However, proposals of chemical structures are often based on the deduction of the molecular mechanism of $HO^{\bullet}$ attack towards melatonin or on the comparison to known *in vivo* metabolites of melatonin (as AFMK, Harthe et al., 2003 or c3HO-MLT, Tan et al., 1998), and are not supported accurately by the experimental spectra because of the lack of specific information for identification.

Advanced mass spectrometric and chromatography-coupled methodologies are required to identify the oxidation products of melatonin with a better accuracy. Liquid chromatography coupled to tandem mass spectrometry with hydrogen–deuterium exchange (LC-HDX-MS/MS) is one of them and is susceptible to provide more valuable information for identification. HDX of protonated melatonin and its *in vitro* oxidation end-products have been examined by liquid chromatography coupled to electrospray ionization (ESI)/ion-trap mass spectrometry (Collin et al., 2009). In deuterated medium (D_2O/MeOD, 1/1 v/v), melatonin is able to exchange 3 times and is thus detected at *m/z* 236 as a pseudo-molecular monocharged ion. In these conditions, an hydrogen–deuterium scrambling was found to occur between deuterium and hydrogen on position C2 and C4 of the indole ring during the fragmentation process. The spectrum showed multiple consecutive fragment ions corresponding to the loss of variably deuterated neutral molecules. This characteristic, also observed for tryptophan (Lioe et al., 2004), was used to identify the hydroxylated oxidation products of melatonin *in vitro*, after a chromatographic separation on a reverse-phase C18 column by eluting with deuterated solvents (D_2O/acetonitrile, 80/20 v/v). Two chromatographic peaks were detected and further fragmented: by looking at the multiplicity of product ions, HDX methodology showed that the hydroxyl group of radiolytically hydroxylated melatonin was located on position C6 or C7 for the first product and on position C2 or C4 for the second (Fig. 4). The same strategy was used for the identification of the other radio-induced oxidation products of melatonin *in vitro*: N^1-acetyl-5-methoxykynurenin (AMK), N^1-acetyl-N^2-formyl-5-methoxykynurenin (AFMK), known as minor metabolites of melatonin *in vivo* (Harthe et al., 2003; Reiter et al., 2000; Silva et al., 2004), and dehydro-AFMK.

5. Results from melatonin analyses

Efficient detection methods of melatonin, together with optimized extraction protocols from plant samples, allowed to confirm the presence of melatonin in several plant products, but also showed that its concentration varies widely on the species and on plant parts (Paredes et al., 2009). The role of melatonin in plants may be a protection against oxidative stress (Tan et al., 2002), especially in seeds where melatonin has been hypothesized to protect germ from oxidative damage due to UV light, drought, temperature variations, and environmental toxins (Manchester et al., 2000).

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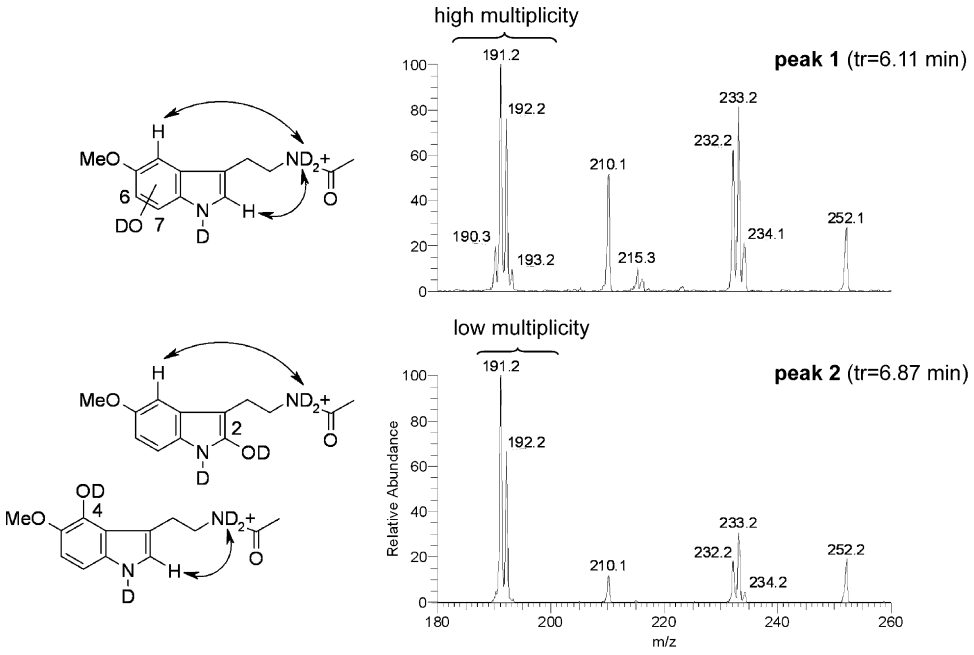

Fig. 4. Hydrogen Deuterium Exchange of hydroxylated melatonin (HO-MLT). During MS fragmentation, H/D scrambling occurs between deuterium of the amide terminal group and protons on positions C2 and C4 of the indole ring. The replacement of one of these protons by the deuterated hydroxyl group is responsible for quenching one of the possible H/D back exchange during fragmentation, and thus leads to a lower multiplicity for consecutive fragment ions. After chromatographic separation, two peaks were detected for HO-MLT (at tr = 6.1 min and tr = 6.87) and fragmented: the peak multiplicity was used as a reliable information regarding the position of the hydroxyl group on the indole ring of HO-MLT (adapted from Collin et al., 2009).

Table 1 presents some dietary sources of melatonin, with a special mention to Graminae that exhibit high contents of melatonin (rice, barley, sweet corn, oat, tall fescue) (Hattori et al., 1995). This suggests that it is possible to ingest sufficient quantities of melatonin in edible plants to perhaps influence physiological processes. As an example, food-derived melatonin could also be involved in post-prandial sleepiness after ingestion of melatonin-rich food, since melatonin is considered as a sleep inducing molecule (Dollins et al., 1994).

The fate of melatonin in circulation is still poorly understood, but intra-arterial infusion of melatonin in rats showed that the half-life of melatonin was only 20 min (Gibbs and Vriend, 1981). After ingestion of 1 g melatonin in human, about 90% melatonin was converted into metabolites (especially 6-hydroxymelatonin) with a renal elimination of sulphated or glucuronidated conjugates (Leone et al., 1987). A recent study in critically ill patients showed that oral melatonin supplementation resulted in a rapid enteral absorption, with pharmacological levels reached within 5 min and a serum peak (11,040 pg mL^{-1}, or 47,582 pmol L^{-1}) after 16 min and a half-elimination time of about 1.5 h (Mistraletti et al., 2010). A pharmacokinetic study in rats showed that about 500 ng melatonin should be continuously infused per hour to freely moving catheterized rats to maintain a 10-fold elevation of their plasma levels (Huether et al., 1992); under these conditions, concentrations of melatonin (plus melatonin metabolites) have been monitored in several tissues in order to appreciate partitioning of melatonin and the results showed large amounts in small intestine (duodenum, jejunum, ileum) and lower gut (caecum, colon) at the end of a 2h-infusion, and in feces after 6 h (Table 2) (Messner et al., 1998). Indeed, about 45% of the melatonin administered during the 2 h infusion period were found in the urine and 20% in the small intestine whereas 6 h after the end of the infusion, the majority of melatonin and its metabolites initially present in the small intestine had been eliminated in the feces. The gut so appears not only as a site of melatonin synthesis (Lee et al., 1995) but also as a site where melatonin concentrates, possibly suggesting a role of melatonin in the gastrointestinal tract, especially for ensuring protection against inflammation and ulceration of the intestinal mucosa (Melchiorri et al., 1997; Bubenik et al., 1998). The study of Messner et al. (1998) also demonstrated that a certain proportion of circulating melatonin must enter tissues and become covalently bound to amino acids of tissue proteins. Apart from this non oral supplementation in rats, Table 2 shows melatonin amounts in the body resulting from oral melatonin intakes. It clearly shows that increased amounts of ingested melatonin (at low doses, i.e., 0.1–10 mg) resulted in enhanced serum melatonin concentration, as performed in healthy volunteers (Dollins et al., 1994); under these conditions, this orally administrated melatonin has been shown to be a potent hypnotic agent; this study also suggested that the physiological increase of melatonin level at night may initiate the normal sleep onset.

6. Therapeutic potential of melatonin

As melatonin is produced according to a circadian rhythm, it has been used mostly to correct insomnia and jet lag (Petrie et al., 1993; Dahlitz et al., 1991; Haimov and Lavie, 1997) as it plays a major role in the synchronization of the sleep/wake cycle (Reiter, 1980). Indeed, circulating melatonin concentration is significantly decreased in elderly insomniacs than in age-matched controls and their onset and peak times are delayed (Haimov and Lavie, 1997). Moreover, the regulation of central and peripheral clocks in humans has been shown to be based on clock genes (Cermakian and Boivin, 2009), and circadian clock gene expression in peripheral tissues could be uncoupled from the activity of the central clock during periods of acute systemic inflammation, as recently reported in blood leukocytes (Haimovich et al., 2010). Under these conditions, the realignment of the central and peripheral clocks could also be of help for recovery from disease in humans. Increasing evidence suggests that melatonin rhythmicity plays by itself key roles in several metabolic func-

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Table 1
Dietary sources of melatonin.

Source	Melatonin content (pg/g)	Reference
Alfalfa (seed)	16000[b]	Manchester et al. (2000)
Almond (seed)	39000[b]	Manchester et al. (2000)
Anise (seed)	7000[b]	Manchester et al. (2000)
Apple	48	Hattori et al. (1995)
Asparagus	10	Hattori et al. (1995)
Banana	466	Dubbels et al. (1995)
Barley	378	Hattori et al. (1995)
Barley	82	Hernandez-Ruiz et al. (2005)
Barley (roots)	24	Arnao and Hernández-Ruiz (2009)
Beetroot	2	Dubbels et al. (1995)
Black mustard (seed)	129000[b]	Manchester et al. (2000)
Cabbage	107	Hattori et al. (1995)
Canary grass	27	Hernandez-Ruiz et al. (2005)
Carrot	55	Hattori et al. (1995)
Celery (seed)	7000[b]	Manchester et al. (2000)
Chinese cabbage	113	Hattori et al. (1995)
Chungiku	417	Hattori et al. (1995)
Coriander (seed)	7000[b]	Manchester et al. (2000)
Cucumber	25	Hattori et al. (1995)
Cucumber	86	Dubbels et al. (1995)
Fennel (seed)	28000[b]	Manchester et al. (2000)
Fenugreek (seed)	43000[b]	Manchester et al. (2000)
Flax (seed)	12000[b]	Manchester et al. (2000)
Ginger	584	Hattori et al. (1995)
Grape skin	5–965	Iriti et al. (2006)
Green cardamone (seed)	15000[b]	Manchester et al. (2000)
Indian spinach	39	Hattori et al. (1995)
Japanese ashitaba	624	Hattori et al. (1995)
Japanese butterbur	50	Hattori et al. (1995)
Japanese radish	657	Hattori et al. (1995)
Kiwi fruit	24	Hattori et al. (1995)
Milk thistle (seed)	2000[b]	Manchester et al. (2000)
Oat	1796	Hattori et al. (1995)
Oat	91	Hernandez-Ruiz et al. (2005)
Olive oil (extra virgin)	71–119[a]	De La Puerta et al. (2007)
Onion	32	Hattori et al. (1995)
Pineapple	36	Hattori et al. (1995)
Poppy (seed)	6000[b]	Manchester et al. (2000)
Rice	1006	Hattori et al. (1995)
Strawberry	12	Hattori et al. (1995)
Sunflower (seed)	29000[b]	Manchester et al. (2000)
Sweet corn	1366	Hattori et al. (1995)
Tall fescue	5288	Hattori et al. (1995)
Taro	55	Hattori et al. (1995)
Tart Cherries	2060–13460	Burkhardt et al. (2001)
Tomato	32	Hattori et al. (1995)
Tomato	112–506	Dubbels et al. (1995)
Tomato	2–16	Van Tassel et al. (2001)
Tomato	1067–1399	Pape and Lüning (2006)
Turmeric	120000[b]	Chen et al. (2003)
Walnuts	3500	Reiter et al. (2005a,b)
Welsh onion	86	Hattori et al. (1995)
Wheat	125	Hernandez-Ruiz et al. (2005)
White mustard (seed)	189000[b]	Manchester et al. (2000)
Wolf berry (seed)	103000[b]	Manchester et al. (2000)

[a] pg/mL.
[b] pg/g dry tissue (1 mol melatonin = 232 g).

tions as an antioxidant, anti-inflammatory chronobiotic and even possibly as an epigenetic regulator, via mechanisms including nuclear receptors, co-regulators, histone acetylating and DNA-methylating enzymes (Korkmaz and Reiter, 2008; Korkmaz et al., 2009a).

It is worth noting that oral administration of melatonin (dosages from 1 to 300 mg) (Vijayalaxmi et al., 2004) or 1 g melatonin daily for 30 days (Nordlund and Lerner, 1977) resulted in no negative side effects.

The following review of the therapeutic potential of melatonin will distinguish between the ways to increase dietary intakes of melatonin, i.e., to obtain physiological concentrations, and supplementation with supraphysiological dosages of melatonin that leads to pharmacological concentrations.

6.1. Increasing melatonin dietary intakes

As mentioned above, relevant amounts of melatonin have been found in many roots, leaves, fruits and seeds of several plant species, although the concentration of melatonin is greatly variable in species and plant tissues (Table 1) (Paredes et al., 2009). It may be of great interest to identify beneficial plant properties related to the presence of melatonin, and whether the positive health effects observed in humans could be due to melatonin consumed in the diet, and whether melatonin may be working synergistically with other antioxidant molecules present in plants. An efficient uptake of melatonin from food should be expected to influence blood plasma concentration, which is basically very low (200 pg mL^{-1} (862 pmol L^{-1}) at the maximal night level, and below

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Table 2
Examples of exogenous melatonin intakes and resulting amounts found in the body.

Species	Body part	Physiological value (pg/mL)	Oral intake amounts	Concentration (pg/mL)	Delay after intake	Reference
European see bass	Plasma	18	2.5 mg/kg[a] 0.1 mg/kg[a]	464 170	45 min	Rubio et al. (2004)
Human	Saliva	–	5 mg	4199	30 min	Wirz-Justice et al. (2002)
	Serum	3 to 20	–	–	–	Reiter (1991)
		15	0.1 mg 0.3 mg 1 mg 10 mg	50 120 410 6825	4 h	Dollins et al. (1994)
Chick	Plasma	19	3.5 ng/g plant food[b]	33	90 min	Hattori et al. (1995)
Wistar rat	Small intestine Lower gut Feces	–	1 μg[c]	13900[d] 720[d] 9400[d]	immediately after a 2 h-infusion period 6 h	Messner et al. (1998)

[a] mg/kg BW (Body Weight).
[b] Corn, milo, beans, rice.
[c] 500 ng/h continuous infusion, for 2 h.
[d] Melatonin + metabolites, pg/g wet weight tissue (1 mol melatonin = 232 g).

10 pg mL^{-1} (43 pmol L^{-1}) during the day) (Hardeland and Pandi-Perumal, 2005). The question arises whether the amounts present in the food can be sufficient to increase the plasma level of melatonin, as previously proposed by Hattori et al. (1995). It is worth noting that gastrointestinal tract plays a role not only in melatonin intake, participates in enterohepatic cycling, but also as an extrapineal site of melatonin biosynthesis (Konturek et al., 2007).

6.2. Supplementation with supraphysiological dosages of melatonin

Supplemental melatonin (i.e., use at supra-physiological doses), unlike that derived from natural sources (e.g. "greens" products such as wheatgrass or ryegrass), needs a pre-market authorization since it is regarded as a medicinal product.

To focus on the antioxidant effects of melatonin, several clinical studies have been carried out with patients in an attempt to assess the activity of melatonin towards radical-induced damage (Gitto et al., 2001b; Fulia et al., 2001; Ochoa et al., 2003; Pappolla et al., 2000, 2003). Melatonin turned out to be a modulator of oxidative stress under acute conditions such as surgery (Kücükakin et al., 2009). Under these conditions, there is a massive inflammatory response with production of cytokines (interleukins: IL-1, IL-2, IL-6, IL-8) that play a role in cell activation and in the subsequent formation of ROS and RNS (Gitto et al., 2004). Several clinical trials showed that melatonin could be efficient in preventing cell damage. As an example, Fulia et al. (2001) supplemented 10 newborns with asphyxia with 80 mg melatonin (8 doses of 10 mg, separated by 2 h intervals) per os, within the first 6 h of life, and monitored oxidative and nitrosative stress markers by assaying malondialdehyde (end-product of lipid peroxidation) and nitrite/nitrate (nitric oxide-derived products) levels, before and after melatonin administration. Whereas these markers were elevated in newborns with asphyxia compared with newborns without asphyxia, the patients treated with melatonin exhibited lower levels of malondialdehyde and nitrate/nitrite than in the placebo group without melatonin. Moreover, no death was observed in the group with melatonin, vs. 3 deaths in the placebo group. These results indicate that melatonin may be beneficial in the treatment of newborn infants with asphyxia, and these beneficial effects may be related both to the antioxidant properties of melatonin and to its ability to increase the efficiency of mitochondrial electron transport. Similarly, Gitto et al. (2001b) determined the serum concentration of lipid peroxidation products (malondialdehyde, 4-hydroxyalkenals) in 10 septic newborns given 20 mg melatonin (two oral doses of 10 mg each, with a 1-h interval) or placebo; the concentrations of these oxidation products in newborns with sepsis were significantly higher than those in healthy infants without sepsis. In contrast, there was a significant reduction of these concentrations in septic newborns treated with melatonin to the levels observed in the healthy controls. Melatonin also improved the clinical outcome of the septic newborns, as 10 out of all septic children who were not treated with melatonin died within 72 h after diagnosis of sepsis whereas none of the 10 septic newborns treated with melatonin died. Finally, in neonates undergoing surgery, where clinical outcomes seems to depend on oxidative stress-induced damage, ten newborns (group 1), 5 newborns with surgical malformations and respiratory distress (group 1a) and 5 with isolated abdominal surgical malformations (group 1b) received a total of 10 doses of melatonin (10 mg/kg) at defined times interval for 72 h, within 3 h after the end of the operation. Ten surgical neonates (group 2) did not receive melatonin, and twenty healthy neonates (group 3) served as controls (Gitto et al., 2004). Surgical stress markers (IL-6, IL-8, TNF-α, nitrite/nitrate levels) were measured at the end of the operation, before treatment (melatonin or placebo) and respectively 24 h, 72 h, and 7 days after treatment. Postoperative value of cytokines and nitrite/nitrate levels of groups 1 and 2 were significantly higher than in group 3. Compared with group 1b, group 2 displayed significantly higher cytokines and nitrite/nitrate levels at 24 h, 72 h, and at 7 days intervals. In group 1a the immediate postoperative values of cytokines were significantly higher than in groups 1b and 2, but a significant improvement was observed after administration of melatonin with significantly lower levels of IL-6 and IL-8 with respect to group 2. This study showed that melatonin reduced cytokines and nitrate/nitrite levels, showing potent antioxidant properties with improvement in clinical outcome. Melatonin, via its antioxidant properties, could thus modulate oxidative stress, which may improve organ function and reduce morbidity and mortality, at least as tested in newborns where the drug was able to decrease oxidative stress induced by surgery (Kücükakin et al., 2009). Melatonin has been investigated in a wide range of diseases, such as heart disease, Alzheimer's disease, AIDS, diabetes, depression, cancer (Beyer et al., 1998).

As an example of use of melatonin in chronic diseases, it has been shown that melatonin administration in patients with Alzheimer disease significantly delayed the progression of the disease and decreased brain atrophy as assessed by MRI (Brusco et al., 1998). It is now admitted that the neuronal loss in Alzheimer disease

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could result from radical-induced apoptosis of neuronal cells. It thus seems that the antioxidant properties of melatonin could protect neurons from the degeneration leading to cell death. Moreover, no toxicity or adverse effect has been reported consecutively to the daily administration of melatonin (1–300 mg, or 4.3–1291.5 μmol) (Jan et al., 2000; Seabra et al., 2000), which could encourage the long-term administration of melatonin in patients suffering a neurodegenerative disease such as Alzheimer disease. Melatonin has been reported to inhibit the intrinsic apoptotic pathways in neurodegenerative diseases including stroke, Alzheimer disease, Parkinson disease, Huntington disease, and amyotrophic lateral sclerosis (Wang, 2009).

Experimental studies in rodents showed that melatonin (5 mg or 21.5 μmol/kg body weight, 5 day/week, for 50 days) decreased the oxidative stress induced by D-galactose (DG) treatment to mimic natural aging; melatonin thus lowered the formation of protein carbonyls in the liver, kidney and brain of DG treated mice, decreased TBARS production in serum and brain, prevented DG-induced increase of soluble receptors for advanced glycation endproducts (sRAGE), decreased the expression of the pro-apoptotic bax and caspase-3 proteins in splenocytes, and lowered Aβ protein expression in the brain (Hsieh et al., 2009). It has been suggested that the anti-aging effect of melatonin may be related to the reduction of Aβ-induced lipid peroxidation *in vitro* (Feng and Zhang, 2004). More specifically, melatonin (10 mg or 43 μmol/kg in the drinking water, for 9 months, starting 1 month after birth) seemed able to modulate the expression of alpha-secretase in a model of senescence-accelerated prone mice (SAMP8), thereby contributing to the decrease of the high levels of aggregating Aβ protein in this model (Gutierrez-Cuesta et al., 2008). Moreover, in this model exhibiting a marked acceleration of aging in relation with oxidative stress (Caballero et al., 2009), melatonin improved pro-survival signals and reduced pro-death signals, as shown by the decrease of Bid and the increase of Bcl-2 levels, in comparison with non-treated SAMP8. Melatonin also exhibited antiapoptotic properties after ischemic neuronal injury in the rat, via enhancement of Bcl-2 induction and DNA repair capacity (Sun et al., 2002). The influence of melatonin on the antioxidant role of Bcl-2 and more generally against the oxidative stress-related impairments has been demonstrated, paving the way for the treatment of age-induced neural processes. Moreover, a significant decrease in the fluidity of synaptosomal and mitochondrial membranes of SAMP8 mice was observed, as assessed by fluorescent polarization after incorporation of a probe (TMA-DPH). In this model, melatonin could prevent the rigidity observed in mitochondrial membranes, and it could slow down the aging process by maintaining membrane fluidity and structural pathways (García et al., 2010).

More generally, there is a search for any therapeutic agent improving the quality of life of the elderly, and melatonin administration may improve the temporal organization during aging (Karasek, 2004). Although recommendations of melatonin supplementation in elderly should be considered, there is a need for extensive studies on the use of melatonin in order to accede to a better quality of life in advanced age.

Beneficial antioxidant effects of low doses of melatonin have also been shown in several chronic diseases, such as rheumatoid arthritis (10 mg/day) (Forrest et al., 2007), primary essential hypertension in elderly patients (5 mg/day) (Kedziora-Kornatowska et al., 2008), type 2 diabetes in elderly patients (5 mg/day) (Kedziora-Kornatowska et al., 2009) or females suffering infertility (3 mg/day) (Tamura et al., 2008). Melatonin attenuates molecular and cellular damages resulting from cardiac ischemia/reperfusion (a massive release of free radicals is involved in the tissue damage following the reperfusion process); anti-inflammatory and antioxidative properties of melatonin also seem to be involved

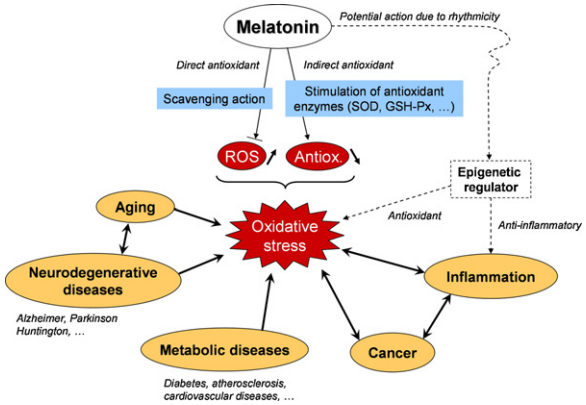

Fig. 5. Melatonin: action on oxidative stress and potential applications in human disease and aging. GSH-Px: glutathione peroxidase; ROS: reactive oxygen species; SOD: superoxide dismutase.

in the protection against vascular disease and atherosclerosis development (Dominguez-Rodriguez et al., 2009). Its protective action in ischemia/reperfusion processes could also be beneficial in limiting damage following organ transplantation (Fildes et al., 2009).

Melatonin may be useful for the treatment of inflammatory disease, as it reduces inflammatory injury by blocking transcription factors and NFκB (Li et al., 2005), thereby decreasing further ROS formation within cells. In the same way, melatonin seems able to inhibit the activation of cyclooxygenase 2 (COX-2) and of the inducible NO synthase (iNOS), both activated in chronic inflammation disorders (Deng et al., 2006). Physiologic data suggest that melatonin is an important regulator of both inflammation and motility in the gastrointestinal tract, and some studies in humans suggest that supplemental melatonin may have an ameliorative effect on ulcerative colitis (Terry et al., 2009).

There has also been evidence of suppressive effects of melatonin on carcinogenesis (Anisimov et al., 2006). Indeed, the positive effects of melatonin treatment have been demonstrated in patients with advanced cancer. Mills et al. (2005) conducted a systematic review of ten randomized controlled trials of melatonin in 643 solid tumor cancer patients and its effect on survival at 1 year. Melatonin reduced the risk of death after 1 year (relative risk: 0.66, 95% confidence interval: 0.59–0.73), without any severe adverse events. A pilot phase II study (Lissoni et al., 1995), conducted in women with metastatic breast cancer who had progressed in response to tamoxifen alone, would suggest that the concomitant administration of melatonin, which was given orally at 20 mg/day in the evening, may induce objective tumour regressions in metastatic breast cancer patients refractory to tamoxifen alone, and this effect was observed with a good tolerance.

More specifically, a very recent study reported a beneficial action of high doses of melatonin (20 mg/kg) for inhibiting apoptosis and liver damage resulting from the oxidative stress in malaria, which could be a novel approach in the treatment of this disease (Srinivasan et al., 2010).

To conclude, a growing body of evidence suggests that melatonin could have significant potential for beneficial properties, especially on chronic diseases, as recently reported (Reiter et al., 2006; Korkmaz et al., 2009b; Jung-Hynes et al., 2010). By contrast with classical antioxidants that often failed to exhibit beneficial effects in metabolic disease and aging (Vivekananthan et al., 2003; Miller et al., 2005; Bjelakovic et al., 2007), melatonin displays protective effects, especially related to its antioxidant and anti-inflammatory properties (Fig. 5). This should lead to future clinical research with the aim to improve public health.

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