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REVIEW ARTICLE

Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin

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Abstract

The discovery of melatonin and its derivatives as antioxidants has stimulated a very large number of studies which have, virtually uniformly, documented the ability of these molecules to detoxify harmful reactants and reduce molecular damage. These observations have clear clinical implications given that numerous age-related diseases in humans have an important free radical component. Moreover, a major theory to explain the processes of aging invokes radicals and their derivatives as causative agents. These conditions, coupled with the loss of melatonin as organisms age, suggest that some diseases and some aspects of aging may be aggravated by the diminished melatonin levels in advanced age. Another corollary of this is that the administration of melatonin, which has an uncommonly low toxicity profile, could theoretically defer the progression of some diseases and possibly forestall signs of aging. Certainly, research in the next decade will help to define the role of melatonin in age-related diseases and in determining successful aging. While increasing life span will not necessarily be a goal of these investigative efforts, improving health and the quality of life in the aged should be an aim of this research.

Keywords: *Melatonin; free radical; hydroxyl radical; oxidative stress; nitrisative stress*

Introduction

Because they were dermatologists with an abiding interest in abnormal skin pigmentation in humans, Lerner and colleagues (1958; 1959a; 1960) worked diligently to isolate and characterize the pineal molecule that lightened the skin of tadpoles (McCord and Allen, 1917). After identifying the methoxy derivative of serotonin, the molecule now known as melatonin was found to be ineffective in causing the accumulation of the pigment granules around the nucleus of human melanophores. Despite this, the extensive effort required to isolate the indoleamine from an estimated 250,000 bovine pineal glands was not wasted and it is likely that they (Lerner *et al.*, 1959b; Lerner and Wright, 1960) did not envisage what the wide-ranging actions of melatonin would contribute to biology and clinical medicine. While the effort to isolate and identify melatonin was monumental, it was made even more difficult by the fact that the pineal glands from which melatonin was extracted were presumably from tissues collected from cattle killed during the day, when melatonin levels are at their trough. Only after its discovery was it shown that melatonin synthesis in the pineal is much higher at night than during the day (Axelrod *et al.*, 1964; 1965; Quay, 1964) although there were earlier morphophysiological indications that the pineal gland was more active in darkness than in light (Quay, 1956; Mogler, 1958).

While it had long been suspected that the pineal gland was somehow linked to reproductive physiology (Kitay and Altschule, 1954; Thieblot and LeBars, 1955), the first incontestable evidence for this came when it was discovered that surgical removal of the pineal gland of a photoperiodic species, the Syrian hamster, prevented the dramatic shutdown of the reproductive system that occurred when this species was exposed to short days (Hoffman and Reiter, 1965a; 1965b; Reiter and Hester, 1966). The implication of these findings was clear, namely that the pineal gland, via its secretory product melatonin, likely signaled the seasonally

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changing light:dark environment and regulated annual cycles of reproduction accordingly; this was definitively documented less than a decade later in a study in which hamsters were maintained under natural photoperiodic and temperature conditions (Reiter, 1973a). The integrated role of photoperiod, the pineal gland, and melatonin in seasonal reproduction is true for both short-day and long-day breeding species (Reiter, 1973b; 1974; Tamarkin *et al.*, 1985; Lincoln *et al.*, 2003).

The functional repertoire of melatonin, however, extends well beyond its control of annual cycles of sexual physiology in photoperiodic animals. The circadian rhythm of melatonin has been unequivocally linked to biological rhythmicity (Arendt, 2005; Masson-Pevet, 2007), sleep (Gorfine and Zisapel, 2009; Jan *et al.*, 2009), immune function (Cardinali *et al.*, 2008; Maldonado *et al.*, 2009), blood pressure (Simko and Paulis, 2007; Reiter and Korkmaz, 2008), diabetes (Peschke, 2008; Korkmaz *et al.*, 2008), neurodegenerative diseases (Pappolla *et al.*, 2000; Reiter *et al.*, 2004), ischemia/reperfusion injury (Reiter *et al.*, 2005a; Tengattini *et al.*, 2008), cell physiology (Benitez-King, 2006), and cancer inhibition (Blask *et al.*, 2005; Shiu, 2007; Korkmaz *et al.*, 2009a), among others. Lerner and colleagues (1958) would surely be pleased to learn that the role of melatonin in skin physiology is also coming into focus (Slominski *et al.*, 2007; Fischer *et al.*, 2008). Many of the actions of melatonin described in the reports mentioned here are a result of its interactions with cell membrane receptors for the indole (Barrett *et al.*, 2003; Dubocovich and Markowska, 2005); perhaps in some cases, however, melatonin's actions may additionally involve its association with binding sites in the nucleus (Acuna-Cashoviejo et al., 1994; Weisenberg *et al.*, 1995; Tomas-Zapico and Coto-Montes, 2005) or with molecules in the cytosol (Pozo *et al.*, 1997; Benitez-King, 2006).

In 1993, an additional discovery was made which further broadened the functional role of melatonin in physiology. Tan and co-workers (1993) reported that melatonin functioned as a direct free radical scavenger, an action that is receptor-independent. This unexpected finding opened a large new field of investigation because free radicals, which are neutralized by antioxidants, are involved in a vast number of diseases (Cerutti, 1994; Halliwell, 1997; Siu *et al.*, 2006; Khansari *et al.*, 2009). The current review primarily summarizes data related to receptor-independent and free radical scavenging effects of melatonin which reduce oxidative stress.

Melatonin: an antioxidant and the antioxidant cascade

In 1993, Tan and co-workers made the novel observation that melatonin had the capability of donating electrons *in vitro* to reduce the reactivity of molecules with an unimpaired electron in their valance orbital, i.e. free radicals. Thus, melatonin, in addition to its actions via receptors on the limiting membrane and within the nuclei of cells, also apparently directly interacted with potentially damaging agents without the necessity of first binding to a receptor. These *in vitro* observations were quickly supported by *in vivo* findings which showed the melatonin reduced molecular damage associated with massive free radical generation (Melchiorri *et al.*, 1994; Tan *et al.*, 1994). These non-receptor-mediated actions of melatonin have proven important in the ability of this indoleamine to protect against damaging oxygen and nitrogen-based reactants under many different high oxidative stress conditions and in many different species (Escames *et al.*, 1997; Hardeland *et al.*, 2006; Hardeland, 2008; Tamura *et al.*, 2008a; Gitto *et al.*, 2009).

The study of Tan *et al.* (1993) used a highly reliable method to document the ability of melatonin to scavenge the devastatingly reactive hydroxyl radical (•OH). When melatonin was added to a mixture of hydrogen peroxide (H_2O_2) and the spin trapping agent, 5, 5-dimethyl-pyrroline N-oxide (DMPO), it very significantly reduced the formation of the DPMO-OH adduct. The presence of the adduct was identified using electron spin resonance (ESR) spectroscopy. Since melatonin markedly reduces the DMPO-OH adduct, these findings provided direct proof that melatonin scavenges the •OH making it unavailable to form adducts with DMPO (Figure 1). Molecules that had a chemical structure similar to that of melatonin were either less effective or totally ineffective in reducing DMPO-OH adduct formation. Similarly, other well known antioxidants, i.e.

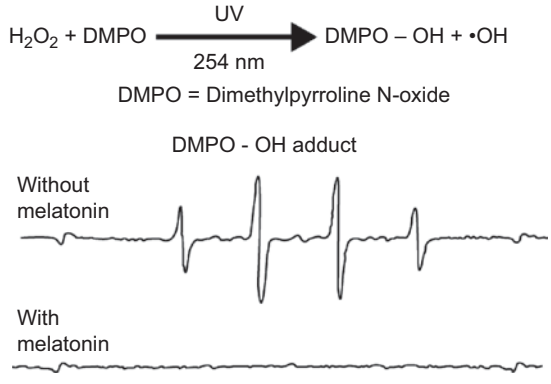

Figure 1. A summary of the methodology used by Tan *et al.* (1993) to document the hydroxyl radical (•OH) scavenging activity of melatonin. DMPO is a spin trapping agent. The spin trap forms adducts with the •OH (DMPO-OH) which are quantified by electron spin resonance spectroscopy (ESR). In the absence of melatonin, numerous DMPO-OH adducts were formed, as indicated by the ESR spectrum. When melatonin was added to the H_2O_2 + DMPO mixture, it quenched the •OH and reduced the DMPO-OH signal. UV = ultraviolet light.

mannitol and glutathione, also were less efficient than melatonin in reducing the formation of the DMPO-OH. The authors speculated that the unique chemical structure of this highly lipophilic resonance-stabilized molecule accounts for the ability of melatonin to function as an •OH scavenger. Any molecule that interferes with the ability of the •OH to mete out molecular damage is extremely important given that this radical species, among many that are generated, accounts for a significant portion of the total molecular damage that radicals and related products produce. Matuszak and co-workers (1997) also used ESR and the spin trap, DMPO, to document that melatonin detoxities the •OH.

Ebelt *et al.* (2000) used ESR to confirm the efficacy of melatonin in quenching the •OH. These investigators, using a different spin trapping agent than that of Tan *et al.* (1993) (i.e. 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide; DEPMPO) showed that melatonin quenches, in a dose-response manner, the formation of the OH-DEPMPO adduct. The •OH in this case were generated via the Fenton reaction in a non-buffered aqueous solution. The DEPMPO spin trap is somewhat more sensitive for the detection of oxygen-centered radicals than is DMPO which was utilized by Tan and colleagues (1993). Besides testing the ability of melatonin to scavenge the •OH in an aqueous solution, Ebelt *et al.* (2000) found that melatonin also prevented •OH-mediated lipid peroxidation, showing that the indole functions as a radical scavenger in both aqueous and lipid environments. These observations are consistent with many earlier reports documenting the ability of melatonin to ameliorate the oxidation in lipids both *in vitro* and *in vivo* (Sewerynek *et al.*, 1995a; 1995b; Giusti *et al.*, 1996; Livrea *et al.*, 1997).

The •OH scavenging activity of melatonin has been repeatedly confirmed using other highly reliable methodologies as well (Stasica *et al.*, 1998; 2000; Turjanski *et al.*, 1998; Bandyopadhyay *et al.*, 2000; Brömme *et al.*, 2000; Qi *et al.*, 2000a; 2000b; Li *et al.*, 2002; Fukutomi *et al.*, 2006; Zavodnik *et al.*, 2006; Velkov *et al.*, 2009) and the studies have been extended to show that this indoleamine also neutralizes other reactive oxygen and nitrogen-based reactants (Gilad *et al.*, 1997; Zhang *et al.*, 1998; Ceraulo *et al.*, 1999; Noda *et al.*, 1999; Blanchard *et al.*, 2000; Tan *et al.*, 2000; 2002; Reiter *et al.*, 2001; 2003; 2008a; Turjanski *et al.*, 2001; Allegra *et al.*, 2003; Rosen *et al.*, 2006). Some of the methods used to estimate the scavenging actions of melatonin included pulse radiolysis, salicylate trapping, reduced oxidative damage, chemiluminescence and functional theory computational tools. In these studies, melatonin was found to scavenge nitric oxide ($NO^{\bullet}$), the peroxynitrite anion ($ONOO^{-}$), singlet oxygen ($^1O_2$), superoxide anion radical ($O^{\bullet}_2$), hydrogen peroxide (H_2O_2) (see below, however), and hypochlorous acid (HOCl) (Reiter *et al.*, 2001; Allegra *et al.*, 2003; Matuszak *et al.*, 2003; Pandi-Perumal *et al.*, 2006). These investigations were conducted using pure chemical systems, *in vitro* cultured cells, *in vivo* and *in silico* methodologies. In terms of the •OH, the rate at which melatonin scavenges this radical is $2.8–7.1 \times 10^{10}$ $m^{-1}s^{-1}$. This is a rate constant similar to that of other highly effective •OH scavengers. The findings regarding the ability of melatonin to neutralize H_2O_2 are in conflict (cf. Tan *et al.*, 2000; Fowler *et al.*, 2003).

One of the metabolites that are formed when melatonin detoxifies the •OH is cyclic 3-hydroxymelatonin (c-3OHM) (Tan *et al.*, 1998a). This metabolite was generated in a cell-free chemical system and it was structurally identified using a combination of mass spectrometry, proton nuclear magnetic resonance (^{2}H-NMR), COSY ^{1}H-NMR analysis, and calculations on the relative thermodynamic stability. c3-OHM was also measured in the urine of rats and humans using high performance liquid chromatography (HPLC) (Ma *et al.*, 2006). Also, when rats were challenged by exposure to ionizing radiation, a procedure which generates massive levels of •OH, the amount of urinary c3-OHM increased dramatically. These findings indicate that melatonin scavenges the •OH *in vivo* and that the quantity of c3-OHM in the urine is an index of free radical detoxification by the indoleamine. Thus, c3-OHM is a footprint of melatonin's action as a •OH scavenger. The results also suggested that melatonin would serve as a potent radioprotective agent, a speculation that has been repeatedly confirmed in subsequent investigations (Vijayalaxmi *et al.*, 1996a; 1999a; 2004; Karbownik and Reiter, 2000; Shirazi *et al.*, 2007). It has also been shown that c3-OHM is probably produced when melatonin scavenges other reactive oxygen species as well, e.g. $^1O_2$ (Siwicka *et al.*, 2008). In addition to its presence in the urine of humans, c3-OHM is also excreted, as expected, via the urine in rats (Ma *et al.*, 2006). Besides being produced when melatonin scavenges two •OH, others have also reported its presence after the interaction of melatonin with $ONOO^{-}$ (Zhang *et al.*, 1999; Peyrot *et al.*, 2003).

Subsequent investigations have now shown that melatonin is actually a prodrug for a family of other molecules that also have the capability of neutralizing oxygen and nitrogen-based reactants (Hardeland and Pandi-Perumal, 2005). Hence, when c3-OHM is formed during the scavenging of two radicals by melatonin, it is not the terminal agent in this metabolic pathway. Rather, c3-OHM is itself an effective scavenger and in doing so it generates N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) (Tesoriere *et al.*, 2001). Until recently, c3-OHM and AFMK were not available for use as standards for their measurement, e.g., by HPLC, or to test their biological activity. Recently, a method was published for the

synthesis of the cyclic derivative of melatonin (Siwicka *et al.*, 2004) and AFMK can be purchased from Sigma Chemical Company (St. Louis, MI, USA).

Although c3-OHM was discovered rather recently (Tan *et al.*, 1998a), the existence of AFMK has been known for decades. In 1974, Hirata and colleagues described the presence of AFMK in the brain and thought it was exclusively formed by the enzyme 2,3-indole dioxygenase (Hirata *et al.*, 1974). It is now known that AFMK is also non-enzymatically generated when melatonin interacts with H_2O_2 (Tan *et al.*, 2000). This could be an important function of melatonin in protecting cells from oxidative stress, given that H_2O_2 is the precursor of the •OH. If this reaction occurs *in vivo*, melatonin would function like glutathione peroxidase which enzymatically also removes H_2O_2 thereby reducing the generation of the •OH. In numerous experimental models, melatonin has been found to produce AFMK (Hardeland *et al.*, 1995; 2003; Silva *et al.*, 2000; Ximenes *et al.*, 2001; de Almeida *et al.*, 2003). Some of these conversions require enzymatic processes while others do not. Given that AFMK exists in evolutionarily ancient unicellular organisms, whereas 6-hydroxymelatonin is a major hepatic metabolite of melatonin in mammals, we have speculated that the formation of AFMK predated the hepatic metabolism of melatonin to 6-hydroxymelatonin (Tan *et al.*, 2007a).

Leucocytes are also an important venue for the formation of AFMK. When activated, these cells can produce AFMK at levels five-fold above what is generated under basal conditions (Silva *et al.*, 2004). AFMK is also produced in the rat retina with peak levels during darkness; this rise coincides with the nocturnal elevation in retinal melatonin production (Rozov *et al.*, 2003). In skin as well, AFMK is a major metabolite of melatonin after this tissue is exposed to ultraviolet B radiation (Fischer *et al.*, 2006). Moreover, preliminary evidence suggests that in plants as well, AFMK is likely a melatonin metabolite (Tan *et al.*, 2007b), probably being formed when melatonin scavenges free radicals (Tan *et al.*, 2007c).

AFMK is not the terminal molecule in melatonin's antioxidative cascade. Rosen and co-workers (2006) have documented that it interacts with ROS/RNS to form N1-acetyl-5-methoxykynuramine (AMK). Moreover, AMK collaborates with the ABTS cation radical to produce oligomers (Than *et al.*, 2006) while 3-acetamidomethyl-6-methyoxycinnolinone and N1-acetyl-5-methoxy-3-nitrokynuramine are formed when AMK scavenges the $ONOO^-$ (Guenther *et al.*, 2005).

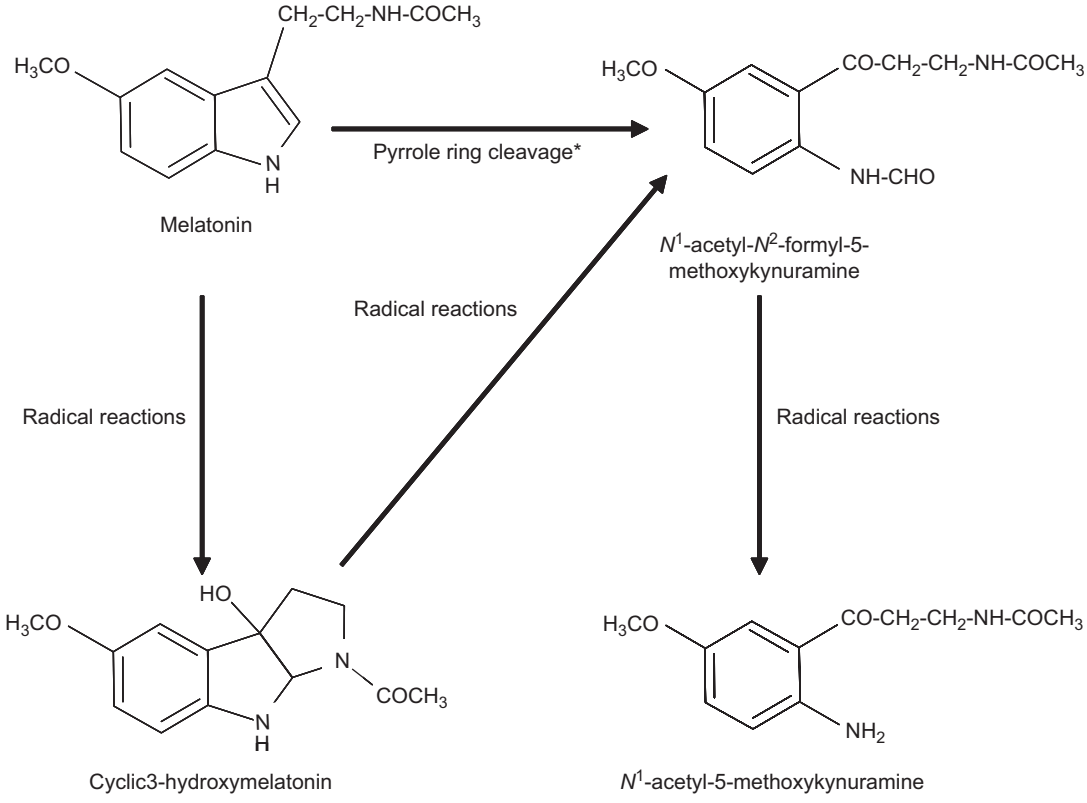

Figure 2. The antioxidant cascade of melatonin. When melatonin interacts with oxidants it generates cyclic 3-hydroxymelatonin (c3OHM) and N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK). Both c3OHM and AFMK are likewise scavengers leading to the formation of N1-acetyl-5-methoxykynuramine (AMK); this latter molecule is a radical scavenger as well. This cascade of reactions greatly increases the effective concentration and scavenging efficacy of melatonin. Each of the metabolites has been specifically identified using nuclear magnetic resonance.

The scheme illustrated in Figure 2 summarizes the antioxidative cascade of melatonin; in this pathway the functions of melatonin are amplified by the fact that all of its metabolites are also free radical scavengers. In this context, melatonin serves as a prodrug. Each of the products in this pathway has been identified and is known to be formed during their respective interactions with radicals and radical derivatives. We have estimated that, as currently described, this cascade of reactions may neutralize up to 10 radical products (Tan *et al.*, 2007a). At this point the primary, secondary, tertiary and quaternary products of melatonin are believed to have the capability of neutralizing toxic reactants. Whether the series of reactions indentified is the complete cascade or whether other potential detoxifying molecules are yet to be described remains unknown. Clearly, the proposed reactions of melatonin greatly increase the effective concentration of this pluripotent antioxidant. Not uncommonly, non-scavenger metabolites of antioxidants are recycled, e.g., ascorbic acid recycles oxidized vitamin E; that one melatonin metabolite may also be converted back to melatonin has also been proposed (Mahal *et al.*, 1999), although this observation requires confirmation.

A large number of antioxidants have been identified and, because of the rather brief history of investigation into melatonin, most are better known than melatonin. For example, the classic vitamin antioxidants have been studied for many decades and even much of the lay public is cognizant of their ability to detoxify free radicals or their derivatives.

While there is a remarkably large amount of information regarding the substantial efficacy of melatonin in reducing oxidative stress, there are several publications claiming melatonin is either not a relevant scavenger of at least the peroxyl radical or it is only modestly effective in the detoxification of free radicals. These studies were performed *in vitro* and the direct extrapolation of the results to the *in vivo* system is problematic.

Soon after the report appeared in which melatonin was found capable of scavenging the •OH (in a pure chemical system) (Tan *et al.*, 1993), we continued these studies by examining the ability of melatonin to trap a variety of toxic reactants in an *in vitro*/chemical environment (Marshall *et al.*, 1996). Using a series of classic tests, which theoretically identify potent antioxidants, melatonin was found to protect catalase and also reduce the oxidation of 5-thio-2-nitrobenzoic due to its ability to scavenge HOCl. Melatonin was also found to limit the oxidation of ox-brain phospholipids with a calculated IC_{50} of 210 μM. Melatonin reacted with trichloromethylperoxyl radical with a rate constant of 2.7×10^{10} $M^{-1}s^{-1}$, failed to scavenge the $O_2^{\cdot-}$ and only weakly protected DNA from free radical damage in a ferric-bleomycin system. On the basis of these exclusively *in vitro* data, there was hesitancy about claiming that melatonin was an aggressive *in vivo* free radical scavenger (Marshall *et al.*, 1996). This feeling was also voiced in a recent review (Halliwell, 2009) although the author of this report acknowledged that melatonin has beneficial actions in protecting against oxidative stress without offering an explanation as to how this protection is achieved if not via antioxidative processes.

Antunes and co-coworkers (1999) ostensibly thoroughly tested the *in vitro* activity of melatonin as a peroxyl radical ($LOO^{\bullet}$) scavenger, i.e. as a chain breaking antioxidant during the propagation of lipid peroxidation. In their system, they claimed melatonin was ineffective in directly neutralizing the $LOO^{\bullet}$ although they found melatonin did retard iron-catalyzed oxidation of lipids and, as a consequence, they classified melatonin as a preventive antioxidant of the metal ion deactivating subclass. Due to its limited ability to function as a $LOO^{\bullet}$ scavenger and their presumption of low *in vivo* tissue concentrations, Antunes *et al.* (1999) concluded that melatonin has little value in the intracellular environment as an interrupter of lipid peroxidation once the process is initiated. In regard to the earlier reports indicating melatonin is a significant antioxidant, Antunes *et al.* (1999) cautioned about translating those *in vitro* findings to the *in vivo* situation; that limitation would presumably also apply to their own findings which were exclusively *in vitro* and outside the context of an intact cell. As will be seen below, in living animals melatonin compares very well with other antioxidants in terms of reducing free radical damage, including that to lipid molecules (some of these data are summarized in Table 1).

The reluctance of some researchers to accept that melatonin is an antioxidant is based on the outcomes of a small number of *in vitro* pure-chemical studies. This conclusion is drawn against a backdrop in excess of one thousand publications illustrating the ability of melatonin to reduce oxidative molecular damage both *in vitro* and *in vivo*. If the premise is accepted that melatonin is, in fact, insignificant as a free radical scavenger, then investigators will have to come up with a novel alternative mechanism(s) to explain the high efficacy of the indoleamine in limiting free radical damage. This challenge awaits further investigations of this important protective molecule.

Oxidative stress in cells and tissues: reduction with melatonin

Numerous oxygen- and nitrogen-based radicals as well as several non-radical species attack and destroy essential molecules intracellularly (Figure 3). The number of

Table 1. A multitude of studies have compared the relative efficacies of melatonin with other antioxidants in terms of their radical scavenging activities or their protective actions against oxidative stress. Some of these findings are summarized in this table. The majority of these studies have shown melatonin to be superior to other antioxidants under the specific conditions of the experiments. The preponderance of *in vivo* evidence especially indicates that melatonin is often better than other protective molecules in limiting free radical damage. As well as the reports summarized in the table, other data are reviewed in the text.

Antioxidants compared (reference)	Antioxidant dose	Free radical generator(s)	Species/tissue/ medium	Endpoints	Outcome
Melatonin, glutathione, mannitol(Tan *et al.*, 1993)	Mel = 5–90 μM GSH = 70–170 μM Man = 100–160 μM	Ultraviolet light + H_2O_2	Pure chemical system	Disappearance of DMPO-OH adducts	Melatonin was more effective than GSH or mannitol in scavenging the ·OH
Melatonin, vitamin C, vitamin E (trolox), N-acetylcysteine (Martin *et al.*, 2000a)	Mel = 100 μM Vit C = 1 mM Vit E = 1 mM NAC = 1 mM	t – BHP	Isolated rat brain mitochondria	GSH, GPx, GRd	Melatonin, at lower concentrations, protected against GSH oxidation and GPx and GRd inhibition
Melatonin, vitamin C, vitamin E (trolox) (Qi *et al.*, 2000b)	Mel = 0.25–10 μM Vit C = 1–250 μM Vit E = 1–250 μM	H_2O_2 + chromium	Purified calf thymus DNA	8-OHdG	Melatonin was more effective (lower IC_{50}) than vit C and E in reducing DNA damage
Melatonin, mannitol, vitamin E (trolox) (Qi *et al.*, 2001)	Mel = 0.01–4 μM Man = 0.01–4 μM Vit E = 0.01–4 μM	ALA + Fe^{2+}	Purified calf thymus DNA	8-OHdG	Melatonin was more effective (lower IC_{50}) than mannitol or vitamin E
Melatonin, vitamin E, N-acetylcysteine (Sener *et al.*, 2003)	Mel = 10 mg kg^{-1} Vit E = 30 mg kg^{-1} NAC = 150 mg kg^{-1}	Acetaminophen	*In vivo* rat (blood, liver, kidney)	BUN, ALT, AST, GSH, MDA, oxidized protein	Melatonin provided greater protection, at a lower dose, than vit E or NAC
Melatonin, vitamin C, 2-lipoic acid, xanthurentic acid, reserveratrol, EGCG (Lopez-Burrillo *et al.*, 2003)	Mel = 0.5–10 μM Vit C =0.5–200 μM LA = 0.5–500 μM XA = 0.5–200 μM Res = 0.5–100 μM EGCG = 0.5–20 μM	Chromium + H_2O_2	Purified calf thymus DNA	8-OHdG	Melatonin was more effective (lower IC_{50}) than other antioxidants
Melatonin, vitamin C, glutathione, vitamin E (trolox), NADH, NADPH (Tan *et al.*, 2003)	Mel = 2.5 μM Vit C = 5 μM GSH = 5 μM Vit E = 5 μM NADH = 5 μM NADPH = 5 μM	$ABTS^{\cdot+}$	Pine chemical system	Scavenging of the $ABTS^{\cdot+}$	Melatonin was more effective (lower IC_{50}) than other antioxidants
Melatonin, vitamin E (Jou *et al.,* 2004)	Mel = 0.1–10 mM Vit E = 0.1–10 mM	H_2O_2	Cultured rat astrocytes	ROS fluorescence	Melatonin better than vitamin E in quenching fluorescence
Melatonin, vitamin C, vitamin E (Guha *et al.*, 2007)	Mel = 10–20 mg kg^{-1} Vit C = 100–400 mg kg^{-1} Vit E = 100–400 mg kg^{-1}	*Plasmodium yoelii*	Rat liver	MDA, protein, carbonyl, GSH	Melatonin at lower doses provided better protection than vitamins C or E
Melatonin, vitamin E, β-carotene (Sadir *et al.*, 2007)	Mel = 10 mg kg^{-1} Vit E = 40 mg kg^{-1} Car = 40 mg kg^{-1}	Cyclo-phosphamide	Rat urinary bladder and urine	MDA, nitrites/nitrates	Melatonin, even at lower does, was as good as vitamin E and better than β-carotene
Melatonin, vitamin E (Kara *et al.*, 2008)	Mel = 10 mg kg^{-1} Vit E = 60 mg kg^{-1}	Cadmium	Rat liver and kidney	MDA, SOD	Protection was equivalent but melatonin was half the dose of vitamin E
Melatonin, N-acetylcysteine (Hong *et al.*, 2009)	Mel = 2.5–10 mg kg^{-1} NAC = 100 mg kg^{-1}	CCl_4	Rat liver and serum	MDA, GPx, hydroxyproline, hyaluronic acid, laminia, PIII NP	Melatonin, at lower doses, provided better protection in most cases
Melatonin, glutathione, vitamin E (trolox) (Liepnitz *et al.*, 2009)	Mel = 200 μM GSH = 100 μM Vit E = 1.5 μM	Glycine	Rat cortical homogenates	MDA	All equally prevented lipid peroxidation but melatonin given at a lower dose
Melatonin, vitamin E (Sharma and Haldar, 2009)	Mel = 5 mg kg^{-1} Vit E = 10 mg kg^{-1}	Phenyl-hydrazide	Palm squirrel spleen	MDA, SOD	Melatonin was more effective even though given at a lower dose

DMPO-OH + 5,5-dimethyl-pyrroline-N-oxide-hydroxy radical adduct (identified by electron spin resonance spectroscopy); t-BHT = *tert*-butylhydroperoxide; GSH = reduced glutathione; GPx = glutathione peroxidase; GRd = glutathione reductase; 8-OHdG = 8-hydroxy-2-deoxyguanosine (a damaged DNA product); ALA = delta-amino levulinic acid; BUN = blood urea nitrogen; ALT = alanine aminotransferace; AST = aspirate aminotransferase; MDA = malondialdehyde (a product of lipid peroxidation); $ABTS^{\cdot+}$ = 2,2[1]-azinobis-(3-ethylbenzothiozoline-6-sulfonic acid cation ion; SOD = superoxide dismutase; CCl_4 = carbon tetrachloride; P III NP = procollagen III N-terminal peptide.

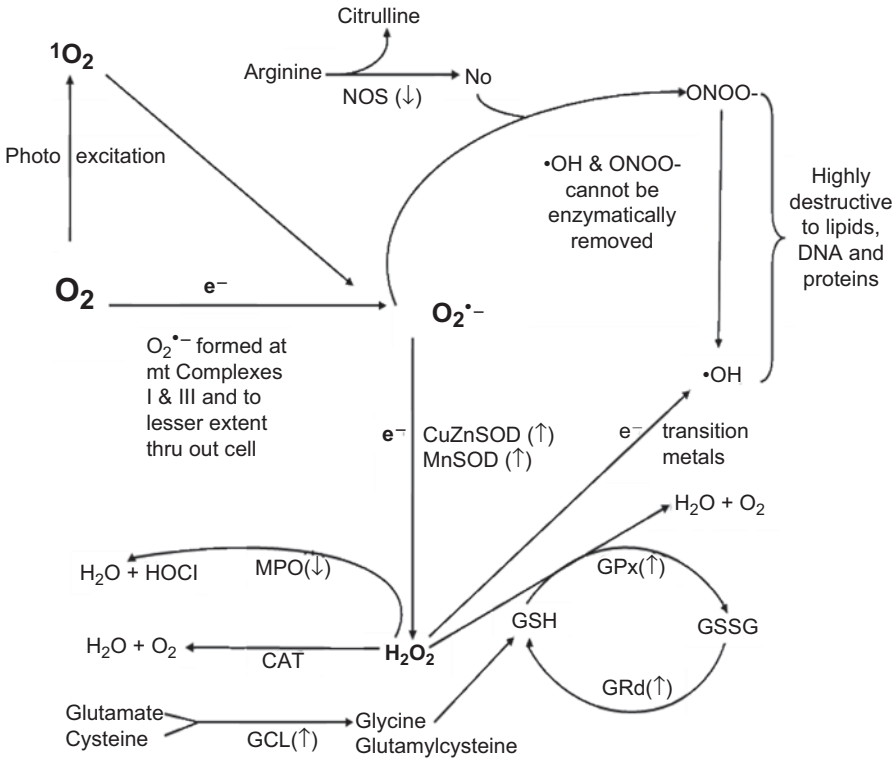

Figure 3. The toxic oxygen and nitrogen-based derivatives which are formed when oxygen is metabolically reduced. The most reactive metabolites, i.e. •OH and $ONOO^-$, are not enzymatically removed and the only way to protect against them is by scavenging. The figure also illustrates the antioxidative enzymes that are stimulated (↑) by melatonin as well as the pro-oxidative enzymes which are inhibited (↓). CAT = catalase; CuZnSOD = copper/zinc superoxide dismutase; GCL = glytamyl cysteine ligase; GPx = glutathione peroxidase; GRd, glutathione reductase; GSH = reduced glutathione; GSSG = oxidized glutathione; MnSOD = magnesium superoxide dismutase; MPO = myloperoxidase; NOS = nitric oxide synthase.

publications confirming the ability of melatonin to protect against both oxidative and nitrosative stress under *in vitro* and *in vivo* conditions is massive. These actions have been the subject of several reviews which discuss limited aspects of this field of research (Hardeland *et al.*, 1993; Reiter, 1995, 1998; Cheung, 2003; Reiter *et al.*, 2005a; Maldonado *et al.*, 2007). Only examples of the remarkable degree of protection afforded by melatonin will be summarized herein.

Melatonin protects against ischemia/reperfusion injury

A primary example of melatonin's ability to reduce oxidative stress comes from a plethora of studies using the ischemia/reperfusion (I/R) model. Anoxia, which occurs during ischemia and reperfusion with oxygenated blood when a previously obstructed vessel is reopened, results in large-scale free radical mediated damage and leaves enormous amounts of molecular rubbish in its wake. A major portion of the mutilation is a result of free radicals generated during these processes. Organs in which this damage is of greatest concern include the myocardium (as in a heart attack) and in the brain (as a stroke, or brain attack) because of the high morbidity and mortality associated with interruption of the blood supply to these organs. Experimentally, the administration of melatonin has been shown to highly significantly reduce tissue damage and abnormal physiology resulting from I/R both in the heart (Tengattini *et al.*, 2008) and in the brain (Reiter *et al.*, 2005a), including the spinal cord (Nesic *et al.*, 2008; Samantaray *et al.*, 2008).

Cardiac arrhythmias are a frequent consequence of a transitory interruption of the blood supply to the heart. Using the Langendorf *ex vivo* heart model, Tan *et al.* (1998b) showed that melatonin infused during the period of coronary artery occlusion and re-opening significantly reduced premature ventricular contractions during reperfusion; it was surmised, albeit not proven, that the beneficial actions of melatonin in this study related to its free radical scavenging and antioxidative properties. The results of this study and others were used as a rationale for an investigation on the application of melatonin in patients undergoing angioplasty during which myocardial arrhythmias sometimes occur (Dominguez-Rodriguez *et al.*, 2007).

Many other studies have provided biochemical and molecular biological evidence that melatonin benefits the heart during periods of anoxia/hypoxia and reoxygenation and, additionally, these experiments confirmed that the antioxidative properties of melatonin are involved in its protective actions (Kaneko *et al.*, 2000; Lagneux *et al.*, 2000; Lee *et al.*, 2002; Dobsak *et al.*, 2003). Not only pharmacological concentrations, but physiological levels of melatonin also are reported to improve myocardial function which is reduced by I/R or doxorubicin, a drug that is toxic to the heart because it generates free radicals in this organ (Sahna *et al.*, 2002; 2003).

During acute myocardial infarction or unstable angina pectoris, elevated blood concentrations of soluble adhesion molecules are measured. These rises could contribute to the recruitment, adhesion and subsequent transendothelial migration of circulating leucocytes which are accepted as important processes in atherosclerotic disease (Ross, 1999). Dominguez-Rodriguez *et al.* (2008) examined the relationship of blood melatonin levels to those of vascular cell adhesion molecule-1 (VCAM-1) in patients with ST-segment elevation myocardial infarction (STEM1) and compared them to levels in healthy control subjects. Both groups of patients exhibited day (0900h)/night (0200h) differences in blood melatonin concentrations, while the nocturnal rise in melatonin was significantly less in the STEM1 patients. Also, whereas nighttime VCAM-1 levels were higher in both groups of subjects, they were highest in the patients with cardiac pathology. The augmentation of nighttime VCAM-1 concentrations in the STEM1 subjects was presumed to be related to a diminished rise in nocturnal melatonin levels since it is known that melatonin reduces adhesion molecules. One implication of these findings is that the loss of melatonin may contribute to cardiovascular disease by encouraging the adhesion and transepithelial migration of leucocytes which would promote the formation of atherosclerotic deposits. The findings related to the protective effects of melatonin in the brain and spinal cord during I/R are equally as impressive as those in myocardial tissue (Cuzzocrea and Reiter, 2001; Reiter *et al.*, 2005a; Koh, 2008).

Melatonin as a radioprotector

Clinical, experimental or accidental exposures to ionizing radiation are classic means that result in the generation of free radicals within cells and tissues. Because of this, radiation is used to kill cancer cells; however, at the same time, normal cells in the path of the radiation are also damaged. Additionally, there are some situations where individuals may be accidentally exposed to toxic or even lethal levels of ionizing radiation. Examples include the nuclear reactor accident at Chernobyl or a nuclear explosive device on the battlefield. The ramifications of the exposure of large numbers of people to ionizing radiation could be severe and it would be helpful under those conditions for the populace or war fighter to have something to potentially protect themselves from the radiation exposure.

One such agent used for this purpose is amifostine [2-(3-aminoproplamino) ethylsulfanyl phosphonic acid] (also known as WR-1065) (Brizel, 2007; Kouvaris *et al.*, 2007). Amifostine is an organic thiophosphate product which is dephosphorylated *in vivo* by alkaline phosphatase to the active cytoprotective thiol metabolite. While amifostine is an effective agent against damage produced by ionizing radiation, there are a number of drawbacks to its use particularly in the event where a large number of subjects would be exposed to toxic levels of ionizing radiation. Firstly, the drug must be given intravenously, a serious shortcoming if hundreds of individuals were simultaneously exposed to a high dose of ionizing radiation in a remote area. Also, the side effects cannot be considered insignificant and include hypotension (62% of patients), hypocalemia, diarrhea, nausea, vomiting, erythema multiforme, among others (Hosseinimehr, 2007; Valeyrie-Allanore *et al.*, 2008). For a fighting force in a battlefield situation, amifostine would greatly reduce their ability to carry out their assignments, i.e. it may render these individuals ineffective as a fighting force.

In contrast to amifostine, melatonin is a molecule with very low toxicity over a wide range of doses and it is a highly effective protector against molecular damage due to ionizing radiation exposure (Vijayalaxmi *et al.*, 1999c; 2004; Karbownik and Reiter, 2000; Karbownik *et al.*, 2000). As noted above, in 1993 Tan and co-workers documented that melatonin was useful as a scavenger when rats were subjected to whole body ionizing radiation. While these workers did not specifically measure molecular damage in the irradiated animals, they did show that the product, c3-OHM, which is formed when melatonin scavenges two ·OH was elevated in the urine documenting that the highly destructive ·OH, which is a primary culprit in mediating radiation damage, was being neutralized. Since melatonin scavenged radicals resulting from ionizing radiation exposure, it was presumed that damage to essential macromolecules was also reduced.

In the decade following the discovery by Tan *et al.* (1993), many studies were carried out showing that melatonin, given in advance of ionizing radiation exposure, reduced the associated molecular damage. The group of Vijayalaxmi *et al.* (1995a; 1995b; 1996a; 1996c) and others (Blickenstaff *et al.*, 1994; Karbownik and Reiter, 2000; Monobe *et al.*, 2005) have confirmed the cytoprotective actions of melatonin. Vijayalaxmi

and colleagues (1995a, 1995b) exposed human lymphocytes for 20 minutes to 150 cGy gamma radiation. Of special interest in this study was the damage to nuclear DNA; melatonin limited the number of abnormal cells expressing genetic damage, i.e. exchange type of aberrations, accentric fragments and the formation of micronuclei which are usual consequences of high energy radiation exposure. In general, the abnormal changes were reduced by an estimated 60–65% when melatonin was used as a radioprotective agent. In one *in vivo*/*in vitro* study, half of a group of adult humans was given melatonin orally, after which a blood sample was collected; lymphocytes were harvested and then exposed to 150 cGy gamma radiation (Vijayalaxmi *et al.*, 1996b). Moreover, serum concentrations of melatonin were measured in both groups of subjects. Those who had received melatonin had much higher circulating levels of the indoleamine; this correlated with reduced levels of lymphocytic DNA damage as estimated by the lower numbers of chromosomal aberrations and micronuclei.

Survival is a commonly used end point to check the efficacy of a radioprotector against the damaging effects of ionizing radiation. Within 12 days after a radiation dose of 950 cGy, all mice that had not received melatonin had died; conversely, in the melatonin-treated group 40% of the mice were still alive 30 days later (Blickenstaff *et al.*, 1994). In a similar study by another group, the 30 day survival rate of mice given a single radiation dose of 815 cGy was also significantly improved by melatonin administration (Vijayalaxmi *et al.*, 1999a). Moreover, in a related study the destruction of bone marrow cells was evaluated after ionizing radiation exposure and, again, the indoleamine attenuated the damage done to blood precursor cells in the marrow (Vijayalaxmi *et al.*, 1996a). The cells comprising the bone marrow are especially sensitive to ionizing radiation and their loss contributes to the debilitation and lethality of high energy radiation. Thus, the fact that melatonin attenuates damage to these cells has high clinical relevance.

Like the cells of the bone marrow, those lining the intestinal cysts are readily destroyed by ionizing radiation; this leads to sloughing of the gastroendothelial lining cells of the gut which causes severe diarrhea and possibly mortality. Monobe *et al.* (2005) showed that orally administered melatonin protected against intestinal damage following the exposure of male mice to doses of radiation (CS^{137} gamma-rays; 0.98 Gy min^{-1}) ranging from 7 to 21 Gy. The doses of melatonin used ranged from 1 to 20 mg kg^{-1} with the degree of protection of the epithelial lining cells positively correlating with the dose.

That the genome is protected from ionizing radiation damage by melatonin was verified by Karbownik and colleagues (2000). The elevation of hepatic 8-hydroxy-2-deoxyguanosine levels observed after whole body radiation (800 cGy) of rats was highly significantly counteracted by pre-treating the animals with 50 mg kg^{-1} melatonin (given intraperitoneally). Likewise, lipid peroxidation products were also depressed in the liver due to melatonin administration and the fluidity of microsomal membranes was preserved; membranes become rigid when their intrinsic polyunsaturated fatty acids are oxidized.

Clearly, in each study where melatonin has been employed as a radioprotector, it has been proven to be effective. The results of the studies summarized herein as well as others can be located in several reviews of this subject (Vijayalaxmi *et al.*, 1999c; 2004; Karbownik *et al.*, 2000; Blickenstaff *et al.*, 1994; Shirazi *et al.*, 2007). Given the low toxicity of melatonin, it would seem to be better suited than amifostine for use in the event of the exposure of a large population to high level of irradiation or in the battlefield situation where it is important that the treated individuals do not suffer incapacitating side effects.

Application of the knowledge of melatonin's antioxidative cascade, as described above, led Manda and co-workers (2007), in lieu of melatonin itself, to test one of its metabolites, AFMK, as a radioprotector against x-ray irradiation. Male mice were exposed to a 6 Gy dose (exposure duration, 10.9 min) of whole body radiation at 200 kV and 20 mA with half of the mice being given an intraperitoneal injection of 10 mg/kg AFMK 30 minutes before the radiation exposure occurred. At 24 hours after x-ray radiation onset, the quantities of cerebral cortical damaged DNA (as 8-OHdG), protein carbonyl and products of lipid peroxidation (malondialdehyde + 4-hydroxy alkenals) (MDA + 4-HDA) were markedly lower in the brains of melatonin-treated mice compared to those in the diluent-injected animals. Additionally, these workers verified that AFMK dose-dependently scavenged the ·OH as shown by the *in vitro* ESR measurement of the reduction of DMPO-OH adducts following the radiolysis of water with 10 Gy x-irradiation.

Melatonin would seemingly be particularly useful as a radioprotector in situations in which exposure to radiation is either expected or suspected (e.g. on the battle field). Since melatonin can be orally self-administered and it does not functionally compromise the fighting force, its prophylactic use should be considered. Its ease of administration and its lack of side effects are in contrast to those of amifostine. The latter can only be administered by someone with at least a minimal amount of experience in giving intravenous injections and, moreover, once administered, the fighting capability of the individual is diminished because of the substantial side effects of amifostine. Finally, in the event of an impending or actual exposure of a large group of individuals to a serious ionizing radiation dose, the

number of intravenous injections required becomes problematic.

Melatonin would also have utility in situations in which ionizing radiation exposure is predictable, e.g. from sun spots. This is a consideration for astronauts on deep space flights where serious, although intermittent and predictable, radiation hazards can occur. In addition to using procedures to shield the individuals from space irradiation, melatonin could be taken immediately prior to the irradiation exposure to provide protection by means of an "internal shield." This could be done since the ingestion of melatonin would not seriously compromise the decision-making or task-performing abilities of the astronauts. The only caveat may be that melatonin may induce sleepiness although the ability of melatonin to do so is circadian time dependent.

A significant component of space radiation is formed of protons and high-mass, high-atomic number particles (Z) and high energy particles known as HZE particles. When these particles pass through tissues they generate reactive oxygen and reactive nitrogen species (ROS/RNS), agents that are obviously capable of imparting massive molecular damage leading to debilitation, illness and, in the most serious cases, death.

Given the protective actions of melatonin against ROS/RNS, scientists at the National Institute of Radiological Sciences in Japan tested the efficacy of melatonin in combating molecular damage due to exposure of male mice to high-LET (linear energy transfer) ^{56}Fe particle irradiation (Manda *et al.*, 2008). In this study, whole-body irradiation was performed using a high-Let ^{56}Fe beam (500 MeV/nucleon) with a mono-energetic beam with a narrow Bragg Peak (MONO). The dose was 2 Gy per mouse with a dose rate of 0.88 Gy min^{-1}. Melatonin (10 mg kg^{-1}) was given intraperitoneally 30 minutes before the animals were irradiated. The mice were killed 60 days post irradiation and brain tissue was collected for analysis of free radical-mediated damage. Among a variety of measures, Manda and co-workers (2008) examined cerebellar DNA (8-OHdG), protein (carbonyl) and lipid (MDA + 4-hydroxyalkenals) damage as well as the incidence of apoptosis and/or necrosis of cerebellar Purkinje cells. Regardless of the parameter measured, melatonin provided highly significant protection against high-LET ^{56}Fe irradiation. The authors concluded under the conditions of this study that melatonin proved highly effective in mitigating neural damage resulting from LET irradiation and they feel that the findings provide hope for the possible use of melatonin as a neuroprotective strategy for astronauts during deep space flights. It seems imperative to expand these studies by examining the beneficial actions against LET particle irradiation to other tissues and species with the intent of establishing the use of melatonin, an endogenously-produced and non-toxic molecule, as a radioprotective agent in space.

Melatonin as a protection against ocular diseases

Given the fact that many disease states have, as part of their etiology, free radical-mediated damage, it was assumed that melatonin may be helpful in either delaying the progression or reducing the severity of these conditions. In the case of ocular structures, there are a variety of disease states where melatonin may be of benefit. For example, retinopathy of prematurity is known to be associated with free radical damage and may be in part attributable to the fact that certain antioxidants, e.g. glutathione and vitamin E, are relatively deficient in pre-term infants. Sepsis, which is not uncommon in premature infants, or exposure to a high oxygen atmosphere are common predictable risk factors for retinopathy of prematurity. While the efficacy of melatonin in modifying the course of retinopathy of prematurity has not been examined in humans, Gitto *et al.* (2001a; 2001b; 2009) have found that the indoleamine markedly attenuated the severity of sepsis and reduced the incidence of septic shock in premature newborns. The action of melatonin in this case was likely related to the antioxidant actions given that the circulating levels of lipid peroxidation products were reduced subsequent to melatonin administration.

One of the most common ocular diseases and one that unquestionably involves free radial damage is cataract. Cataract is the leading cause of blindness in many countries. The crystalline lens becomes opaque as a consequence of the local generation of the $^{\bullet}$OH and their excessive production is considered a common cause of non-congenital cataract. Both H_2O_2 and $NO^{\bullet}$ levels are elevated in the aqueous humor in individuals with cataractous eyes (Spector and Garner, 1981) and the numbers of oxidized macromolecules in the opaque lens are well above those in the normal lens.

A commonly used model of cataracts in newborn rats is to inject them with buthionine sulfoxamine (BSO) on day 1 after birth. This glutathione synthesis inhibiting agent causes the animals to develop cataracts by the time they are two weeks of age. In two successive studies from the same laboratory, the daily subcutaneous administration of melatonin to BSO-treated rat pups led to a highly significant reduction in the incidence of cataracts (Abe *et al.*, 1994; Li *et al.*, 1997). Moreover, melatonin reduced the quantity of oxidized lipid in the lens. Glutathione, the synthesis of which was inhibited by BSO, is normally an essential antioxidant in the lens which defers the development of cataracts. In the studies of Abe *et al.* (1994) and Li and co-workers (1997) melatonin clearly was an adequate substitute

for glutathione since it dramatically reduced cataracts resulting from glutathione depletion.

Melatonin protects against free radical toxicity in humans

The ultimate goal of basic research is obviously to translate the data into useful applications in humans. Although not yet common, melatonin has been given to humans, especially newborns, for the purpose of reducing oxidative stress. Many of these studies have come from the group of Gitto *et al.* (2001a; 2002) where melatonin has been found to be highly effective in attenuating the biomarkers of oxidative stress.

Cellular damage and compromised functions are common during the immediate pre-, peri- and postnatal periods in humans. The oxidative damage accumulates as a consequence of a number of situations including inflammation due to infections (e.g. sepsis), transitory asphyxia resulting from a difficult delivery, pediatric surgery and hyperoxia exposure to assist in the survival of newborns with respiratory distress syndrome (Gitto *et al.*, 2001a; 2002; 2009). In each of these conditions, when infants were treated with melatonin the quantity of oxidatively-damaged molecules in the blood was reduced and the ability of the babies to survive and thrive was enhanced (Fulia *et al.*, 2001; Gitto *et al.*, 2004a; 2004b). Importantly, in none of these studies were untoward side effects of melatonin noted and in these situations melatonin was judged to be safe for use in infants. Considering the positive results obtained to date and, if these findings are confirmed by additional investigators, it is anticipated that melatonin will become a frequently-used drug in neonatal units. In surgically-treated adults, melatonin has also been shown to attenuate oxidative stress (Kucukakin *et al.*, 2008).

Summarized above are only a small fraction of the plethora of studies that have verified the ability of melatonin to mitigate oxidative/nitrosative stress in both animals and humans. What is clear from these studies is that melatonin and/or its metabolites protect all major molecules, i.e. DNA, proteins and lipid, from free radical-mediated damage. Considering the differential distribution of these molecules within cells, the implication is that melatonin is widespread intracellularly and that it can accumulate in sufficient concentrations in the vicinity of each of these molecules to fend off the massive numbers of free radicals that normally attack essential molecules in many of these experimental situations. Considering these findings, a high priority should be given to studies designed to examine the uptake of melatonin by cells as well as how it is distributed within the membranes, cytosol and nucleus, as exemplified in a recent publication by Hevia and colleagues (2008).

Melatonin as a pro-oxidant

Not every report has documented the antioxidant activity of melatonin. There are a few publications indicating that it may have pro-oxidant effects as well (Osseni *et al.*, 2000; Clapp-Lilly *et al.*, 2001; Wolfler *et al.*, 2001). This would not be unexpected since redox cycling molecules usually are capable of promoting pro-oxidant actions. An excellent example of one such molecule is vitamin C; in the presence of free iron, vitamin C is capable of producing extensive oxidative stress. Several groups have failed to document the ability of melatonin to induce oxidative damage, but the reports listed above claim to have done so. Both Osseni and co-workers (2000), using a human liver cell line (HepG2), and Clapp-Lilly *et al.* (2001), using an organotypic mouse brain slice culture system, readily observed the antioxidative potential of melatonin but under some conditions the pro-oxidative potential was reported. This action was especially apparent at high melatonin concentrations, e.g. 1 mM or, in one case, when the concentration in a solution containing liver cells was 0.1–10 μM. These findings are difficult to reconcile in light of the very large number of studies where such effects have not been observed.

The study of Wolfler and colleagues (2001) used a human leukemia Jurkat cell line. They reported that both μM and mM melatonin concentrations depleted glutathione levels and counteracted the effects of other antioxidants. If melatonin is, in fact, pro-oxidant in leukemia cells, it could be beneficial in the treatment of this cancer type.

Considering these three publications, investigators and clinicians should be attentive to the possibility that under some unique or unusual circumstances, melatonin may have the ability to promote free radicals. It does remain to be seen, however, whether these observations were spurious or in fact can be routinely confirmed; to date confirmations have been difficult to achieve.

Features of melatonin as an antioxidant

One issue that is frequently raised when melatonin is espoused as an antioxidant is its circadian rhythm. In all animals, blood melatonin levels are uniquely elevated during the daily dark period (Reiter 1991); the implication is that antioxidative protection by melatonin would be greater at night than during the day. The lack of a close temporal relation between maximal nocturnal circulating melatonin levels and the daily interval (day time in diurnally active species) during which free radical generation is at its peak seems to argue against the indoleamine contributing in a meaningful way to

antioxidant protection. While the concentrations of melatonin in the blood do correlate with the antioxidative status of this fluid (Albarran *et al.*, 2001; Reiter *et al.*, 2005b), at any one time there are numerous circulating antioxidants so it is difficult to ascribe the antioxidant status of this fluid to melatonin alone.

Free radical production and the associated protection from these oxygen and nitrogen derivatives primarily occur intracellularly. Hence, blood concentrations of the melatonin are irrelevant in terms of intracellular protection. Rather little is known concerning the levels of melatonin within cells. There is preliminary evidence that the mitochondria may accumulate melatonin against a concentration gradient (Martin *et al.*, 2000a). This would certainly be advantageous given that these organelles are a major site of free radical generation (Starkov, 2008; Whiteman *et al.* 2008). Melatonin reduces electron leakage from the respiratory chain complexes (Jou *et al.*, 2002; Leon *et al.*, 2004; 2005), which decreases the likelihood of nearby oxygen molecules being reduced to radical products. Certainly the ability of melatonin to scavenge any radicals generated in mitochondria has been visualized immunocytochemically when appropriate fluorescent probes have been used (Jou *et al.*, 2004). Additionally, however, melatonin scavenges radicals generated in the cytosol (exclusive of its actions in mitochondria) and nucleus (Jou *et al.*, 2007).

The conspicuous melatonin rhythm that exists in the blood is not generally believed to manifest itself intracellularly, although data related to this issue is meager. Thus, it is assumed that within cells the amount of melatonin over a 24-hour light:dark cycle may be persistently high and only disappears as the indole is used as a scavenger. In this regard, it is interesting that under high oxidative stress conditions, the normally elevated nocturnal blood melatonin levels are rapidly depressed to daytime values even though pineal production of the indoleamine is at a maximum (Troiani *et al.*, 1988a; 1988b). Rapidly depressed circulating concentrations of melatonin also occur even when exogenous melatonin is given to animals experiencing elevated free radical generation (Wu *et al.*, 1987; 1988). In other words, when animals are stressed to the point where free radicals are being abundantly produced intracellularly, blood levels of melatonin rapidly fall, presumably due to the fact that its transport into cells is hastened where it is needed to combat free radical-mediated molecular mutilation. These observations are consistent with the idea that melatonin is rapidly taken into cells when it is needed to protect against a free radical onslaught and there are obviously times when pineal melatonin synthesis cannot keep up with demand, i.e. when free radical generation exceeds antioxidant availability, oxidative damage results.

Another matter that is often mentioned when melatonin is considered to play an essential role in antioxidant protection is its concentrations within cells. As mentioned above, rather little is known about the levels of this indoleamine within most cells. Under any circumstances, it would not seem to be at the same levels as, for example, glutathione, an antioxidant that is often in millimolar concentrations. The question then arises of how an antioxidant at an apparently much lower intracellular concentration successfully competes with glutathione as a scavenger. If concentration is the only important parameter, then at any one time there would only be one functioning antioxidant within cells, i.e. the one in greatest concentration. It is not, however, the overall concentration of an antioxidant within a cell that is most important in providing protection against free radicals since a variety of antioxidants of different concentrations function simultaneously within cells. Given that the damage inflicted by a highly destructive free radical, e.g. the •OH, is site-specific (the site at which the radical is generated), the only concentration of an antioxidant that is relevant is that at the specific site of •OH generation. It is presumed, although unproven, that melatonin may have a positional advantage to protect against highly reactive radicals. Again, this is consistent with the reportedly high levels of melatonin within mitochondria (Martin *et al.*, 2000a), a location that would allow melatonin and its metabolites to neutralize the large number of radicals that are normally generated in these organelles. Moreover, the fact that several of melatonin's metabolites are scavengers also increases the effective concentration of this indoleamine at the site where free radicals are being produced (Tan *et al.*, 2007a; Peyrot and Ducrocq, 2008).

Finally, given that melatonin induces a number of enzymes which metabolize radicals and/or their derivatives to innocuous products (Barlow-Walden *et al.*, 1995; Pablos *et al.*, 1995; Reiter *et al.*, 2000; Rodriguez et al., 2004), the presumed high concentrations required for direct free radical scavenging may be less important. This is especially so considering the ability of melatonin to stimulate both CuZn- and Mn-superoxide dismutase and glutathione peroxidase. The actions of melatonin in promoting the activities of antioxidative enzymes may be mediated by an action of the indoleamine on both membrane and nuclear receptors (Tomas-Zapico and Coto-Montes, 2005). The related signal transduction mechanisms would greatly exaggerate the response to a small number of ligands, thereby markedly improving the ability of melatonin to arrest free radical damage. While melatonin has been repeatedly shown to stimulate the activity of glutathione peroxidase, this action does not account for the ability of melatonin to protect the heart from ischemia/reperfusion injury, a situation in which free radical production is highly elevated (Chen *et al.*,

2009). The antioxidative enzymes that have been shown to be stimulated by melatonin are identified in Figure 3.

$ONOO^-$ is a highly toxic reactant that is formed when $NO^\bullet$ couples with the $O_2^{\cdot}$-. Since AMK, a melatonin derivative, inhibits the pro-oxidative enzyme, i.e. inducible nitric oxide synthase (iNOS) (Leon *et al.*, 2006), it also greatly reduces $ONOO^-$ formation since $NO^\bullet$ is not available for coupling with the $O_2^{\cdot}$-. This could be a significant factor in the total antioxidant protection provided by melatonin. A number of *in vivo* studies have shown that melatonin also has an inhibitory action on NOS (Pozo *et al.*, 1994; Chang *et al.*, 2008; Esposito *et al.*, 2008). Considering the results of Leon *et al.* (2006), the apparent actions of melatonin in inhibition of NOS in these studies may have been a consequence of its metabolite, AMK.

In many studies, cellular glutathione levels are preserved under conditions of high oxidative stress if melatonin is available in high concentrations. This suggests that melatonin may be used in preference to glutathione as an antioxidant and/or, alternatively, melatonin may induce glutathione synthesis. Indeed, the rate limiting enzyme in glutathione production, i.e. glutamyl cysteine ligase, has been shown to be upregulated by melatonin (Urata *et al.*, 1999; Winiarska *et al.*, 2006). Thus, whereas melatonin may be used as an antioxidant preferentially over the tripeptide, melatonin seems also to stimulate the production of this important antioxidant.

Another facet of melatonin that could make it highly relevant as an intracellular antioxidant relates to the possibility that it may be induced in many cells in response to elevated oxidative stress. While the evidence for this in multicellular organisms is sparse, this is known to occur in the one-celled algae, *Gonyaulax polyedra* (Fuhrberg *et al.*, 1997). In these cells, melatonin is upregulated in response to cold temperature up to the mM range (compared to maximal levels of low nM values in the blood of mammals.). These highly-elevated levels are considered physiological in this species and they are capable of preventing death of *Gonyaulax* exposed to normally lethal levels of oxidative stress (Antolin *et al.*, 1997). In the rat, many cells have been shown to contain the mRNA for the enzymes that synthesize melatonin in the pineal gland (Stefulj *et al.*, 2001). Thus, in vertebrates, many cells, theoretically at least, may have the capability of producing melatonin for their own use as an antioxidant or for other reasons.

Melatonin: a physiological or pharmacological antioxidant

It could be argued that melatonin is not likely a relevant antioxidant *in vivo* because its physiological concentration is too low to successively compete with other antioxidants, which are often presumably in much higher concentrations, for the detoxification of radicals and radical products (Reiter and Tan, 2003; Reiter *et al.*, 2005c). This judgment is always based on blood levels of the indoleamine which, even at night when melatonin concentrations in the circulation are at their peak, are in the low nanomolar range. It is also often tacitly assumed that melatonin levels in blood are reflective of its concentrations within other tissues and cells, i.e. that melatonin is in equilibrium within an organism.

Melatonin, however, is clearly not in equilibrium in organisms. Other bodily fluids, e.g. ovarian follicular fluid (Tamura *et al.*, 2008b; 2009), cerebrospinal fluid (Skinner and Malpaux, 1999), bile (Tan *et al.*, 1999a; Messner *et al.*, 2001; Koppisetti *et al.*, 2008), etc., contain much higher concentrations of melatonin than does the blood. Moreover, as knowledge in this field continues to accumulate, it is apparent that a very large number of tissues/cells have the capability of producing melatonin. Thus, melatonin is now known to be synthesized in retinal photoreceptors (Pang and Allen, 1986; Cahill and Besharse, 1992), bone marrow cells (Tan *et al.*, 1999b; Conti *et al.*, 2000), lens of the eye (Abe *et al.*, 1999; Itoh *et al.*, 2007), enterochromaffin cells of the gastrointestinal tract (Bubenik *et al.*, 1999; Bubenik, 2002), airway epithelium (Kvetnoy, 2002), skin (Slominski *et al.*, 1996; 2002; Fischer *et al.*, 2008), ovary (Itoh *et al.*, 1999), etc. Within these cells, melatonin levels are likely elevated well above those in the circulatory fluid.

It is anticipated that continuing investigations may document that melatonin is produced in almost every cell in multicellular organisms. While melatonin was initially thought to be exclusively produced in the vertebrate pineal gland (King and Steinlechner, 1985), this is obviously not the case and there is reason to believe this should not be expected. Non-vertebrate species, e.g. insects which lack a pineal gland, produce melatonin (Vivien-Roels and Pevet, 1993; Tilden *et al.*, 1997; Markowska *et al.*, 2009) as do slime molds (Hardeland and Poeggler, 2003), bacteria (Manchester *et al.*, 1995; Tilden *et al*, 1997) and unicells (Hardeland *et al.*, 1995; Antolin *et al.*, 1997). Thus, evolutionarily melatonin did not evolve as an exclusive secretory product of the pineal gland and its synthetic activity may be at least residually retained by all cells. This is consistent with the observation that attempts to identify mRNAs for the melatonin enzymes, i.e. HIOMT and AANAT, have been uncovered in many cells of rats (Stefulj *et al.*, 2001). This implies that these cells may themselves be capable of producing melatonin.

Of even greater interest is that in the unicellular organism, *Gonyaulax polyedra*, melatonin synthesis is inducible under conditions that generate high oxidative stress (Antolin *et al.*, 1997). The question then remains,

could melatonin produced in individual cells of multicellular organisms be inducible when free radical generation is elevated? Currently, there is no evidence that this is the case in vertebrates, but the molecular machinery for this seems to be present in individual cells. If this is the case, intracellular concentrations could be sufficiently high to compete in the scavenging of toxic radical species.

Even if melatonin is not generated in response to stressful conditions, it should not be dismissed as a potentially important physiological antioxidant. Certainly, in numerous studies summarized above melatonin, frequently in lower doses than other antioxidants, proved more effective in reducing the quantity of free radical-damaged intracellular molecules than did the premier free radical scavengers, e.g. vitamin C, vitamin E, β-carotene, NAC, etc.

Antioxidants are obviously differentially soluble and their concentrations are not uniform throughout cells. Lipid-soluble vitamin E localizes in highest concentrations in cellular membranes (Bjorneboe *et al.*, 1990); yet melatonin, which is also lipid-soluble, counteracts lipid peroxidation in membranes equivalent to or better than vitamin E (Jou *et al.*, 2004; Liepnitz *et al.*, 2009). Does this necessarily prove that melatonin is in higher concentrations in cellular membranes?

If the intracellular concentrations argument is used to explain the efficacy of a molecule as a free radical scavenger, then only the antioxidant in the highest concentration within a cell would function as such, i.e. a cell would only have a single functional free radical scavenger. In many cases this molecule would be GSH since its intracellular levels are often in millimolar concentrations. It seems likely that at any one time there are several, perhaps numerous, free radical scavengers operating within a given cell and/or a subcellular compartment with each being at a different concentration. This being the case, the total intracellular concentration obviously does not necessarily determine the free radical scavenging action of a molecule. What may be more important is the local concentration of an antioxidant at the site of free radical generation. The most highly-reactive and damaging radicals travel a miniscule distance before oxidizing a bystander molecule. Hence, the concentration of an antioxidant at the site of free radical generation, e.g. in mitochondria, becomes critical. As mentioned above, little is known regarding the subcellular distribution of melatonin and its concentration within specific cellular organelles (Martin *et al.*, 2000b; Reiter *et al.*, 2008a; 2008b). Thus, whether it has the positional advantage (at the site of free radical generation) referred to previously which allows it to successfully compete as a scavenger remains unknown but deserves investigation.

At the present time, melatonin has been shown to be capable of the deactivation of toxic radicals both *in vitro* and *in vivo* and to very effectively reduce oxidative damage in cultured cells and in virtually every tissue in multicellular organisms. Whether direct free radical scavenging processes are the exclusive or major means by which melatonin abates radical-mediated molecular destruction or whether other processes are also involved, e.g. stimulation of antioxidative enzymes (Rodriguez et al., 2004; Tomas-Zapico and Coto-Montes, 2005) and inhibition of pro-oxidative enzymes (Pozo *et al.*, 1994, 1997; Hardeland, 2008), remains to be determined. It cannot be denied, however, that melatonin markedly reduces excessive oxidative damage under many experimental and clinical conditions where the molecular destruction occurs as a consequence of a high free radical-related disease or to aging (Poeggeler, 2005; Shiu, 2007; Reiter *et al.*, 2008c). Whether this protection is physiological, or can only be achieved with pharmacological doses of the indoleamine, continues to be debated.

Melatonin: comparison with other antioxidants

In the initial demonstration of the ·OH scavenging ability of melatonin, Tan *et al.* (1993) also compared the indoleamine to mannitol and glutathione, two molecules that are widely accepted as being effective scavengers, in terms of their relative efficacies in neutralizing the highly toxic ·OH. In their study, H_2O_2 was irradiated with 254 nm ultraviolet light to generate the ·OH which were captured by the spin trapping agent, DMPO, to form OH-DMPO adducts (Figure 1). By adding an antioxidant to the mixture, i.e. melatonin, mannitol or glutathione, they differentially reduced the formation of the adducts with IC_{50} of 21, 123 and 283 μM, respectively, indicating that at least under these circumstances melatonin was far superior to mannitol and glutathione as a ·OH scavenger. These observations were considered important inasmuch as the ·OH is so reactive it can damage any molecule it encounters intracellularly and, moreover, this radical is believed to account for in excess of 50% of all oxidative damage that occurs within cells and organs. In addition to comparisons with mannitol and glutathione in terms of its antioxidative capability, melatonin has been tested against other structurally-diverse radical scavengers as well (Figure 4).

Melatonin has been most frequently compared with vitamin E in terms of its relative efficacy in protecting against free radicals and the accompanying molecular damage. In addition to the studies summarized in Table 1, these comparisons were made in the following *in vivo* studies in relation to the cardiotoxicity of doxorubicin (Abdel Wahab et al., 2000), cholestasis induced by extra hepatic duct ligation (Montilla *et al.*, 2001),

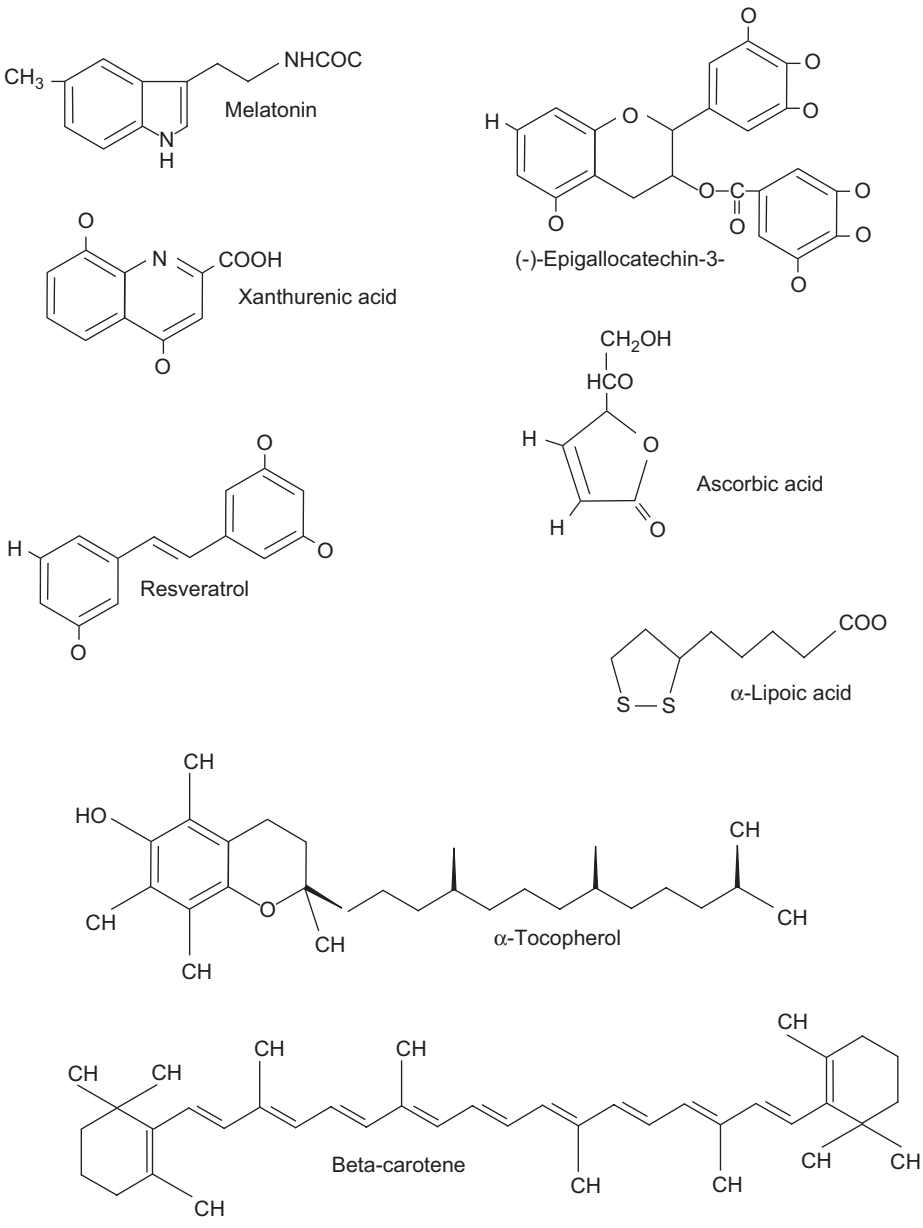

Figure 4. Melatonin and structurally diverse antioxidants with which melatonin has been compared. These comparisons were made in pure chemical, *in vitro* and *in vivo* systems by a variety of different laboratories. In most cases melatonin was equivalent to or better than the other antioxidants in scavenging radicals and/or reducing oxidative/nitrosative stress.

erythrocyte toxicity mediated by chlorpyrifos-ethyl (Gultekin *et al.*, 2001), hepatic damage resulting from ethanol administration (Mansouri *et al.*, 2001), tissue damage due to phenylketonuria (Martinez-Cruz *et al.*, 2002), streptozotocin-induced diabetes (Baydas *et al.*, 2002a), basal levels of lipid peroxidation (Baydas *et al.*, 2002b), retinal ischemia-reperfusion injury (Yilmaz *et al.*, 2002), brain lipoperoxides induced by amyloid-β peptide (Rosales-Corral *et al.*, 2003), neural and hepatic lipid peroxidation and changes in GSH levels mediated by thioacetamide (Tunez *et al.*, 2007), caerulein-induced pancreatic and hepatic oxidative damage (Esrefoglu *et al.*, 2006), malondialdehyde levels and alterations in antioxidative enzymes resulting from exposure to 720 cGy ionizing radiation (Yilmaz and Yilmaz, 2006), and mitochondrial damage induced by fetal hyperphenylalaninemia (Martinez-Cruz *et al.*, 2006), etc. The animals of choice in these studies were rats, mice, and guinea pigs. Both melatonin and vitamin E are highly lipid-soluble molecules and, as a result, they would be expected to be especially effective antioxidants in the lipid-rich portions of cells. In most of the studies mentioned above melatonin was equal to or better than vitamin E in curtailing the breakdown of lipids (Figure 5).

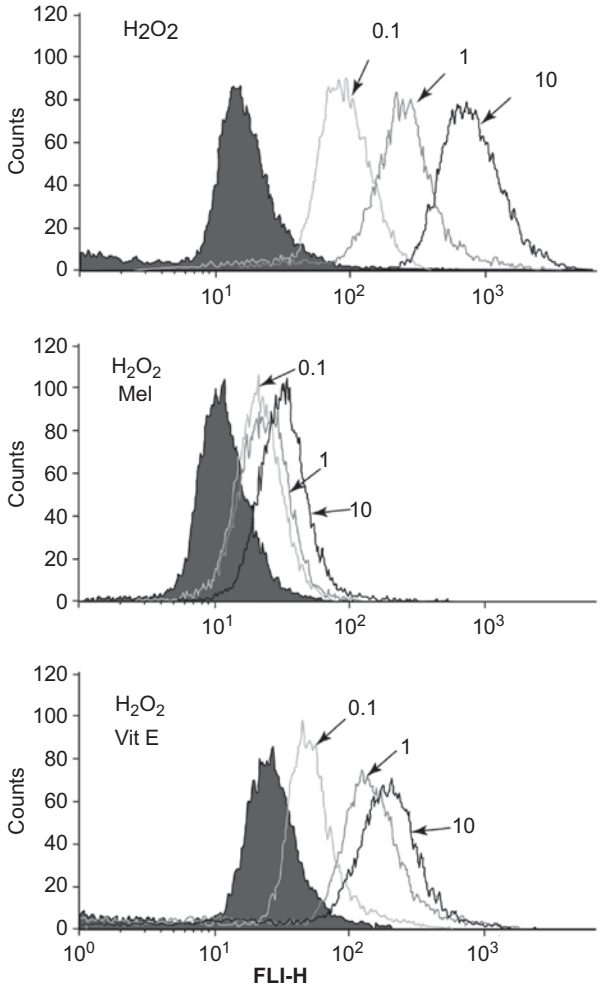

Figure 5. A combination of flow cytometry and fluorescence microscopy was used to generate this figure. Primary rat brain astrocytes in culture were challenged with increasing concentrations of H_2O_2 (0.1, 1 or 10 mM (top panel). The curves shifted to the right are quantifications of mitochondrial free radical generation, revealed as fluorescent intensity using dihydrorhodamine-123. Both melatonin (middle) and vitamin E (both at 100 μM concentrations) (bottom) functioned as antioxidants and shifted the curves to the left. However, the shifts induced by melatonin were greater than those resulting from vitamin E treatment, indicating greater scavenging by the former molecule (modified from Jou *et al.* 2004).

Potassium bromate ($KBrO_3$) is an oxidizing agent that is commonly used as a food additive. When given orally or when injected intraperitoneally into rats, elevated levels of the DNA damaged product, 8-hydroxydeoxyguanosine (8-OHdG) as well as by-products of lipid peroxidation are found in the kidney (Kurokawa *et al.*, 1983; Sai *et al.*, 1991). Given that the most likely explanation for renal damage is that the metabolism of $KBrO_2$ results in the generation of free radicals, Cadenas and Barja (1999) attempted to reduce the destruction of DNA by giving a variety of antioxidants (melatonin, resveratrol, vitamin E, butylated hydroxytolvene (BHT) and 2-mercaptoethylamine) or a spin trapping agent, α-phenyl-N-tert-butyl nitrone (PBN). Because of the different routes of administration (intraperitoneally injected or provided in the diet), multiple or single injections, and different doses of the reducing agents examined, it is difficult to determine which molecule provided the best protection against $KBrO_2$. If one ignores all the variables mentioned and considers only the mean renal 8-OHdG levels, with the exception of 2-mercaptoethylamine, all agents roughly equally reduced damage to kidney DNA induced by $KBrO_3$, although statistical analysis suggested resveratrol provided the greatest protection and it was somewhat better than that provided by melatonin. In this case, however, resveratrol had been given at a dose of 16 mg kg^{-1} BW for 7 days (112 mg kg^{-1} total) while the total melatonin dose was 48 mg kg^{-1} (four doses of 12 mg kg^{-1} each over a 24 hour period); thus, the claim that resveratrol was the most potent antioxidant may be premature.

After obtaining the results of a series of cell-free *in vitro* experiments in which melatonin was compared to glutathione, ascorbic acid and vitamin E (trolox, water soluble vitamin E), the group of Sofic *et al.* (2005) was especially enthusiastic about melatonin's ability to resist oxidative damage when they stated that "melatonin may be the premier molecule to protect against oxidative stress". They drew this conclusion after allegedly showing that melatonin, of the four antioxidants compared, was the most potent LOO• and •OH scavenger. Indeed, according to their findings melatonin was twice as effective as vitamin E, an essential chain breaking antioxidant, in scavenging the LOO• and four times more potent than vitamin C or GSH. A similar claim regarding the LOO• scavenging activity of melatonin relative to that of vitamin E had also been made earlier (Pieri *et al.*, 1994). In light of the findings of Antunes *et al.* (1999), however, it would seem that the status of melatonin as a functionally relevant direct detoxifier of the LOO• is still in limbo. Certainly, satisfactory resolution of what appear to be almost diametrically opposed findings of Sofic *et al.* (2005), Pieri *et al.* (1994) and Winston *et al.* (1998) on one side and Antunes and co-workers (1999) on the other must seemingly await further experimentation.

Since melatonin is quite capable of limiting lipid peroxidation, especially in the *in vivo* situation, and if it is only a weak chain-breaking antioxidant (i.e. direct LOO• scavenger), then its ability to reduce the oxidation of lipids likely stems from its function as a preventive antioxidant, e.g. as a scavenger of the •OH and the $ONOO^-$ (Tan *et al.*, 1993; Matuszak *et al.*, 1997; El-Sokkary *et al.*, 1999; Yin *et al.*, 2006). This assumption, however, may also have to be modified considering the findings of Winston *et al.* (1998). Using a gas chromatographic assay for evaluating the oxyradical scavenging activity of antioxidants, this group reported that melatonin is equivalent to vitamin E (trolox) and better than vitamin C as a LOO•

radical scavenger. Vitamin E, of course, is considered among the most efficient LOO• scavengers and the optimal chain breaking antioxidant; in this study melatonin was equivalent to vitamin E.

That oxidative stress is a major contributor to the development of the systemic complications of malaria has been known for almost two decades (Clark *et al.*, 1989; Siddiqi and Pandey, 1999). In the liver of *Plasmodium*-infected mice, elevated levels of the activity of xanthine oxidase, a free-radical generating enzyme, and lipid peroxides are known to occur (Guha *et al.*, 2006; Dey *et al.*, 2009). Based on these findings, it was surmised that melatonin may be useful to reduce the obvious oxidative damage that occurs during malarial infections (Guha *et al.*, 2007). To test this, mice were inoculated intraperitoneally with *Plasmodium yoelii* (MDR strain) and the degree of parasitemia was monitored by means of repeated blood smears.

Once parasitemia was established, infected mice were treated with either melatonin, vitamin E or vitamin C. Melatonin, in a dose-response manner scavenged •OH generated in the liver and markedly reduced lipid hydroperoxides and protein carbonyl that were a consequence of the malarial infection while elevating hepatic glutathione concentrations (Figure 6). The effective dose of melatonin required to achieve these beneficial changes was roughly 20-fold lower than those of either vitamin C or E. Moreover, melatonin provided hepatoprotection by almost completely suppressing the mitochondrial apoptosis pathway including restoration of the mitochondrial membrane potential, preventing caspase 3 activation, limiting the over-expression of Bax, preventing the down regulation of bcl-2, and reducing DNA fragmentation and apoptosis (evaluated using the TUNEL assay).

Considering the marked protective effects of melatonin on all aspects of liver function during malarial infection, Guha and co-workers (2007) suggest that melatonin, in preference to either vitamin C or vitamin E, may well be the most effective agent to combat free radical-mediated molecular mutilation resulting from a malarial infection. This damage is considered to be a major contributor to the pathophysiology of this debilitating condition.

While melatonin was previously shown to be a highly protective agent against ionizing radiation (Vijayalaxmi *et al.*, 1996b, 1999b), a comparative study with vitamin E re-emphasized how effective the indoleamine is in this regard. In the Yilmaz and Yilmaz (2006) study which compared the radioprotective capability of vitamin E and melatonin, rats were treated for 5 days with 100 mg kg^{-1} of either molecule. The animals were then exposed to 720 cGy total body irradiation in two fractions (separated by a 12 hour interval; dose rate of 32 cGy min^{-1}); 5 days later skeletal muscle and the femurs of the rats were removed, and the homogenates of these tissues were used for the measurement of lipid peroxidation (represented by MDA) as well as the activities of two antioxidative enzymes, glutathione peroxidase (GPx) and catalase (CAT). In both tissues from animals

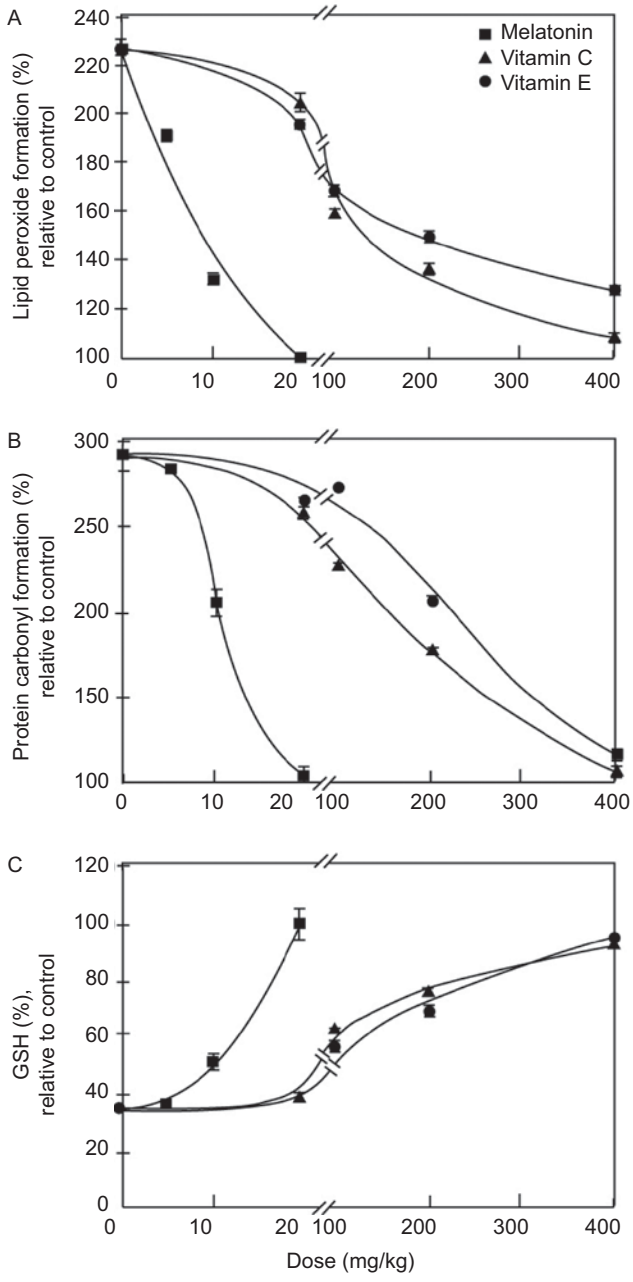

Figure 6. Comparative efficacies (points are means ± SEM) of melatonin, vitamin C and vitamin E in reducing oxidative damage to lipids (top panel) and to proteins (middle panel) and stimulating glutathione (bottom panel) levels in the liver of mice infected with *Plasmodium yoelii*. Malarial infection is normally associated with elevated oxidative stress and mitochondrial mediated apoptosis of hepatocytes. Clearly, melatonin was superior to either of the antioxidant vitamins in reversing these changes (modified from Guha *et al.*, 2007).

given equivalent doses of melatonin or vitamin E (i.e. 100 mg kg^{-1} day^{-1}) melatonin was far more protective than the vitamin in limiting the damage to cellular lipids and the changes in the antioxidative enzymes. In their concluding remarks the authors (Yilmaz and Yilmaz, 2006) suggest that melatonin may be a valuable drug for protection against ionizing radiation, a recommendation that is certainly in line with earlier observations of the use of melatonin against the damaging effects of radiation (Vijayalaxmi *et al.*, 1996b, 1999b).

While melatonin has been shown to attenuate cyclophosphamide (CP) toxicity to the urinary tract (Topal *et al.*, 2005; Zupanic et al., 2008), Sadir and co-workers (2007) used the CP model to compare the relative efficacies of β-carotene, vitamin E (α-tocopherol) and melatonin in reducing oxidative damage to the urinary bladder of rats. The detrimental effects of CP in this tissue were shown to be, at least in part, due to the fact that the alkylating antineoplastic drug stimulates inducible nitric oxide synthase (iNOS), thereby elevating intracellular levels of NO and making it available for coupling with $O_2^{\cdot-}$ with the resulting rise in $ONOO^-$ levels (Beckman and Koppenol, 1996; Korkmaz *et al.*, 2008). ONOO is a potent oxidizing agent and additionally it may degrade into the •OH (Pacher *et al.*, 2007). In the Sadir *et al.* (2007) study, in an attempt to abrogate CP toxicity, drug-treated rats were given either β-carotene, vitamin E (both at a dose of 40 mg kg^{-1} daily) or melatonin (10 mg kg^{-1} daily). As endpoints of oxidative stress, the authors measured MDA and iNOS levels in the bladder, and the urinary excretion of nitrite/nitrate (NO_x). In this system β-carotene provided weak protection against CP toxicity while melatonin and vitamin E reduced bladder damage significantly; however, vitamin E was given at a four-fold greater dose than was melatonin.

There is one report suggesting that carotenoids are no longer used in radical detoxification when melatonin is abundantly available (Bertrand *et al.*, 2006). When male zebra finches were given a drinking fluid enriched with melatonin, a non-pigmentary antioxidant, their bill developed a brighter orange/red pigmentation, reflecting a higher carotenoid concentration. One implication of this finding is that melatonin is used in lieu of carotenoids as an antioxidant, making the latter available as a pigment.

Melatonin safety

Melatonin has been available as an over-the-counter supplement for more than a decade in many countries. As expected, when it initially became available for use, there was concern about its safety and potential toxicity (Arendt, 1997; Guardiola-LeMaitre, 1997; Weaver, 1997). In the three reports cited, the alleged toxicities were in reality speculations concerning the presumed potential of melatonin for having harmful effects rather than a documentation of actual damage the ingestion of melatonin had done. These reports, also, justifiably cautioned against the use of melatonin during pregnancy and in autoimmune diseases.

With regard to pregnancy, a large study conducted under FDA/GLP guidelines and specifically designed to examine the toxicity of melatonin during pregnancy was performed by Jahnke *et al.* (1999). In this very large, carefully controlled experiment, pregnant rats on days 6–19 were treated daily with diluent or with large pharmacological doses of melatonin (50–200 mg kg^{-1} BW). After the pups (a total of 1118 offspring) were delivered, they were examined using a plethora of morphological and biochemical parameters. On the basis of their findings, the authors concluded that melatonin, even at these exceptionally high doses, had "no significant embryo/maternal toxicity." The maternal LOAEL (lowest observable adverse effect level) was ≥ 200 mg kg^{-1} day^{-1} while the maternal NOAEL (no observable adverse effect level) was found to be ≥ 100 mg kg^{-1} day^{-1}. In this report the embryonic NOAEL was ≥ 200 mg kg^{-1} day^{-1}. The melatonin doses used in this experiment were tens of thousands of times greater than the usual supplemental doses taken by humans.

Subsequent studies in animals have also dispelled fears concerning the possible toxicity of melatonin during pregnancy (Ishizuka *et al.*, 2000; Abecia *et al.*, 2002). Moreover, in situations where there is exaggerated free radical damage in developing embryos, maternally-administered melatonin was found to attenuate oxidative damage in the fetus (Wakatsuki *et al.*, 2001; Welin *et al.*, 2007). This is consistent with the ability of melatonin to rapidly cross the placenta (Okatani *et al.*, 1998).

Most recently, Tamura and co-workers (2008b; 2009) found that melatonin levels in ovarian follicular fluid negatively correlated with DNA damage and lipid peroxidative products in human oocytes. Consistent with this, when women who were to undergo *in vitro* fertilization and embryo transfer (IVF-ET) were given melatonin prior to oocyte collection, fertilization and successful pregnancy rate were increased relative to women who underwent the same procedure but who had not received melatonin. Since successful implantation and pregnancy requires an oxidatively undamaged oocyte, Tamura and colleagues (2008b) feel that the antioxidative actions of melatonin accounted for the greater success of IVF-ET in the women treated with the indoleamine. There are a variety of other ovarian and uterine conditions where melatonin will likely be beneficial (Paul *et al.*, 2008; Tamura *et al.*, 2008a, 2009) although, in these cases, the appropriate studies remain to be performed.

Collectively, the precautions introduced regarding the use of melatonin because of its presumed harmful effects on reproductive physiology and pregnancy seem unwarranted. Melatonin has been used regularly by humans for almost two decades and negative reproductive consequences have not been reported. Likewise, the pharmaceutical industry has developed patentable melatonin receptor agonists which have not been shown to have negative reproductive consequences.

Numerous other studies have noted an absence of toxicity of melatonin in studies performed in both prepubertal and adult humans (Fulia *et al.*, 2001; Gitto *et al.*, 2001b; 2002; Weishaupt *et al.*, 2006; Dominguez-Rodriguez *et al.*, 2007; 2008; Jan *et al.*, 2007). When all the experimental and clinical studies are considered collectively, melatonin has been shown to be essentially devoid of untoward consequences. While the studies to date indicate melatonin is an especially safe molecule, there may still be special conditions or disease states where it may not be useful. Recently, we urged the use of melatonin in clinical trials to identify under what conditions it would be most beneficial, the optimal doses and the best routes of administration (Korkmaz *et al.,* 2009b).

Concluding remarks

The direct free radical scavenging and indirect antioxidative actions of melatonin are unusually complex and, in some ways, perplexingly effective. The multiplicity of actions by which melatonin reduces oxidative mutilation of essential molecules is remarkable and seemingly unusual for conventional antioxidants. As pointed out herein, not only melatonin but a series of its metabolites are also capable of detoxifying free radicals and related species in what is referred to as the antioxidative cascade. Such a cascade has not been documented for any other free radical scavenger. The presumed direct scavenging is, however, only one of several actions of melatonin that allows it to reduce oxidative damage. Thus, it activates a variety of antioxidative enzymes allowing them to rapidly metabolize toxic species to innocuous molecules. Moreover, the synthesis of glutathione, an essential intracellular antioxidant, is promoted by melatonin. Finally, melatonin actions at the level of the mitochondrial respiratory chain allows for the reduction of electron leakage and the consequential free radical generation, an action referred to as radical avoidance.

While the ability of melatonin to reduce free radical damage is no longer debated, what is discussed is which of the actions of melatonin listed above is the key function of this ubiquitously-acting indoleamine in terms of reducing oxidative stress. It is likely that this varies with the specific situation and tissue. In the future additional details will surely be provided concerning why and how melatonin exerts such remarkable protection against oxygen and nitrogen-derived toxic agents. Certainly, in the context of the antioxidative defense system, melatonin should no longer be considered arcane. Rather, it should be looked at as a key element in mainstream antioxidative medicine.

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