MINIREVIEW

When Melatonin Gets on Your Nerves: Its Beneficial Actions in Experimental Models of Stroke

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This article summarizes the evidence that endogenously produced and exogenously administered melatonin reduces the degree of tissue damage and limits the biobehavioral deficits associated with experimental models of ischemia/reperfusion injury in the brain (i.e., stroke). Melatonin's efficacy in curtailing neural damage under conditions of transitory interruption of the blood supply to the brain has been documented in models of both focal and global ischemia. In these studies many indices have been shown to be improved as a consequence of melatonin treatment. For example, when given at the time of ischemia or reperfusion onset, melatonin reduces neurophysiological deficits, infarct volume, the degree of neural edema, lipid peroxidation, protein carbonyls, DNA damage, neuron and glial loss, and death of the animals. Melatonin's protective actions against these adverse changes are believed to stem from its direct free radical scavenging and indirect antioxidant activities, possibly from its ability to limit free radical generation at the mitochondrial level and because of yet-undefined functions. Considering its high efficacy in overcoming much of the damage associated with ischemia/reperfusion injury, not only in the brain but in other organs as well, its use in clinical trials for the purpose of improving stroke outcome should be seriously considered. Exp Biol Med 230:104–117, 2005

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Stroke—also referred to as ischemia/reperfusion, a cerebrovascular accident, or brain attack—often has devastating neuropathological, neurophysiological, and biobehavioral consequences that commonly result in permanent disability or death. Certain individuals are at increased risk for neurological stroke; this group includes individuals with hypertension, atherosclerosis, heart disease and other cardiovascular disorders, and those who smoke cigarettes (1–3). Also, obesity, advanced age, and diabetes mellitus are well-known risk factors for stroke.

In the context of this review, the term "hypoxia/anoxia" is interchangeable with the term "ischemia." There are essentially two types of ischemia that occur in the brain: global ischemia and focal ischemia (4). Global ischemia occurs when the oxygen (O_2) to supply to the entire brain is interrupted or severely diminished; this occurs, for example, during cardiac arrest, asphyxia, or carbon monoxide poisoning. Normally, in humans cerebral blood flow is on the order of 50 ml/100 g brain/min. When flow is reduced to less than 18 ml/100 g brain/min, neurological injury results (5). Given the high requirement of the central nervous system (CNS) for O_2, the oxygenated blood supply to the brain must be reestablished within minutes to prevent severe neurological damage. Unfortunately, reperfusion and reoxygenation of O_2-deprived tissue also causes extensive molecular destruction resulting in neurological deficits.

Global ischemia for brief periods differentially damages neurons. In particular, resuscitation of individuals after a prolonged interval of cardiac arrest (several minutes), typically leads to extensive damage to the pyramidal neurons of the CA1 region of the hippocampus. Somewhat less easily damaged neurons are found in layers 3, 5, and 6 of the cerebral cortex and neurons in the striatum,

hypothalamus, and cerebellum (6, 7). By contrast, many neurons seem to be capable of tolerating 30 mins or more of ischemia/anoxia. Even when neurons are damaged, death of these cells typically does not occur until 1–5 days following reperfusion/reoxygenation. When neural ischemia (global or local) is prolonged by 60 mins or more, most cells in the ischemic area die and an infarct develops.

Local (focal) ischemia occurs when the blood supply to a portion of the brain is interrupted, such as when a blood vessel ruptures (hemorrhagic stroke) or when a thrombus (clot), often in an atherosclerotic vessel, occurs. Hemorrhagic stroke is further complicated by the bleeding that percolates into the brain since hemoglobin is neurotoxic. It is not uncommon that rupture of a blood vessel in the brain leads to cerebral vasospasm and secondary ischemia. During local ischemia, the neurons and glia nearest the center of ischemic zone, sometimes referred to as the ischemic core or umbra, suffers the greatest amount of damage, while the surrounding area, the ischemic penumbra, which receives some oxygenated blood, becomes relatively less hypoxic and therefore shows less extensive damage.

Stroke is the third most frequent cause of medically related deaths in North America and Europe and the second leading cause of morbidity. Treatments or actions that reduce the incidence of stroke include the use of drugs to reduce hypertension and consuming diets rich in fruits and vegetables that are high in antioxidants; a high-antioxidant diet seems to reduce the incidence of atherosclerosis, thereby ameliorating the likelihood of developing both a hemorrhagic or a thrombotic ischemic event.

The neural damage that results during ischemia is a consequence of a variety of negative factors. To prevent extensive damage, it is most important to restore the blood supply to the ischemic tissue as quickly as possible; however, in doing so, reperfusion with oxygenated blood causes further molecular destruction and neuronal/glial loss. Factors generally believed to be culpable for ischemia/reperfusion (I/R) injury include a variety of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The oxygen-derived reactants of particular interest include the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the highly toxic hydroxyl radical (•OH). The most damaging RNS include nitric oxide (NO•) and especially the peroxynitrite anion ($ONOO^-$; Fig. 1; Refs. 8, 9).

Many of these reactants are generated as a consequence of events that occur during I/R injury, including excessive glutamate release, which produces excitotoxicity (10, 11), acidosis (9, 12), release of "catalytic iron," increases in intracellular Ca^{2+} levels, (13) and the accumulation of free fatty acids (notably arachidonic acid) due to the activation, *via* Ca^{2+}, of phospholipase A_2 (14). Since the final common pathway of these processes is the generation of excessive ROS/RNS, many attempts to ameliorate the neural damage resulting from I/R have included the use of antioxidants and free radical scavengers that neutralize these toxic reactants.

As already alluded to, the brain is particularly susceptible to damage by processes that involve free radicals. There are several reasons for this:

1. O_2 consumption by the brain is much greater than that in other tissues. Although only roughly 2% of total body weight, the brain utilizes 20% of the inhaled O_2. Thus, proportionately, the brain generates many more bellicose ROS than any other tissue.

2. The brain is rich in polyunsaturated fatty acids (PUFA), which are easily oxidized by toxic reactants. This, coupled with elevated radical generation, makes the brain highly vulnerable to damage.

3. The brain contains elevated amounts of non-heme iron. In the event iron becomes liberated and encounters H_2O_2, it generates, *via* the Fenton reaction, the highly reactive and destructive •OH.

4. In addition to iron, certain areas of the brain are rich in ascorbic acid (vitamin C). In the presence of free iron, vitamin C becomes a potent pro-oxidant.

5. Finally, the CNS has a relatively inefficient enzymatic antioxidative defense system. Given these features, it is not totally surprising that, during a lifetime, the CNS suffers more than its share of oxidative mutilation, be it due to stroke or other age-related diseases.

The current review summarizes a series of studies that have used the newly discovered direct free radical scavenger (15–20) and indirect antioxidant (21–24) melatonin to combat the morphological, physiological, biochemical, and molecular damage that is a result of I/R injury in the CNS. The extensive data relating to the specific mechanisms by which melatonin reduces tissue damage that is a consequence of interruption and reestablishment of the blood supply to an organ are beyond the scope of the current review, and the reader is directed elsewhere for this information (15, 19, 20, 25). Suffice it to state, melatonin's ability to reduce molecular damage during I/R likely involves the receptor-independent direct detoxification of ROS/RNS (15, 19, 20) as well as receptor-mediated actions such as the modulation of antioxidative enzymes (21–24). Beyond these presumably primary protective actions, melatonin may well have other beneficial effects, such as limiting electron leakage from the electron transport chain (ETC) in the inner mitochondrial membrane (22); by reducing electron leakage from the ETC free radical, generation would likewise be lessened.

Of importance is that the high efficacy of melatonin in reducing the structural and functional deficits associated with transitory interruption and reestablishment of the blood supply is not uniquely confined to the CNS. Indeed, melatonin's ability to limit I/R damage has been documented in many organs, such as heart (26), liver (27, 28), gastrointestinal tract (29), lung (30), eye (31, 32), kidney (33), and others.

Melatonin: Relation to the Brain

While melatonin is produced in the CNS, specifically in the pineal gland (34), its production is by no means limited

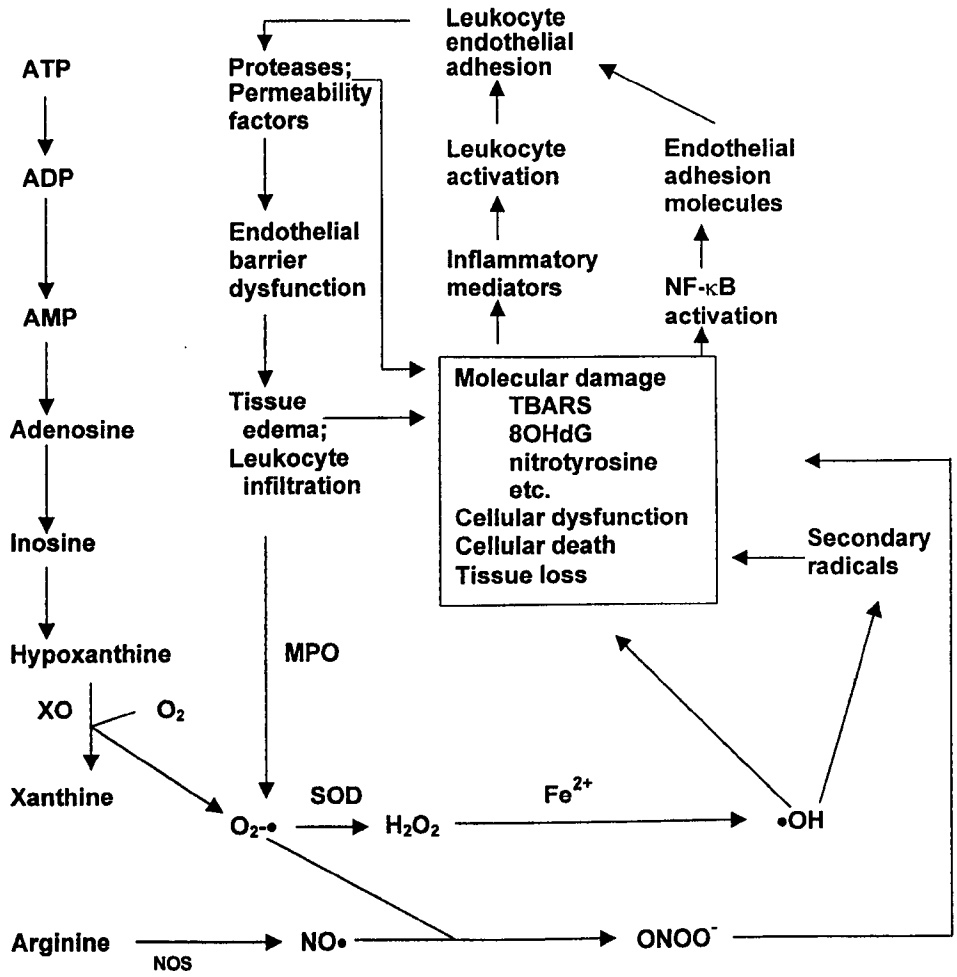

Figure 1. The processes associated with neural damage during ischemia/reperfusion injury are highly complex as illustrated in this figure. Of note is that elevated free radicals and related reactive oxygen species (ROS)/reactive nitrogen species (RNS) are documented culpable agents in this damage. Additionally, tissue damage also results from the extensive edema as well as from the activation of endothelial adhesion molecules that result in leukocyte infiltration into the damaged tissue. Another result of the cellular damage resulting from ischemia/reperfusion injury is a drop in ATP levels that contributes to additional cell death. In the current review, the ability of melatonin to scavenge ROS/RNS as well as to limit edema and reduce adhesion molecules is discussed. Each of these actions of melatonin contribute to its ability to reduce the severity of stroke. XO, xanthine oxidase; NOS, nitric oxide synthase; SOD, superoxide dismutase; MPO, myloperoxidase; TBARS, thiobarbituric acid reactive substances (lipid peroxidation products); 8OHdG, 8-hydroxy-2-dexoxyguanosine (damaged DNA product).

to this organ. Melatonin synthesis has also been documented to occur in the retinas (35), gastrointestinal tract (36, 37), some bone marrow cells (38, 39), peripheral lymphocytes (40), skin (41), and possibly in many other cells as well (42–44). At least in the pineal gland and retina, melatonin production is light:dark dependent with highest levels of the indoleamine being generated during the dark phase of the light:dark cycle. Besides its endogenous production, it is also ingested in the diet since melatonin is present in plants including edible foodstuffs (45, 46). Consumption of foodstuffs containing melatonin is followed by its absorption into the blood (45).

Although its synthesis occurs in a number of cells, the release of melatonin, at least in large amounts, seems to be restricted to the pineal gland. Certainly, surgical removal of the pineal gland eliminates the nocturnal increase in blood melatonin concentrations (34). The mechanisms of melatonin release remain enigmatic, although it is usually assumed that it is a consequence of simple diffusion of the indoleamine out of the pinealocytes. While a similar secretory process for melatonin may exist in other cells where it is produced, in these tissues its actions are confined to the immediate vicinity of its cells of production, and significant amounts typically do not escape into the systemic circulation. In these organs, melatonin has autocrine, intracrine, and pararine actions (40, 47).

In reference to the pineal gland, it has long been suspected that, in addition to its discharge into the rich capillary plexus in the pineal gland, melatonin may also be released directly into the cerebrospinal fluid (CSF) of the third ventricle. Certainly, there are a variety of morphological characteristics of the structures in the vicinity of the pineal gland that would make secretion *via* this route a possibility (48). Recent studies in the sheep support the

release of melatonin from the pineal gland directly into the third ventricle (49, 50). In a series of elegant experiments, this group convincingly showed that CSF melatonin concentrations are diminished when the pineal recess of the third ventricle of the sheep is obstructed; these data strongly imply that melatonin, released from the pineal gland, enters the CSF *via* the recess that invaginates the pineal gland in the posterodorsal aspect of the third ventricle of the sheep (50). If this ventricular route of secretion if further validated in the sheep, it would seem unusual if it were a unique feature of this species. The human pineal, as in the sheep, sits in a subcallosal position and possesses a pineal recess and other morphological modifications that would certainly allow the escape of pineal-derived melatonin directly into the third ventricle. The other important observation that Skinner and Malpaux (49) made was that nocturnal third ventricular CSF melatonin levels are several orders of magnitude higher than simultaneous concentrations measured in the peripheral blood (Fig. 2). This finding by itself implies a direct release of melatonin into the third ventricle or a very active mechanism for the transfer of melatonin from the blood through the choroid plexus and into the CSF. This latter possibly seems unlikely considering the reported data (50).

Elevated concentrations of melatonin in the CSF would certainly provide a number of advantages to the CNS. From this fluid, melatonin could readily be absorbed into the surrounding brain tissue to protect it from oxidative damage and/or for other actions. Furthermore, the indoleamine would avoid being rapidly metabolized by the liver, thereby allowing its more efficient utilization in receptor-mediated as well as receptor-independent actions, for example, as a direct free radical scavenger.

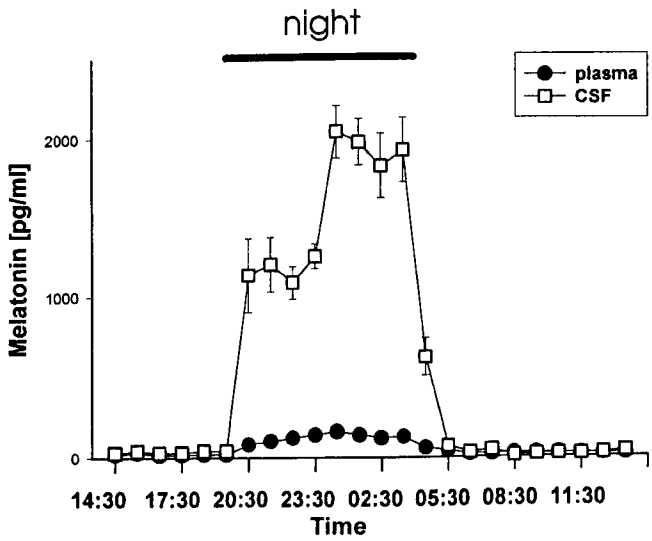

Figure 2. Concurrent melatonin levels in the plasma and the cerebrospinal fluid (CSF) of a sheep over a 24-hr light:dark cycle. As with all mammals, blood melatonin concentrations in the sheep rise from a value of <10 pg/ml during the day to 50–150 pg/ml at night; in contrast, the nocturnal rise in CSF melatonin values are orders of magnitude higher than in simultaneous plasma samples. Likewise, it was shown that in the sheep, melatonin is released from the pineal gland directly into third ventricular CSF. This being the case, the brain is seemingly exposed to much higher concentrations of melatonin at night than are other organs. This is the only species in which a direct release of melatonin into the third ventricle has been convincingly demonstrated, but it is unlikely that it is the only species in which it occurs. From Skinner and Malpaux (49).

The observation of much higher melatonin levels in the CSF than in the blood points out another important feature concerning melatonin; that is, it is not in equilibrium in the body. This has actually been apparent for at least a decade but not widely recognized. In addition to the third ventricular fluid, several other fluids likewise contain concentrations of melatonin that exceed those in the blood when measured at the same time. Like the CSF, bile has levels of melatonin that are orders of magnitude higher than those in the blood (51); similarly, although not equivalent to those in the CSF or bile, ovarian follicular fluid (52) contains melatonin at concentrations that surpass those in the plasma. Likewise, cells that produce melatonin—and there seem to be more than originally anticipated—likely contain high concentrations of the indoleamine. Also, very high intracellular melatonin levels are generally compatible with optimal cellular function as illustrated by millimolar levels (compared to picomolar or low-nanomolar concentrations in mammalian blood) of this indoleamine in the unicell *Gonyaulax polyedra* (53). Furthermore, by the standard of blood levels, the yeast *Saccharomyces cerevisiae* has melatonin concentrations that go well beyond those considered the norm (54). Within the mammalian cell as well, when melatonin was measured in mitochondria of rat liver, they were preliminarily reported to be in the micromolar range (55). Nuclear levels of melatonin in mammalian cells also seem to exceed those in the blood (56). Thus, care must be exercised when defining the physiological concentration of melatonin. Clearly, melatonin concentrations differ among subcellular organelles and bodily fluids as well as between species. Hence, when the phrase "physiological level" is used to describe a particular melatonin concentration, it should be qualified on the basis of a specific organelle, a fluid, and a particular species (57).

For any antioxidant to protect neurons and glia from highly reactive free radicals and associated species, it is imperative that it gets into the brain in sufficient concentrations to do so. In this context, melatonin seems to have an advantage over some antioxidants that are not normally generated in the brain. Vitamins E and C, for example, are not particularly effective in crossing the blood-brain barrier. Conversely, within 10 mins after its peripheral administration, melatonin is already detected in the CNS (56–58).

In addition to its ability to enter the brain, it is important that an antioxidant have wide intracellular distribution and multiple actions to be maximally effective in protecting against oxidative stress. Here again, melatonin may be superior to some other classic antioxidants. Within neurons and glia, vitamin E is restricted, because of its solubility, to

lipid-rich cellular membranes; conversely, the highest concentrations of vitamin C are found in the cytosol. Melatonin's intracellular localization seems to be much more diffused, although the data are still incomplete. Melatonin's high lipophilicity would predict its presence in cellular membranes. However, immunocytochemical and radioimmunoassay data have shown that it is also present in mitochondria (55) and nuclei (58) of cells. Certainly, melatonin has repeatedly been shown to prevent free radical damage to membrane lipids (59–61), to cytosolic proteins (62–64), and to nuclear (65–67) and mitochondrial (68, 69) DNA. Melatonin's wide intracellular distribution seems to relate to its amphilicity, the basis of which may be its *O*-methyl and *N*-acetyl residues (70). Considering the extremely short distances that most highly reactive radicals travel before mutilating a bystander molecule, melatonin (or any free radical scavenger) must be on-site when a radical is produced. In a number of *in vivo* comparative studies, melatonin has been shown to be a more potent protector against free radical—mediated molecular destruction than either vitamin E or vitamin C (66, 71–73).

Use of Antioxidants to Reduce the Consequences of Stroke

As illustrated in Figure 1, ROS and RNS appear to play a central role in the neuronal damage that occurs as a consequence of I/R injury (stroke) in the CNS. Annually, thousands of individuals die or are seriously incapacitated because of a transitory interruption of the blood supply to portions of the brain.

A number of therapeutic interventions have been tested in a variety of animals in an attempt to reduce the neuronal loss and neurophysiologic deficits associated with experimental stroke. Some of these experimental treatments include reducing body temperature, giving inhibitors of NO• synthesis, injecting Ca^{2+} channel blockers, providing agents that reduce the binding of glutamate to its NMDA receptor, and providing either enzymatic or nonenzymatic antioxidants. The intent of the use of each of these treatments is to limit free radical damage in the affected brain region. Some of the naturally occurring and synthetic antioxidants that have been used include superoxide dismutase (SOD or SOD mimics), metal chelators such as desferrioxamine, Ebselen (glutathione peroxidase mimic), α-lipoic acid, vitamins E and C as well as derivatives of these molecules, and lazaroids (e.g., tirilazad mesylate or U-74006F). Additionally, the spin-trapping agent phenyl-tert-butylnitrone (PBN) has been examined as to its efficacy in reducing molecular and cellular damage in the brain due to I/R injury. The perceived success of these treatments is dependent on the specific animal model in which the agent was tested, the duration of the ischemic insult, the length of follow-up after reperfusion was established, the basal levels of endogenous brain antioxidants at the time I/R occurred, and other factors. The intent of each of these treatments was primarily to reduce the free radical damage associated with transient interruption of the blood flow to the brain followed by its reperfusion with oxygenated blood.

Melatonin has also been widely tested as to its ability to reduce molecular and cellular damage in the CNS during I/R injury. The actual number of studies that have been performed using melatonin in its context of an inhibitor of I/R injury is surprisingly extensive considering this indoleamine has been known to function as an antioxidant for only roughly a decade (74). Additionally, several other summaries of this subject have appeared elsewhere and may be consulted for additional details and perspectives (75–78).

As will be apparent in the subsequent paragraphs, melatonin's effects in studies of experimental I/R injury have been unexpectedly successful. The reasons for this are not immediately apparent but presumably relate to its broad-spectrum functions as an antioxidant (19) and to the fact that not only is melatonin highly effective as a direct and indirect antioxidant (20–22), but some of the products that are formed when melatonin interacts with ROS are also highly effective in preventing oxidative damage. For example, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK), both of which are metabolites of melatonin (15, 79–82), are highly efficient radical scavengers (83–86).

The initial study examining melatonin's ability to reduce tissue damage during a transient interruption of the blood supply to the brain followed rather soon after the discoveries that documented the antioxidant properties of melatonin (15, 16, 74, 87). Interestingly, in this report the authors did not determine whether exogenously administered pharmacological levels of melatonin would abate the degree of I/R injury but rather tested the efficacy of depressed endogenous melatonin levels (due to surgical removal of the pineal gland) on neural damage following the temporary interruption of the blood supply to the brain. This would have to be considered a high-risk study in terms of a positive outcome considering that pinealectomy removes only one source of melatonin so that animals, in this case rats (88), were not totally depleted of endogenous melatonin levels, although they were deficient with regard to circulating melatonin concentrations. Despite this, the rats that were deficient in melatonin had larger infarct volumes, more terminal transferase biotinylated-dUTP nick end label (TUNEL)–positive neurons, and fewer surviving cells (Nissl stain) in the brain 4 or 6 hrs after temporary bilateral occlusion of both common carotid arteries (88). The TUNEL assay identifies cells dying because of apoptosis or necrosis after DNA damage. This group quickly followed their initial report with a second study that confirmed that a relative melatonin deficiency is associated with an exaggerated infarct volume and more extensive cellular DNA damage in the brain after a period of transitory focal ischemia followed by reperfusion with oxygenated blood (89). This group also provided data showing that exogenously administered pharmacological levels of melatonin (4

× 2.5 mg/kg body weight [BW] before ischemia onset) reduced the amount of tissue loss in the CNS after I/R.

The ability of endogenous levels of melatonin to contribute protection to the brain during I/R in rats was also confirmed by Kilic and colleagues (31). In this study, both pinealectomized and pineal intact rats were subjected to a reversible 120-min focal brain ischemia. As a consequence of the relative melatonin deficiency that followed pinealectomy, infarct volume after endovascular middle cerebral artery occlusion (MCAO) was exaggerated relative to that in the intact animals, and supplemental melatonin (4 mg/kg BW before ischemia onset and before reperfusion) significantly limited brain damage 22 hrs after reperfusion onset. This report also showed that the neurological deficits associated with I/R injury in pinealectomized rats were ameliorated by giving melatonin exogenously.

The results showing that attenuated melatonin levels enhance neurological damage after an I/R episode have noteworthy implications given that melatonin production and its secretion from the pineal gland diminishes with age (90–92). Hence, considering that older humans have lower endogenous melatonin concentrations than their young counterparts, when they experience a stroke it would be expected that the degree of neural damage would be enhanced in an older population. Certainly, Manev and Uz (93) suggest this in an opinion paper, and an obvious implication is that aged individuals who have reduced melatonin levels and who are prone to stroke may benefit from melatonin supplementation.

Other investigations have also examined melatonin's ability to reduce neurological damage after focal I/R of the brain. For example, Cho and colleagues (94) specifically investigated the consequences of I/R on the CA1 pyramidal neurons of the hippocampus of rats after 10, 20, or 30 mins of ischemia followed by reperfusion for 7 days. The hippocampal pyramidal neurons are frequently lost during stroke since the neurotransmitter glutamate, which is abundant at synapses that end on pyramidal neurons, is massively released during brain injury. The consequence of the release of glutamate is excitotoxicity, a process that is highly damaging to postsynaptic neurons including the pyramidal cells of the hippocampus. Excitotoxicity is deadly to neurons because it ultimately leads to pronounced free radical generation, molecular damage, and cell death. Cho *et al.* (94) found that melatonin (10 mg/kg BW) protected the pyramidal cells from excitotoxicity-induced cell death that followed the induction of experimental stroke. Indeed, in other models of excitotoxicity, melatonin has also proven protective of neurons against glutamate-induced damage (95–97). These observations are of considerable importance given that excitotoxicity-mediated neuronal death, besides being involved in I/R injury, is believed to be a factor in age-related neurodegenerative conditions such as Alzheimer's and Parkinson's diseases as well (98–100).

While the previously summarized findings certainly indicate that one of the mechanisms by which melatonin reduces neural damage after I/R involved its ability neutralize free radicals, actual documentation of this had to await a study by Li *et al.* (101). This group placed microdialysis probes into the ischemic area of the brain of rats and used salicylate trapping to estimate •OH generation in either the presence or the absence of supplemental melatonin administration. In this study, 2,3-dihydroxybenzoic acid (DHBA), a product that is generated when salicylate scavenges an •OH, was found to be elevated in microdialysates recovered from the rat brain during ischemia and reperfusion. These elevated values were significantly reduced when melatonin was given before the dialysates were collected from the brain. In this situation, DHBA levels were lowered because melatonin, more effectively than salicylate, scavenged the newly formed •OH. These findings certainly support the conclusion that melatonin's efficacy in limiting neuronal death and infarct volume that is a consequence of MCAO may well relate to the ability of the indole to directly detoxify the devastatingly toxic •OH. Beyond this action of melatonin, however, it is likely that the indole's protective effects in the brain also involve preservation of mitochondrial physiology (22, 23, 102), stimulation of antioxidative enzymes (24), as well as other actions (76).

Apoptosis is a major process by which cells die in the CNS as well as elsewhere after they are damaged by oxidative stress. Ling and colleagues (103) examined the effects of melatonin on the proto-oncogenes, *bcl-2* and *bax*, in the rat brain during hypoxia and reoxygenation associated with MCAO. The *bcl-2*, which enhances cell survival, was upregulated in the penumbral area of an infarct in the rat brain after MCAO when melatonin was given to protect against free radical damage; *bax* levels, however, were not altered by either a period of ischemia follow by reperfusion or by exogenous melatonin administration. Since the *bcl*-2 levels were elevated as a result of melatonin treatment, the *bcl*-2-to-*bax* ratio was likewise increased. These results are consistent with melatonin's ability to reduce cellular loss in the CNS by inhibition of apoptosis, a process often found to be reduced as a consequence of the actions of melatonin in the brain (104, 105) and in other organs (106).

Sinha *et al.* (107) used another approach to estimate how effectively melatonin protected against infarct volume after induced hypoxia and reoxygenation of the brain. In this report, the infarct volume in the rat brain after a 2-hr occlusion of the middle cerebral artery followed by a 30-min internal of reperfusion was evaluated using magnetic resonance imaging (MRI). In the animals that received melatonin, the lesion size was estimated to be reduced by 50% compared to that in diluent-treated controls. Furthermore, using a number of behavioral tests, they established that the neurological deficits associated with MCAO were also less conspicuous in melatonin-treated rats. The presumptive mechanisms for the protective effects of melatonin in this study, as expressed by the authors (107), related to the ability to the indole to directly scavenge free

radicals as well as its propensity to promote the activities of several antioxidative enzymes.

Kondoh *et al.* (108) and Torii and colleagues (109) also employed MRI signals to assess the amount of damaged tissue in the brain of rats subjected to MCAO (using an intraluminal occlusion technique) with and without concurrent melatonin administration. In this case, melatonin was given orally at doses of 6 mg/kg BW; the initial dose was administered immediately before onset of a 1-hr MCOA, while the second dose was given 24 hrs later. When the brain of rats was visualized, the T2-weighted signals, indicative of the water content of the brain, showed massive edema in the area of the brain that had been subjected to I/R. Oral melatonin treatment reduced the amount of edema by more than 50% with this reduction being greater in the cerebral cortex (59.8%) than in the striatum (34.2%). When the volume of edema was estimated in successive coronal brain slices, melatonin more effectively reduced cerebral cortical edema posterior as opposed to anterior to the bregma (Fig. 3).

This group also used TTC (2,3,5-triphenyltetrazolium chloride)-stained brain slices to calculate the volume of infarcted tissue at 7 days after the ischemic episode. This method documented that the volume of dead tissue was well correlated with the edematous areas as estimated using MRI. When neuronal counts were performed, these also indicated that melatonin, given orally, is highly effective in limiting brain damage resulting from focal I/R. While the report of Kondoh *et al.* (108) claims that behavioral assessments of the brain-damaged rats were performed, no data on these tests were provided. The degree of neural protection ascribed to melatonin in the two studies summarized previously (108, 109)—that is, roughly 60% and 30% in the cortex and striatum, respectively—is equivalent to that reported by some of their colleagues (110). This latter group also showed that not only neurons but also glial cells survive much better in the I/R region of the rat brain after the animals are treated with melatonin. Glial cells commonly respond with reactive gliosis when the brain is damaged. In other models of CNS damage, melatonin has also been found to reduce the increased reactivity of the glial elements (111, 112).

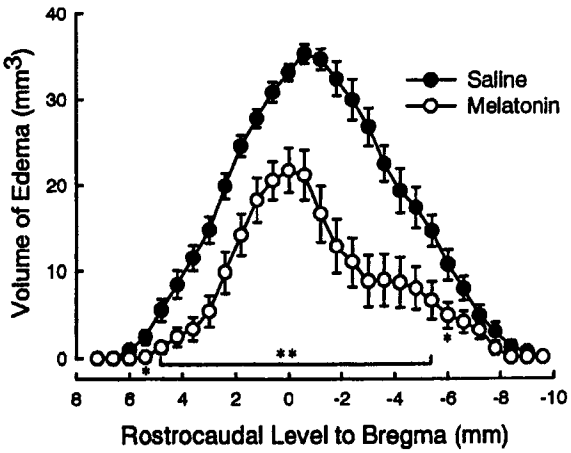

Figure 3. Reduction in cerebral edema in the rat brain after induced ischemia/reperfusion injury with and without melatonin treatment; the volume of edema was estimated using successive, from front to back, T2-weighted magnetic resonance images. Melatonin clearly limited the volume of edema at all cortical levels with the effect being somewhat greater caudal to the bregma. In these brains, the infarct volume correlated with the volume of edema; thus, melatonin treatment reduced not only edema volume but the size of the infarct as well. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared with saline treated controls. The horizontal bar signifies a $P < 0.01$ between melatonin- and saline-treated rats over the length of the bar. From Torii *et al.* (109).

Intraarterial thread occlusion of the middle cerebral artery and its withdrawal after 30 mins was used by Kilic *et al.* (113) to induce I/R injury in mice. Also, in the study melatonin was given with the thrombolytic agent, recombinant tissue-plasminogen activator (t-PA), which is commonly used in humans to dissolve clots within blood vessels. This combination treatment was employed since it may become relevant given that humans with obstruction of a blood vessel supplying the brain are frequently given t-PA for thrombolysis. To test whether there are protective effects of melatonin in this model of I/R, Kilic and co-workers (113) also monitored cerebral flow using laser Doppler (LDF). Melatonin by itself caused a significant reduction in LDF, but when combined with t-PA, it was ineffective in altering blood flow to the cerebral cortex. At 24 hrs postischemic onset, melatonin-treated mice had significantly increased neuronal survival and diminished disseminate cell injury as assessed by cresyl violet and TUNEL staining. Melatonin, when combined with t-PA treatment, was also protective against cell death, although t-PA by itself also provided some neural protection. The protective actions of melatonin were associated with inhibition of caspase 3, an apoptosis-related enzyme. The authors concluded that the brain protection by melatonin in this I/R model was independent of any hemodynamic changes but did involve the inhibition of caspase 3, which was likely a result of melatonin-scavenging radicals at the mitochondrial level and the subsequent diminished release of cytochrome *c* from mitochondria (105).

A commonly used species to investigate the neural consequences of extensive neural hypoxia and reperfusion is the Mongolian gerbil (*Meriones unguiculatus*). The utility of this species in I/R studies stems from the incomplete circle of Willis at the base of the diencephalon. Because of this, when both common carotid or vertebral arteries are obstructed, ischemia is complete in the distribution area of these blood vessels since there is no collateral circulation to supply the region deprived of blood. Two studies that tested melatonin as a protective agent in the CNS have used this species (114, 115). In the first of these studies, bilateral occlusion using vascular clamps of both common carotid arteries followed by reperfusion of the ischemic tissue was followed by increases in cerebrocortical levels of nitric oxide (NO; as estimated by nitrite/nitrate concentrations)

and rises in the second messenger, cyclic guanosine monophosphate (cGMP). It is believed that NO is in part responsible for *N*-methyl-D-aspartate glutamergic toxicity associated with ischemia. Melatonin, given in advance of ischemia, prevented the increases in both NO and cGMP in the frontal cerebral and cerebellar cortices (115). When it couples with $O_2^{\cdot-}$, NO forms the highly toxic $ONOO^-$; this latter molecule is scavenged by melatonin (18–20), and this is one action that was assumed to explain the protective effects of melatonin in this I/R model.

The second study that used the Mongolian gerbil (114) measured more endpoints than did the investigation of Guerrero and co-workers (115). Again, bilateral occlusion of the common carotid arteries was the method employed to induce hypoxia of the forebrain. Melatonin (10 mg/kg BW) was given 30 mins before reperfusion onset and also at 1, 2, and 6 hrs postreperfusion. Cuzzocrea *et al.* (114) measured nitrite/nitrate levels, malondialdehyde concentrations, myloperoxidase activity, neural edema, locomotor activity, nitrotyrosine, and poly (ADP ribose) synthase staining of the hippocampal pyramidal neurons, pyramidal cell counts, and survival of the animals after I/R injury with and without melatonin treatment. For every parameter measured, a significant improvement was seen when the animals had been treated with melatonin (as opposed to diluent). Particularly noteworthy are the survival studies; 5 of 10 diluent-treated, I/R gerbils survived to 24 hrs, while 3 of 10 were alive at 48 hrs; conversely, 10 of 10 and 8 of 10 gerbils survived to 24 and 48 hrs, respectively, when they had been given melatonin.

Perhaps the most extensive series of investigations on the protective actions of melatonin against I/R damage to the rodent brain is that of Cheung and co-workers (77). In two recently published reports where the melatonin injection scheme was simplified (relative to that of the earlier studies), this group (116, 117) documented that a single intraperitoneal injection of melatonin (5–15 mg/kg BW) before 3-hr endovascular MCAO has pronounced cerebroprotective actions. In these studies, infarct volume was reduced by roughly 40%, and an effect of melatonin *via* potential hemodynamic changes was ruled out since these parameters remained unchanged following administration of the indole. Even when a 5-mg/kg BW dose of melatonin commenced within 1 hr of ischemia onset (total duration of ischemia was 3 hrs), the protective actions of the indole were obvious and even more prominent when a second and third dose of melatonin were given at 21 and 45 hrs after reperfusion onset (118). As other investigators had shown, the I/R-induced rises in brain NO concentrations were also reduced in the melatonin-treated rats (119).

Compared to the number of focal I/R models that have been employed to test the cerebroprotective actions of melatonin, many fewer global I/R models have been used. The first of these was conducted by Wakatsuki and co-workers (120). Global I/R was induced in fetal rats by clamping the utero-ovarian arteries bilaterally for 20 mins and then reestablishing the blood flow to the pregnant uterus. A 10-mg/kg-BW melatonin dose was given intraperitoneally to the pregnant dam 1 hr in advance of utero-ovarian artery occlusion. Following I/R, the fetal rat brain had elevated levels of products of lipid peroxidation and damaged DNA, with both changes being attenuated by the single melatonin injection. This study emphasizes another point that was previously established, that is, that melatonin readily passes through the placenta (121) and does so in amounts sufficient to protect the fetus from oxidative damage. The ability of melatonin to enter the fetus when administered to the mother has clear implications for its use in protecting the unborn fetus from free radical damage due to a variety agents.

The other model of global I/R that was used to test melatonin's protective actions in the brain was cardiopulmonary arrest. In this case, cats were the experimental animals, and the duration of blood deprivation to the brain was 15 mins (122). This interruption of blood flow led to extensive loss of CA1 and CA4 hippocampal pyramidal neurons 8 days later, while neurological deficits were also obvious when measured on Days 1–7 post-ischemia. Continuously infused melatonin at a dose of 10 mg/kg BW/hr for 6 hrs commencing at 30 mins after reperfusion onset significantly reduced hippocampal neuronal loss and reduced the severity of the neurobehavioral deficits.

Collectively, the results of the studies summarized here are unambiguous in terms of the ability of melatonin to restrict CNS damage, which is a consequence of I/R. In each of these reports, melatonin administration was usually initiated essentially at the time of ischemia or reperfusion onset. Additional investigations should be designed to examine how soon after an ischemic episode melatonin must be given to be beneficial. As with any potentially protective agents, in a real-life situation it is usually applied at some interval after a stroke patient is identified. To define what is referred to as the "window of opportunity" during which the protective agent provides benefit is important to its most effective use. This information is not yet available for melatonin.

Another aspect of melatonin's use is its potential as a prophylactic agent in reducing the vulnerability of the brain (or any other organ) to I/R damage. As noted previously, because of the gradual reduction in endogenous melatonin production throughout life, circulating values of this protective agent are typically diminished in the elderly (90–92). Since the total antioxidant status of the blood of humans correlates with the melatonin concentration in this fluid (Fig. 4; Ref. 123), it is likely that more extensive oxidative damage occurs, such as in vascular tissue, leading to the likelihood of vessel rupture (which causes hemorrhagic stroke) in the brain (and elsewhere).

Furthermore, since melatonin reduces the production of adhesion molecules (e.g., P-selectin and ICAM, which promote the sticking of leucocytes to the endothelial lining of blood vessels; Ref. 124), the reduction of melatonin

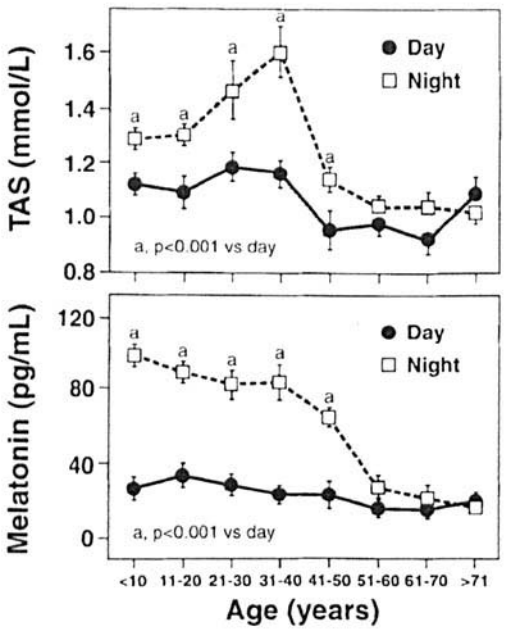

Figure 4. Age-related changes in day and night melatonin levels and the total antioxidant status (TAS) of the blood of humans at various ages. In this study, the individuals, ranging in age from 2 to 89 years, were categorized into 10-year bins. As described previously, increased age is associated with a reduction in nocturnal melatonin values; this drop correlates with a reduction in the TAS of the blood. This suggests that, as melatonin is lost, the ability of individuals to resist oxidative damage is likewise lessened. From Benot *et al.* (123).

levels in the aged may result in an increased frequency of clot development (Fig. 5). Considering these findings, maintaining higher blood levels of melatonin in advanced age may have prophylactic value by reducing the likelihood of an I/R accident. Supplemental melatonin may also be a consideration for individuals who are experiencing elevated oxidative stress, such as smokers; individuals with hypertension, atherosclerosis, or diabetes mellitus; and those who are obese. These are factors that predispose individuals to stroke.

Aspects of Melatonin's Antioxidative Actions

The potential for the use of melatonin in humans for the purpose of combating ROS/RNS-mediated damage during stroke would seem to be great. As summarized herein and elsewhere (77, 78), in the animal studies conducted to date, the indoleamine has proven highly effective in reducing much of the pathomorphology and pathophysiology associated with experimental stroke.

Melatonin is an endogenously generated molecule that is, therefore, not foreign to the body. Under basal conditions, melatonin's primary site of metabolism is probably the liver, where it is enzymatically converted to 6-hydroxymelatonin, which is subsequently conjugated to sulfuric or glucoronic acid, particularly the former. After their release into the blood as 6-hydroxymelatonin sulfate and 6-hydroxymelatonin glucoronate, respectively, these metabolites are excreted into the urine (34). In addition to these enzymatically mediated metabolic pathways, there may well be alternate routes for the breakdown of melatonin that do not require enzymatic interventions and that, under especially high oxidative stress conditions, produce metabolites that have important functions in the organism. Some of these products include cyclic-3-hydroxymelatonin, AMK, and AFMK (79–82, 125). Importantly, these by-products, particularly AMK and AFMK, as well as 6-hydroxymelatonin (and possibly others that remain unidentified), are themselves significant reducing agents that limit

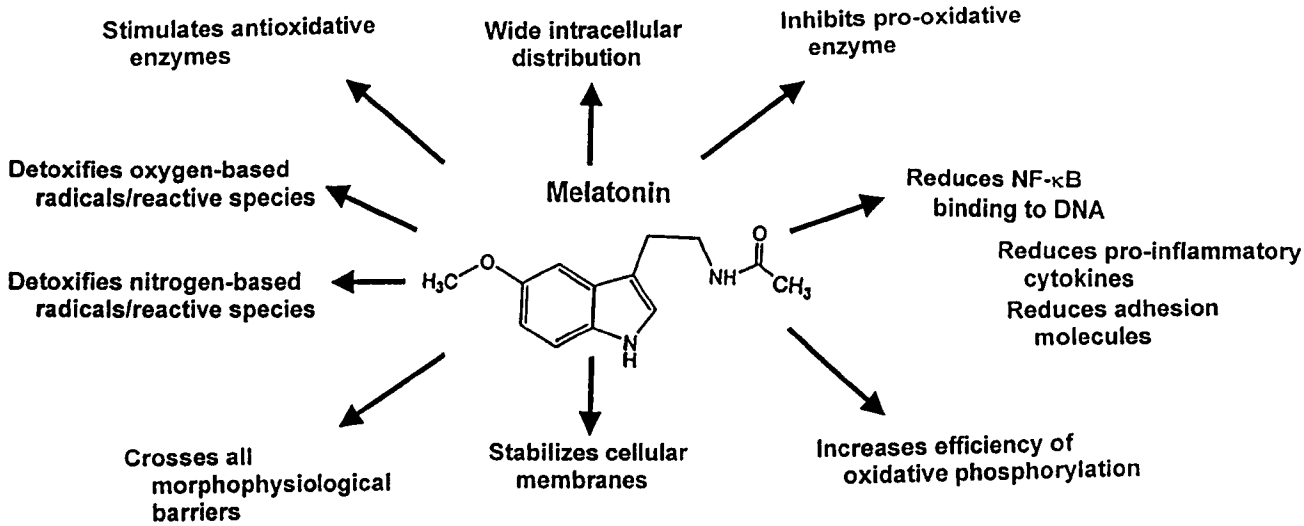

Figure 5. Some of the actions of melatonin that contribute to its ability to limit tissue destruction that is a consequence of ischemia/reperfusion injury in the brain as well as in other tissues. Besides scavenging both oxygen- and nitrogen-based reactants, melatonin stimulates (or preserves) the activities of a variety of antioxidative enzymes, such as superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase. Furthermore, it has beneficial actions at the mitochondrial level, where it seemingly enhances the efficiency of the electron transport chain. Also, it limits adhesion molecule formation, thereby reducing the likelihood of clot formation.

the degree of oxidative damage under a number of circumstances (83–86, 126, 127).

The ability of melatonin as well as some of its metabolites to function in the capacity of free radical scavengers and/or antioxidants has been referred to as an antioxidant cascade (19). Thus, as mentioned previously, not only is the parent molecule melatonin a protective agent against oxidative damage but, likewise, so are its metabolic "offspring." This combination of cascade actions would seem to increase melatonin's efficiency in limiting free radical mutilation.

An argument that could justifiably be levied against melatonin as an important antioxidant *in vivo* is its seemingly low concentrations relative to those of some other free radical scavengers, such as intracellular glutathione. Despite its seemingly low levels, surgical removal of the pineal gland, as noted in this report, a major source of circulating melatonin but by no means the only site of its generation, has repeatedly been shown to aggravate the amount of oxidatively damaged molecules under both basal and elevated oxidative stress conditions (32, 62, 128). This implies that physiological concentrations of melatonin, in conjunction with its metabolites, are sufficiently high to scavenge at least some of the toxic reactants formed or that there are means beyond their scavenging actions that account for some of the protection afforded by the indoleamine. As previously mentioned, subcellular concentrations of melatonin generally correlate poorly with levels in the blood, and certainly a number of fluids have melatonin concentrations that greatly exceed those in the serum (38, 39, 49–53).

It is an interesting possibility that melatonin may actually be inducible under high oxidative stress conditions in mammalian cells. This has been shown to be the case for the unicell *Gonyaulax polyedra,* where the induction of elevated free radical generation (because of their exposure to 0.08 m*M* H_2O_2) was associated with a marked rise in intracellular concentrations of melatonin (53). The exceptionally high intracellular levels of the indoleamine in this case were very likely generated for the purpose of inactivating the inordinately large number of ROS being produced as a consequence of the exposure of the cells to H_2O_2.

The question as to whether some mammalian cells have retained the capacity to respond to high oxidative stress conditions with an augmented production of the antioxidant melatonin has never been examined. Of interest regarding this point are the observations of Stefulj and colleagues (44). This group showed, using the reverse transcription polymerase chain reaction method, that the mRNAs for the two enzymes that convert serotonin to melatonin—serotonin-*N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT)—are present at low levels in a variety of mammalian cells (e.g., in raphe nuclei of the brain stem, spinal cord, and striatum as well as a variety of nonneuronal tissues). One obvious implication of these findings is that, in fact, even under basal conditions, a variety of mammalian cells may be capable of producing melatonin exclusively for their own use and/or that of their neighbors. No one, however, has yet documented whether melatonin is actually produced in these diverse mammalian cells and, if it is, whether levels exhibit a compensatory rise in response to elevated oxidative stress conditions. These are key presumptions that are worthy of investigation.

That melatonin is rapidly used, presumably as a free radical scavenger, when animals are subjected to raised oxidative stress conditions is known. Thus, when rats are forced to swim at night in darkness when the pineal gland is producing and secreting copious quantities of melatonin, the highly elevated plasma values of the indoleamine drop precipitously, as if the animals were acutely exposed to light. Simultaneously, pineal melatonin concentrations drop, but NAT activity, which rate-limits melatonin production, remains high, as it normally is during darkness (129–133). The precipitous drop in melatonin levels in both the blood and the pineal, despite its high level of production within the gland, is consistent with its rapid uptake and utilization by peripheral tissues. A likely possibility is that the augmented production of free radicals resulting from the physical and psychological stress imposed by swimming causes the sudden utilization of the indoleamine as an antioxidant with the result that blood and pineal levels drop even though its synthesis remains elevated.

Concluding Remarks

Numerous studies have now documented that melatonin is a highly effective agent to reduce tissue loss and neurophysiological deficits associated with I/R in animal models. The ability of melatonin to limit cellular destruction as a consequence of the transitory interruption of the blood supply to an organ is not confined to the brain but occurs in other tissues as well, such as the heart (26). Since both stroke and heart attack are very serious conditions that have marked individual and societal consequences, the use of melatonin to potentially protect against these conditions should be seriously considered. The fact that melatonin is an endogenously produced molecule should enhance its safety, given that it is not a foreign substance in the body. Many studies have examined pharmacological doses of melatonin, acutely and chronically in both mammals and humans, as to its toxicity. These investigations have uniformly revealed that the side effects of melatonin are minimal (134–139).

Melatonin's clinical use for the purpose described herein (or for any use) is hampered by the fact that it is a naturally occurring, nonpatentable molecule. Because of this and because it is easily synthetically produced, it is inexpensive. Thus, the profit margin, even for its widespread use, is limited. This prevents companies and/or individuals from promoting or using this molecule. Considering melatonin's high efficacy in reducing oxidative damage in animals and its marked potential for improving human health, it should be earnestly considered for use in

clinical trials, either as a single molecule and/or in combination with other antioxidants (140).

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