

Melatonin and oxidative stress

Oxidative stress plays a key role in the pathogenesis of multiple liver conditions. Such is the case with both alcoholic and nonalcoholic steatohepatitis, hemochromatosis, Wilson's disease, viral hepatitis, and drug-induced liver damage, as well as with liver fibrogenesis and carcinogenesis, apoptosis, and hepatic encephalopathy (1), among many others. In the present issue of the *Spanish Journal of Gastroenterology*, Dr. Cruz and colleagues (2) add chronic experimental cholestasis to the above list. They induced chronic cholestasis in rats by choledocus ligation, and then identified an increase in malonil-dialdehyde (MDA) and a decrease in antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, and catalase in the liver. A number of cardiovascular disorders that may be seen during cholestasis have been attributed to this kind of stress, including hypotension, decreased cardiac output, and reduced myocardial contractility (3-6). The results obtained by Cruz et al. are consistent with those previously reported by this same team (7,8) and other investigators in that experimental cholestasis induced by bile-duct ligation results in oxidative stress, which likely manifests in all organs, including the brain, intestine, kidneys, and liver (1,9-12).

Oxidative stress results from reactive oxygen species (ROS). Free radicals are molecules or atoms with an extra, unpaired electron in their outermost orbit. This negative electric charge renders these molecules highly reactive in an attempt to neutralize the surplus electron by stealing protons from other molecules or atoms. These radicals primarily develop in mitochondria, specifically in the so-called mitochondrial respiratory chain (MRC), during ATP formation from energy contained in carbohydrates and fatty acids. Under normal conditions, 1-4% of oxygen reaching cells is turned into ROS. This small amount can be easily neutralized by antioxidant systems in cells and mitochondria (glutathione, superoxide dismutase, catalase, glutathione peroxidase, etc.). However, on selected occasions, as in deficient MRC functioning, high fatty-acid levels in mitochondria, or increased β -oxidation, ROS formation increases. In such cases many electrons escape from the MRC and directly bind oxygen to form superoxide anion (O_2^-). The latter will give rise to other ROS and also to reactive nitrogen species (RNS), including the powerful hydroxyl radical ($\cdot OH$), hypochlorous acid, peroxyxynitrite ($ONOO^-$) – after binding nitric oxide (NO) (13,14) – and nitrosodioxycarboxylate anion ($ONOOCO_2^-$), which results from $ONOO^-$ binding CO_2 (15).

The toxicity of $ONOO^-$ results from its ability to directly bind hydroxyl radicals in the aromatic rings of amino acids (16), particularly of tyrosine (17), as well as sulfhydryls, zinc thiolates (18), lipids (19), proteins (20), and DNA (21,22). Some of these macromolecules may be destroyed during the aggression. Such effects have an impact on mitochondrial respiration, cell membrane functioning, and genetic ex-

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pression. It is well known that peroxynitrite reduces MRC complex activity (23), and we confirmed that the *in vitro* exposure of normal mitochondrial proteins to ONOO⁻ results in MRC complex degradation and inactivation (24,25). While DNA cleavage may occur at any nucleotide, guanine residues are preferred (26). The action of ONOO⁻ on guanine results in the formation of guanine radicals (\cdot G), which persist in DNA, mainly in mitochondrial DNA, for a long time. Attempts at DNA repair result in *poly-ADP ribose polymerase* (PARP) activation, and such repair is performed at the expense of highly significant energy consumption, which leads to cell death by necrosis (13). However, ONOO⁻ may also destroy or inactivate repair enzymes (27), which would perpetuate DNA damage. Manganese superoxide dismutase (MnSOD) is another enzyme that may be degraded by ONOO⁻, which helps reduce antioxidant capacity in cells (28), and thus originates a vicious circle that increases oxidative stress (29). The binding of protein tyrosine residues may interfere with tyrosine kinase signaling (30,31). Another detrimental effect of ONOO⁻ is its ability to activate transcription factors (NF κ B, AP1), to increase proinflammatory cytokine (TNF α , IL1 β) production (32), and to act as an inflammatory factor. NO is consumed during ONOO⁻ formation, which limits its effects on G protein, as well as its vasodilating effects.

Back to cholestasis, the mechanisms by which this condition induces oxidative stress are uncertain, but bile-duct ligation is known to decrease MRC complex activity (33,34), fatty-acid β -oxidation (33), and antioxidant system functioning (35,36). All this may account for oxidative stress but Esrefoglu et al. provided evidence that bile-duct ligation, in addition to oxidative stress, also results in nitrosative stress by RNS (37). Indeed, hepatic nitrite levels increase during experimental cholestasis (38). Liver damage is likely to induce the inducible nitric oxide synthase (iNOS), to result in NO formation, and hence to give rise to ONOO⁻ formation after NO binding superoxide anions. Bile-duct clearance is followed by a rapid regression of most of the effects induced by biliary obstruction, which suggests that ROS/RNS-related stress results from retention in the liver of substances usually excreted in the bile. These include bilirubin (39), hydrophobic bile salts (34), and cholesterol (40). The latter may modify the physical properties of mitochondrial membranes and increase their rigidity, which has an impact on the activity of enzymes anchored to these membranes (41). Bile acids are most likely responsible for stress. This is suggested by Sokol et al., who demonstrated that hydrophobic bile acids (tauro-chenodeoxycholic acid) give rise to ROS formation, lipid peroxidation, and then liver damage. These effects may be prevented with antioxidants, specifically α -tocopherol (42). Hydrophobic bile acids may directly modify the activity of enzymatic complexes, but it has been suggested that these acids may also solubilize membranes and induce cell death (43), thus leading to oxidative stress, lipid peroxidation, aldehyde formation (MDA, 4-hydroxynonenal), reduced glutathione consumption, and decreased GSH/GSSG ratio (44).

Substances that can neutralize \cdot OH or ONOO⁻ are crucial to protect cells and the body from oxidative and nitrosative stress. In their paper Cruz et al. show that *melatonin* (MT) (N-acetyl-5-methoxytryptamine) may prevent oxidative stress and some of the effects of chronic cholestasis on the body. Interest and understanding in the effects of MT have dramatically increased in recent years. MT is a serotonin derivative released by the pineal gland of vertebrates (45), and to a lesser extent by the retina, gastrointestinal tract (46), and bone marrow (47). It was initially thought to play a role in sleep and circadian rhythm regulation (48), but was subsequently seen

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to also influence the immune system (49), to have oncostatic (50) and anti-inflammatory (51) properties, and to represent a powerful antioxidant that can eliminate both ROS and RNS (52-54). Here we shall discuss the latter effects only.

The administration of pharmacological doses of MT reduces the formation of free radicals, allows the recovery of antioxidant enzymes, and decreases oxidative liver damage (55), including the effects of bile-duct ligation (56-58). The antioxidant power of MT is far greater than that of vitamin E or C, and 5 to 15 times that of glutathione. Such power can be accounted for by the fact that MT targets not only ROS (H_2O_2 , 1O_2 , $\cdot OH$) but also RNS and their derivatives (14,59,60), most particularly the highly potent ONOO⁻. Upon reacting with the latter, 1-nitromelatonin results (61). Many beneficial effects of MT under pathological conditions where oxidative stress is presumed to play a significant role (62,63) likely result from its ability to neutralize peroxynitrite.

Similarly, MT protects both nuclear and mitochondrial DNA from degradation after exposure to ionizing radiation or carcinogens (64,65). This is suggested by the fact that pre-exposure to MT reduces 8-hydroxy-2'-deoxyguanosine, a marker of DNA oxidative degradation. Furthermore, on preventing DNA damage, MT also prevents the huge energy consumption entailed by DNA repair by PARP (66-68), as well as the risk for necrosis.

In addition, MT enhances cellular defense mechanisms –it specifically augments the activity of antioxidant enzymes and decreases that of oxidant enzymes (69,70). In actuality, MT increases messenger RNA levels for superoxide dismutase and gamma-glutamylcysteine synthase (71,72), which enhances the formation of glutathione and glutathione peroxidase (72,73).

MT effects are in many aspects ahead of other antioxidants. As discussed above, MT effects not only counteract ROS but also target RNS. Such power against nitrosative stress is not shared by most other antioxidants. MT is the only agent that can eliminate all the components of the so-called “diabolic triangle” (superoxide anion, NO, ONOO⁻). MT can inhibit iNOS (74-76) and limit NO and peroxynitrite formation. Moreover, no anatomical barriers block its diffusion (77), hence virtually all organs, tissues, and cells are protected by this hormone (78). Other antioxidants commonly encounter impassable barriers. Such is the case with α -tocopherol, which cannot cross the blood-brain and placental barriers. Its penetration power affects not only organs and tissues, but also cell compartments, including the nucleus and mitochondria. It is on these grounds that MT protects cell *membranes*, cell proteins, and both the genomic and *mitochondrial* DNA (65,79). This high diffusibility results from its amphiphilic nature, that is, its high ability to dissolve both in lipids and water. For example, α -tocopherol is a fine fat-soluble antioxidant, hence its protective effects are confined to cell membranes. N-acetyl-cysteine and ascorbate are water-soluble compounds and, as a consequence, cannot protect or even cross cell membranes, or enter the cell.

While MT levels are low in the blood, probably lower than required to exert any antioxidant activity, intracellular concentration is high because of proteins that bind, retain, and concentrate MT (80). MT levels in the various tissues are highly variable depending on these proteins' concentrations within cells, and on receptor expression in their membranes (81).

Furthermore, MT reduces inflammatory response by blocking proinflammatory cytokine transcription (82). These cytokines include TNF α , an iNOS inducer that increases NO synthesis, reduces MRS activity, increases superoxide anion and ONOO⁻ formation, and contributes to both oxidative and nitrosative stress.

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By blocking TNF α synthesis MT prevents this vicious circle from developing, which would aggravate peroxynitrite-induced stress.

An issue shared by many antioxidants, excluding MT, is that under selected circumstances they may become electron donors, behave like oxidants themselves, and worsen oxidative stress. There is no known circumstance where MT may behave in this way. MT may be designated a suicidal antioxidant, as it degrades and disappears after becoming oxidized to reduce other molecules. In contrast to other antioxidants, MT does not use GSH up, but increases GSH cell and tissue reserves (83).

MT effectiveness in vivo has been acknowledged in a number of patients and experimental disease models where oxidative/nitrosative stress is deemed to play a pivotal pathogenic role (65,79,84,85). On the contrary, pineal gland removal, that is, the elimination of the natural source of melatonin, aggravates lesions in these experimental models (79).

From all the above, MT may be considered an exceptional natural substance with multiple beneficial actions on the body that, besides being a regulator of sleep and circadian rhythm, behaves as a powerful antioxidant and antinitrosant to protect the body against illnesses and diseases where oxidative and nitrosative stress play a decisive role. MT acts thus as an anti-inflammatory and anti-malignant agent, delays ageing processes (79,86), and –as stated by Cruz et al. in their article– protects the body against chronic cholestasis effects (2).

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