# Melatonin prevents hydrogen peroxide-induced apoptotic cell death

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**Summary**

Melatonin (MEL) functions in organisms are diverse. The actions considered in the current work relate to its ability to prevent oxidative stress, i.e. molecular damage produced by reactive oxygen species (ROS). Apoptosis is an active form of cell death that is initiated by a variety of stimuli, some of which inducing ROS increase. Hydrogen peroxide ($H_2O_2$) is considered a typical cytotoxic and oxidant agent capable to induce cellular damage through free radical production. In the present work we have investigated its role in the induction of cell death. U937 cells were exposed to 500 µM $H_2O_2$ and MEL behaviour, in the presence of this type of oxidative stress, was evaluated by incubating cells with MEL before and after $H_2O_2$ exposure. Cytometric and microscopy analyses were utilized and revealed that $H_2O_2$ can be considered an apoptotic trigger, if used at proper concentrations. In fact U937 cells, after 500 µM $H_2O_2$ exposure, showed chromatin condensation, micronuclei presence, apoptotic bodies and secondary necrosis. MEL, added before $H_2O_2$ treatment, significantly reduced apoptotic cell number. On the contrary, MEL incubation after $H_2O_2$ induced an increase of apoptotic cell death, and cells in secondary necrosis were detectable. These results indicated that pre-incubation with MEL reduces $H_2O_2$-dependent apoptosis in U937 cells, suggesting a capacity of this hormone to interfere with apoptosis induced by ROS increase.

**Key words:** U937, melatonin, hydrogen peroxide, apoptosis, reactive oxygen species.

## Introduction

Melatonin (N-acetyl-5-methoxytryptamine), the hormone produced by the pineal gland, was shown to have significantly broad actions including oncostatic effects (Bejarano *et al.*, 2009), immune system stimulation (Guerrero and Reiter, 2002) and anti-inflammatory functions (Radogna *et al.*, 2009). Subsequently, MEL was identified as a direct free radical scavenger (Reiter and Korkmaz, 2009) and an indirect antioxidant (Rodriguez *et al.*, 2004). Its function consists in the reduction of oxidative stress, i.e. molecular damage produced by reactive oxygen and nitrogen species (Reiter, 2008). Numerous reports evidenced MEL ability to neutralize free radicals, molecules that have an unpaired electron in their valence orbital, as hydroxyl (•OH) (Li *et al.*, 2002) and oxygen radical ($O_2$•$^-$). Melatonin is also a scavenger of hydrogen peroxide (Tan *et al.*, 2000), a non-radical ROS, generated *in vivo* by several enzyme systems and produced intracellularly by the dismutation of the superoxide anion radical ($O_2$•$^-$). In addition, it is known that MEL acts by indirectly stimulating the antioxidative enzymes, that represent a major defence mechanism against free radical damage.

Literature evidences that MEL, through both antioxidant and scavenger functions, plays an important role in preventing apoptosis: its anti-apoptotic activity was described in several systems, including cerebellar neurons (Baydas *et al.*, 2002) and brain astrocytes (Jou *et al.*, 2004), where it was hypothesized to have an antioxidant

role. A recent study demonstrated MEL capacity to prevent UVB-induced apoptosis in U937 cellular line (Luchetti *et al.*, 2006). In the present work, we have evaluated, by means of cytometric and morphological techniques, cell death induced by $H_2O_2$ treatment in the same cells, investigating, in particular, MEL function in preventing cell damage induced by ROS increase generated by $H_2O_2$ exposure.

## Materials and Methods

### Cell culture

U937 human myelomonocytic lymphoma cell line was grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 25 mM Hepes pH 7.5, 1% antibiotics and maintained at 37°C in humidified air with 5% $CO_2$. Cell viability was assessed by MTT test (Mosmann, 1983).

For the induction of apoptosis, cells (seeded at 1x106 cells/mL) were exposed to 500 µM $H_2O_2$ (Fukamachi *et al.*, 1998) for 3h at 37°C in humidified air with 5% $CO_2$.

MEL (Sigma) was first dissolved in absolute ethanol at initial 100 mM concentration and then diluted at final 1 mM concentration in culture medium. 1 mM MEL treatment (Luchetti *et al.*, 2006) was performed both immediately before and after $H_2O_2$ treatment; subsequently, the treated cells were post-incubated as described above.

### Flow cytometry (FC)

Cell death features (early and late apoptotic, as well as necrotic cells) were evaluated using propidium iodide (PI) staining carried out according to manufacturer's instructions. For FACS analysis $1\times10^6$ cells were collected by centrifugation at 300 g for 5 min at 4°C and washed once in ice-cold PBS. Cells were fixed (and permeabilized) with -20°C cold 70% ethanol overnight at 4°C. Fixed cells were washed in PBS once and resuspended in 1 mL of staining solution (40 µg/mL Propidium Iodide and 100 µg/mL RNase A in PBS). The samples were incubated for 30 min at room temperature in the dark. All samples were analyzed by EPICS XL flow cytometer (Beckman Coulter, Miami, FL, USA) with the appropriate software (System II, Beckman Coulter). Data were analyzed and histograms were plotted using winMDI version 2.9 flow cytometry application software (Scripps Research Institute, La Jolla, CA, USA). Values of PI fluorescence were presented as DNA histograms, showing cell distribution into the main cycle phases (G0/1 =M1, S=M2, G2-M= M3) and where the possible presence of apoptotic cell death can be recognize by typical sub-G1 peak (M4).

### Light and electron microscopy

Differently treated U937 pellets were immediately fixed in 2.5% glutaraldehyde in 0.1 M Sörensen phosphate buffer pH 7.3, postfixed in 1% $OsO_4$ in the same buffer, dehydrated with ethanol and embedded in araldite as previously described (Burattini *et al.*, 2009). Semithin sections were stained at 50-60°C with a mixture of 1% methylene blue and 1% toluidine blue in distilled water. Thin sections were collected on nickel grids, stained with uranyl acetate and lead citrate, and observed with a Philips CM10 electron microscope.

## Results

MTT test (Figure 1) revealed a good cell vitality for control (100%) and MEL alone treated (95.4%) cells, but it decreased to 75% after $H_2O_2$. MEL added before $H_2O_2$ treatment reduced cell death and viability was 83.9%; on the contrary, MEL added after $H_2O_2$ exposure showed a behaviour similar to that with $H_2O_2$ alone, and cell vitality was 74%.

Flow cytometry (Figure 2) evidenced that both control (2A) and cells treated with MEL alone (2B) showed only 1.5% and 2.9%, apoptotic/necrotic cells respectively, revealing a good cell viability.

For both conditions a well preserved cell morphology was confirmed by reverse microscope (RM), semithin sections and ultrastructural analyses (Figure 3A, B, C, D; 4A, B).

PI staining showed that $H_2O_2$ treatment (Figure 2C) induced apoptosis, quantified as 10.6% of PI / FITC ANX-V positive cells. RM and LM showed a variety of apoptotic cells (Figure 3E, F). TEM observations, after $H_2O_2$ treatment, evidenced characteristic apoptotic patterns (Figure 4C), such as chromatin margination towards nuclear periphery with typical cup-shaped patches (Figure 4D). Micronuclei, scattered throughout the cytoplasm and progressively released in

extracellular space, characterize the late apoptotic stage (Figure 4E).

When cells were treated with 1mM MEL before $H_2O_2$, apoptotic cell number was similar to that of the control (Figure 2D). Its percentage appeared, in fact, strongly decreased (2.9%), as confirmed by RM, LM and TEM, that showed a low number of apoptotic cells (Figure 3G, H and 4F, G). Differently, MEL treatment after $H_2O_2$ exposure determined an apoptotic/necrotic cell increase, respect to $H_2O_2$ alone. In fact, percentages detected were 31% of PI/FITC ANX-V positive events (Figure 2E). A higher number of apoptotic cells could also be revealed at RM, LM and TEM, generally characterized by late apoptotic pattern and secondary necrosis (Figure 3I, L and 4H, I).

## Discussion

MEL is an effective antioxidant molecule and a free radical scavenger. Its efficacy is also correlat-

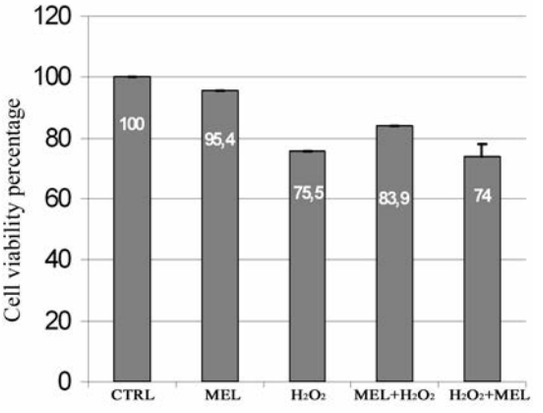


**Figure 1.** MTT assay test. A high percentage of cell vitality appears in both control and MEL treated cells (100% and 95.4% respectively). A reduction of cell viability (75.7%) was obtained after $H_2O_2$ treatment. When cells were pre-treated with 1 mM MEL their vitaility increases to 83.9%, whereas when MEL was added after $H_2O_2$ exposure a behaviour similar to $H_2O_2$ treatment (74%) was observed. Data were collected from three experimental conditions and they were expressed as mean +/- standard error.

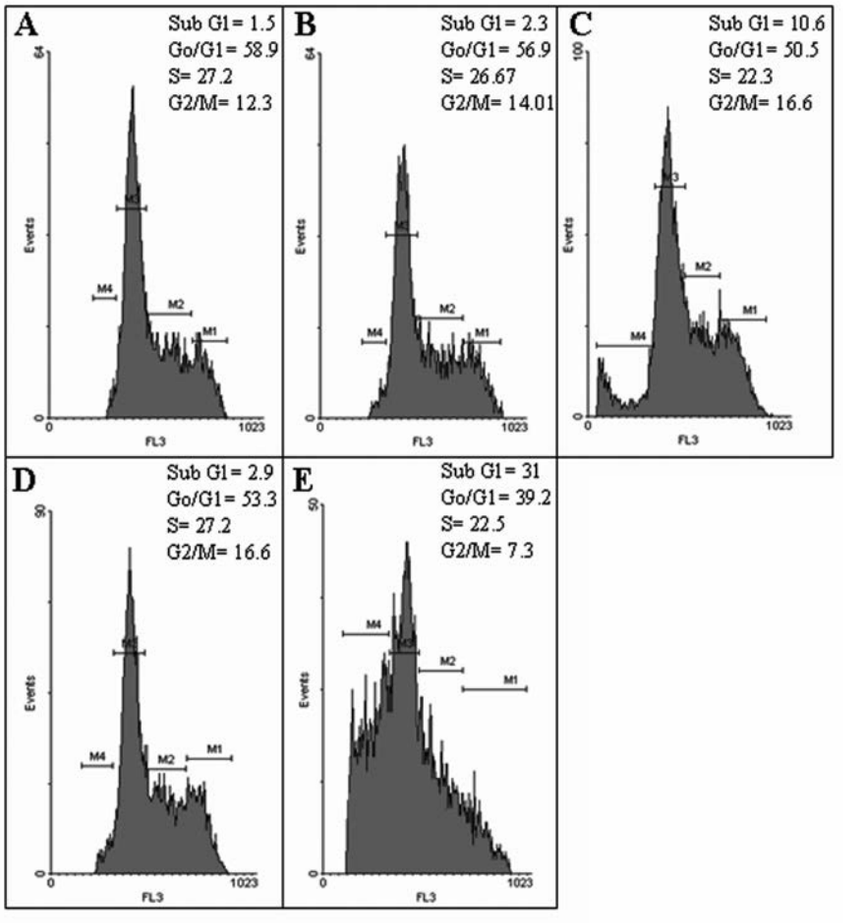


**Figure 2.** FC analysis of DNA content. U937 control cells and cells treated with 1mM MEL alone show a similar distribution of cell cycle phases (A and B, respectively). $H_2O_2$ treated cells reveal a weakly subdiploid peak (C), drastically reduced when cells were MEL-pretreated (D). On the contrary, an evident increase of subG1 events appears when U937 cells are incubated with 1 mM MEL after $H_2O_2$ exposure (E). Representative cytograms are taken from three independent experiments for all experimental conditions

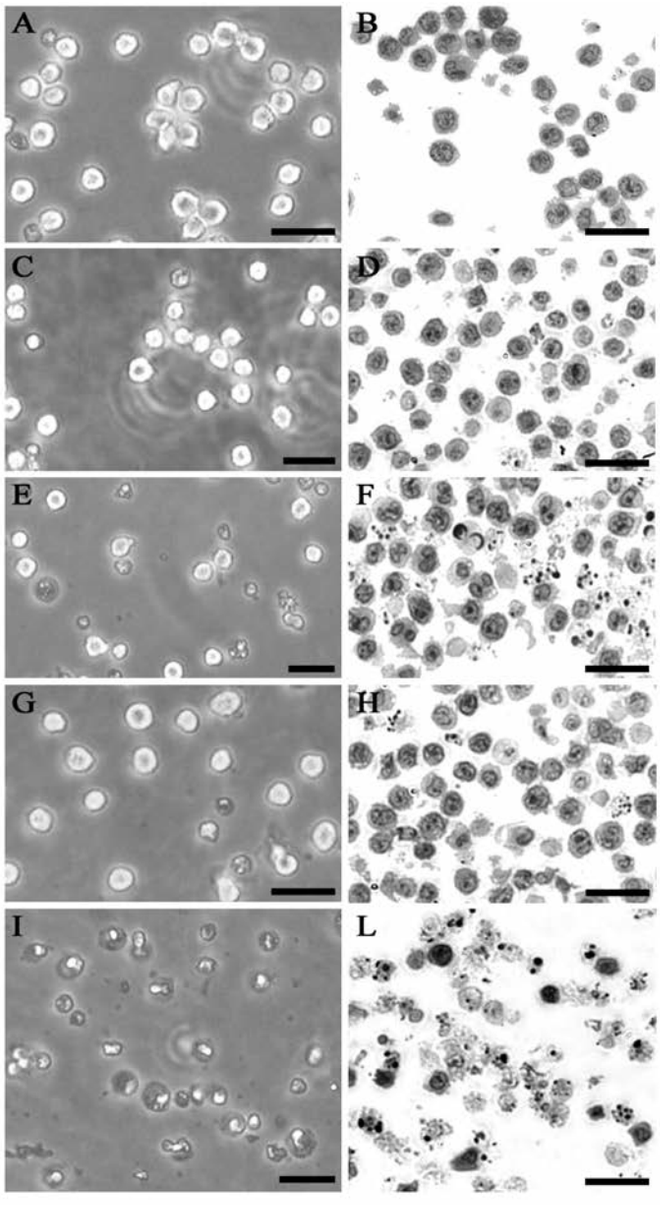


**Figure 3.** U937 cells at RM (A, C, E, G, I) and LM (B, D, F, H, L). Control cells (A, B) and MEL alone treated cells (C, D) show a well preserved morphology. After $H_2O_2$ treatment, apoptotic cell death is evident (E, F). When MEL is added before $H_2O_2$ treatment, only few cells appear apoptotic, being the others similar to the control (G, H). When MEL is added after $H_2O_2$ exposure several apoptotic cells are observed (I, L). Bar = 10 µm.

ed to ability to enhance the activities of a variety of antioxidative enzymes (Rodriguez *et al.*, 2004), stimulatory actions on glutathione synthesis (Winiarska *et al.*, 2006), reduction of electron leakage from the mitochondrial electron transport chain (Leon *et al.*, 2006), and to its synergic interactions with other antioxidants (Lopez-Burillo *et al.*, 2003).

MEL was originally shown to inactivate the highly toxic hydroxyl radical (•OH). Since this discovery, its scavenging repertoire has been demonstrated against $H_2O_2$ (Tan *et al.*, 2000), hypochlorous acid (HOCl) (Zavodnik *et al.*, 2004), singlet oxygen ($O_2$•) (Matuszak *et al.*, 2003), superoxide anion radical ($O_2$ •$^-$), nitric oxide (NO•) (Aydogan *et al.*, 2006), peroxynitrite anion ($ONOO^-$) (Reiter *et al.*, 2001) and others (Hardeland *et al.*, 2005).

In addition, it also favours the defence against oxidative stress by promoting enzymes that metabolize radicals and their products to innocuous agents (Reiter *et al.*, 2008). These indirect MEL antioxidative effects may be mediated *via* membrane and/or nuclear receptors. Examples

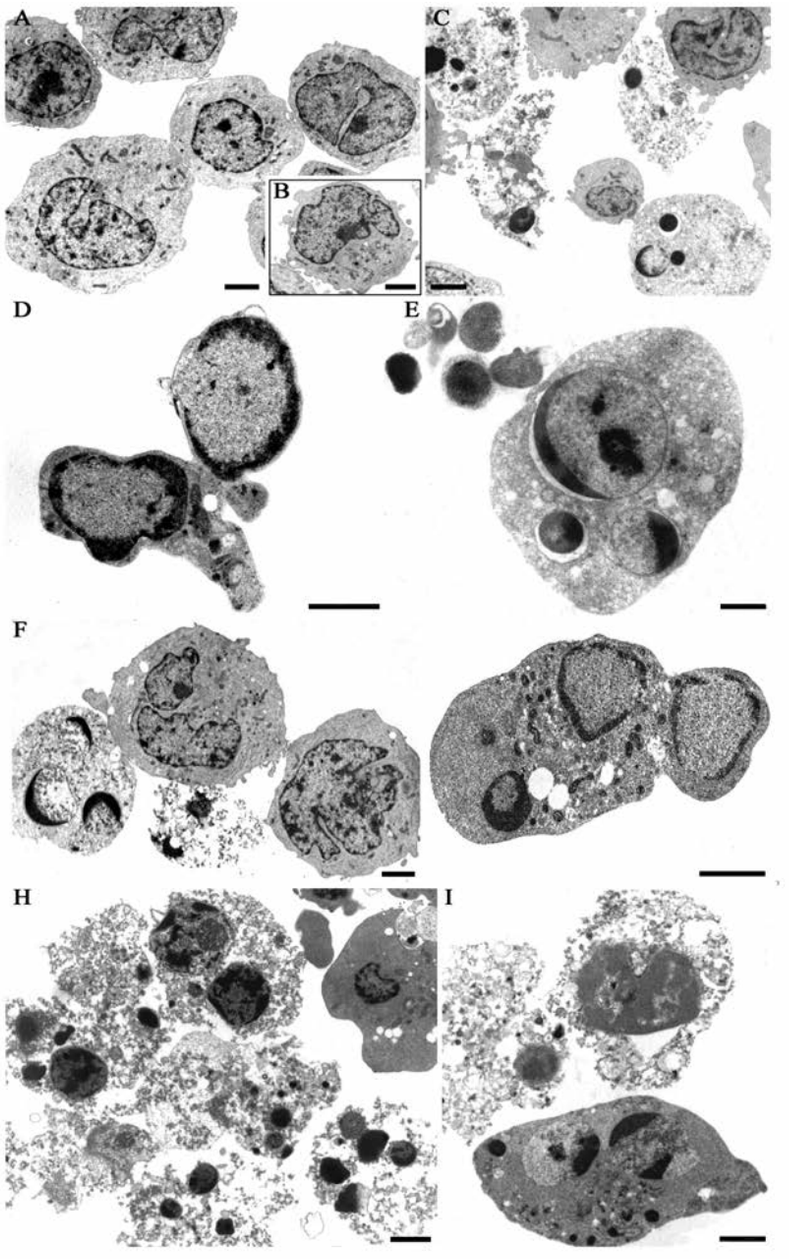


**Figure 4.** TEM analysis. Control cells (A) and U937 treated with MEL alone (B) show a comparable ultrastructural good morphology. After 500 µM $H_2O_2$ treatment, apoptosis appears in all apoptotic stages (C, D, E). Pre-treatment with MEL and successive exposure to $H_2O_2$ evidences a decrease of apoptotic cell number (F,G). When cells are exposed to $H_2O_2$ and then treated with MEL, apoptotic cell number appears strongly increased, with cells in late apoptotic stages, micronuclei, and secondary necrosis (H, I). A, C, F, H, bar = 2.5 µm. B, D, F, G, I bar = 2 µm.

include the widely reported stimulation of glutathione peroxidase (GPx) (Rodriguez *et al.*, 2004), which converts hydroperoxides, including $H_2O_2$, to water and oxygen, while oxidizing GSH.

In this study we have examined the effect of MEL on apoptosis induced via oxidative stress generated by $H_2O_2$. Our data show that $H_2O_2$, while being a free radical inducer, can be considered not only a cytotoxic agent, but an important apoptotic trigger, if utilized at proper concentration i.e. 500 µM. In addition, this study confirms MEL action as an effective antioxidant and free radical scavenger, with a possible protective role from apoptotic /oxidative damage.

These findings are in agreement with our previous study (Luchetti *et al.*, 2006), which reported that MEL was able to prevent UVB-induced apoptosis in U937 cells. Both UVB radiations and $H_2O_2$ lead to ROS generation and cell damage, but UVB seem a more powerful apoptotic trigger. To this

regard, a study on keratinocytes demonstrated that both UVB and $H_2O_2$ led to the increase of intracellular $H_2O_2$ levels, the antioxidants catalase and glutathione monoester, inhibited apoptosis only when induced by $H_2O_2$ and not by UVB (Chang *et al.*, 2003). In addition, DNA damage in the form of cyclobutane pyrimidine dimers was observed after exposure to UVB, but no photoproducts were found in $H_2O_2$-treated cells, suggesting a ROS-independent pathway of UVB-induced apoptosis (Chang *et al.*, 2003). In the present study apoptosis is a weak process if compared with that induced by UVB.

Neverthless, Luchetti *et al.*, (2006) showed that apoptosis was reduced when MEL is added both before and after UVB exposure. Differently, in the present study MEL prevents apoptosis only when added before $H_2O_2$. Differently, MEL incubation after $H_2O_2$ treatment lead to an increase of apoptotic cell death, suggesting that $H_2O_2$ intracellular level increases and free radical production can not be further controlled by MEL. A recent work on mouse mitochondria incubated with MEL, evidenced its capacity to down regulate oxygen consumption, to inhibit oxygen flux increase in the presence of an excess of ADP, to reduce membrane potential, and, consequently, to prevent superoxide anion and hydrogen peroxide production (Lopez *et al.*, 2009).

This study emphasizes MEL role as effective antioxidant and anti-apoptotic trigger. In fact, besides its direct scavenging actions, MEL stimulates several antioxidative enzymes, including superoxide dismutase, glutathione peroxidase and glutathione reductase (Reiter *et al.*, 2002). Therefore, in our model MEL pre-treatment prevents $H_2O_2$ induced apoptosis because of MEL capacity to stimulate protective oxygen radical scavengers. Apoptosis induced by cell damage, via intrinsic pathway, implies redox imbalance or even direct oxidative stress (Gao *et al.*, 2005). Therefore, radical-scavenging MEL property is the mechanism correlated to its anti-apoptotic activity, confirmed by the observation that MEL concentrations required for radical scavenging are closely compared to the anti-apoptotic ones (Radogna *et al.*, 2007). In conclusion, this study is a demonstration of MEL protective role from ROS increase induced by $H_2O_2$, at apoptotic concentration.

## References


Aydogen S, Yerer MB, Goktas A. Melatonin and nitricoxide. J Endocrinol Invest 2006; 29:281-7.

Baydas G, Reiter RJ, Nedvetskii VS, et al. Altered glial fibrillary acidic protein content and its degradation in the hippocampus, cortex and cerebellum of rats exposed to constant light: reversal by melatonin. J Pineal Res 2002; 33: 134-139.

Bejarano I, Redondo PC, Espino J, et al. Melatonin induces mitochondrial-mediated apoptosis in human myeloid HL-60 cells. J Pineal Res 2009;46:392-400.

Burattini S, Ferri P, Battistelli M, et al. Apoptotic DNA fragmentation can be revealed in situ: a morphological approach. Microsc Res Tec 2009;72:913-23.

Chang H, Oehrl W, Elsner P, Thiele JJ. The role of H2O2 as a mediator of UVB-induced apoptosis in keratinocytes. Free Radic Res 2003; 37:655-63.

Fukamachi Y, Karasaki Y, Sugiura T, et al. Zinc suppresses apoptosis of U937 cells induced by hydrogen peroxide through an increase of the Bcl-2/Bax ratio. Biochem Biophys Res Commun 1998;246:364-9.

Gao J, Liu X, Rigas B. Nitric oxide-donating aspirin induces apoptosis in human colon cancer cells through induction of oxidative stress. Proc Natl Acad Sci USA 2005;102:17207-12.

Guerrero JM, Reiter RJ. Melatonin-immune system relationships. Curr Topics Med Chem 2002;2:167-80.

Hardeland R, Pandi Perumal SR. Melatonin, a potent agent in antioxidative defense: actions as a natural food contaminant, gastrointestinal factor, drug and prodrug. Nutr Metab 2005;2: 22-31.

Jou MJ, Peng TI, Reiter RJ, et al. Visualization of the antioxidative effects of melatonin at the mitochondrial level during oxidative stress-induced apoptosis of rat brain astrocytes. J Pineal Res 2004;37:55-70.

Leon J, Escames G, Rodriquez MI, et al. Inhibition of neuronal nitric oxide synthase activity by N1-acetyl-5-methoxykynuramine, a brain metabolite of melatonin. J Neurochem 2006;98:2023-33.

Li XJ, Gu J, Lu SD, Sun FY. Melatonin attenuates MPTP-induced dopaminergic neuronal injury associated with scavenging hydroxyl radical. J Pineal Res 2002; 32:47-52.

López A, García JA, Escames G, et al. Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production. J Pineal Res 2009;46:188-98.

Lopez-Burillo S, Tan DX, Mayo JC, et al. Melatonin, xanthurenic acid resveratrol, EGCG, vitamin C and

alpha-lipoic acid differentially reduce oxidative DNA damage induced by Fenton reagents; a study of their individual and synergistic actions. J Pineal Res 2003;34:269-77.

Luchetti F, Canonico B, Curci R, et al. Melatonin prevents apoptosis induced by UV-B treatment in U937 cell line. J Pineal Res 2006;40:158-67.

Matuszak Z, Bilska MA, Reszka K, et al. Interaction of singlet molecular oxygen with melatonin and related indoles. Photochem Photobiol 2003; 78:449-55.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.

Radogna F, Paternoster L, Albertini MC, et al. Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells. J Pineal Res 2007;43:154-62.

Radogna F, Sestili P, Martinelli C, et al. Lipoxygenase-mediated pro-radical effect of melatonin via stimulation of arachidonic acid metabolism. Toxicol Appl Pharmacol 2009;238:170-7.

Reiter RJ, Tan DX, Manchester LC, Qi W. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species. Cell Biochem Physiol 2001;34:237-56.

Reiter RJ, Tan DX. Melatonin: an antioxidant in edible plants. Ann N Y Acad Sci 2002;957:341-4.

Reiter RJ, Tan DX, Jou MJ, et al. Biogenic amines in the reduction of oxidative stress: melatonin and its metabolites. Neuro Endocrinol Lett 2008;29:391-8.

Reiter RJ, Korkmaz A. Clinical aspects of melatonin. Saudi Med J 2009;29:1537-1547.

Rodriguez C, Mayo JC, Sainz RM, et al. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res 2004;36:1-9.

Tan DX, Manchester LC, Reiter RJ, et al. Melatonin directly scavenges hydrogen peroxide: A potentially new metabolic pathway of melatonin biotransformation. Free Radic Biol Med 2000;29:1177-85.

Winiarska K, Fraczyk T, Malinska D, et al. Melatonin mitigates diabetes-induced oxidative stress in rabbits. J Pineal Res 2006;40:168-76.

Zavodnik IB, Lapshima EA, Zavodnik LB, et al. Hypochlorous acidinduced oxidative stress in Chinese hamster B14 cells: viability, DNA and protein damage and the protective action of melatonin. Mutat Res 2004;559:39-48.