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Melatonin: Quantum-chemical and biochemical investigation of antioxidant activity

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ABSTRACT

Experimental and theoretical investigation of the antioxidant activity of melatonin is carried out. The theoretical approach comprises the evaluation of several appropriate descriptors of scavenging activity with the help of quantum-chemistry methods. The values obtained are compared with available data for substances with established antioxidant properties. One of the most widely used markers for in vivo free radical oxidation processes is malondialdehyde (MDA) as an end product of membrane lipid peroxidation. Experimental support of the computed scavenging parameters is provided by estimation of the effect of supplementary melatonin therapy on the plasma levels of MDA in CRF patients on maintenance HD therapy. Different reaction paths have been considered and related to the obtained data, allowing speculations about the reaction mechanism and the antioxidant potential of melatonin for practical purposes.

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1. Introduction

The aerobic organisms require oxygen to survive. However, during normal metabolism oxygen produces reactive oxygen species such as free radicals and related reactants, or oxidants for brevity, some of which are highly toxic and deleterious for cells and tissues. The oxidants that are not directly scavenged, or in other words not metabolized, attack cellular components producing harmful molecular debris and sometimes causing cellular death [1].

To protect the cells from the damage caused by oxidants, the organisms have evolved several antioxidant defense mechanisms for rapid and efficient removal of reactive oxygen species from the intracellular environment. In normal circumstances, there is a balance between antioxidants and oxidants. When the equilibrium between oxidants and antioxidant defense systems is imbalanced in favor of the oxidants, the condition is known as oxidative stress [1]. There is abundant evidence that the oxidative stress triggers various undesired processes at cellular, tissue and organism levels and plays a major role in the pathogenesis of many human diseases like ischemia/reperfusion syndrome, atherosclerosis, chronic renal failure (CRF), etc. [1].

It has been found that the oxidative stress plays a key role in the development of various complications during continual hemodialysis (HD) therapy of CRF patients [2,3]. Through the repeated contact between the blood and the HD membrane, endogenous inflammatory mechanisms are activated and the production of reactive oxygen species grows [4,5]. Partial elimination of some endogenous antioxidants (uric acid, vitamins, etc.) from the blood during the HD procedure is another factor contributing to the development of chronic oxidative stress [6]. Furthermore, clear indications for increased membrane lipid peroxidation caused by free radicals have been established in CRF patients [7,8]. In the clinical practices, preventive strategies for restricting the negative effects of oxidative stress during HD treatment are applied more and more frequently. However, in the recent years clinicians have become more reserved toward the use of exogenic antioxidants such as vitamins C and E when treating HD patients. This change in the opinion of the medical circles is due to the fact that in high concentrations the exogenic antioxidants turn into prooxidants [1]. This is why research efforts have been made for finding effective physiological antioxidants. In this sense, melatonin has been recognized as an appropriate antioxidant for controlling some endocrine and neurological dysfunctions and decreasing oxidative damages in CRF patients [2,9–11].

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On the other hand, there exist experimental proofs that melatonin is incapable of trapping/scavenging peroxyl radicals in homogeneous solutions and in model heterogeneous systems where metal ions are absent [12,13]. It was found also that the antioxidant activity of melatonin in soybean PC liposomes is much lower than that of α-tocopherol under comparable assay conditions but a combination of melatonin and α-tocopherol resulted in a synergistic antioxidant effect [14].

The neurohormone melatonin (*N*-acetyl-5-methoxytryptamine) is a highly conservative molecule that is produced in all organisms. In humans it is produced in the brain by the pineal gland from the essential amino acid tryptophan. The synthesis and release of melatonin are stimulated by darkness and suppressed by light [15]. Administered orally, melatonin is absorbed quickly by the gastrointestinal tract and easily passes through the morpho-physiological barriers as well as through the cellular membrane structures. Melatonin facilitates various physiologic processes – circadian rhythm, sleeping and being awake, sexual activity and reproductive functions, tumor growth, immune response and aging [16]. Due to its low toxicity, melatonin is used as pharmacological substance for treating sleep disorders [17].

In the current work, we present both experimental and theoretical investigation of the antioxidant activity of melatonin. The theoretical framework comprises several descriptors estimated with the help of quantum-chemistry methods. The values obtained are compared with available data for well-known antioxidants. Thus, we draw conclusions for the scavenging capabilities of melatonin and relate the results to the experimental data. The experimental part of this pilot study was designed to evaluate the effect of supplementary therapy with melatonin on the plasma levels of malondialdehyde (MDA) in CRF patients on maintenance HD therapy. MDA is an end product of membrane lipid peroxidation caused by free radicals and is one of the most widely used markers for in vivo free radical oxidation processes.

2. Theoretical approach

The chemical transformations in the reaction of radical scavenging by an antioxidant can follow three general pathways (Fig. 1) [18–21].

(i) Electron transfer from the antioxidant to the active radical, which produces a cation radical and an anion. The electron transfer is followed by proton transfer from the cation radical to the anion. (ii) Direct hydrogen atom transfer between the antioxidant and the active radical. (iii) Deprotonation of the antioxidant followed by electron transfer from the resulting anion to the active radical. The next step is protonation of the anion produced by the active radical. The mechanisms shown in Fig. 1 address only the formation of the final stable radical Ao• and do not account for any subsequent transformations of Ao•.

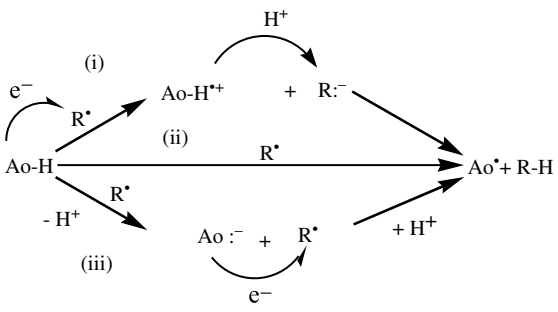

Fig. 1. The main reaction mechanisms of radical scavenging.

The probability of mechanism (i) depends on the reduction capability of the antioxidant which cannot be unambiguously deduced on pure chemical grounds. Mechanisms (ii) and (iii) are only feasible when a dissociable hydrogen atom (or, respectively, a proton) is present. Moreover, the stability of the resulting structure (Ao• or Ao:$^-$) is determined by the possibility of delocalization of the electrons remaining after the dissociation. The delocalization potential of melatonin is favorable for homolytical dissociation of the following bonds: the C–H bond in the methylene group connected to the indole ring (Radical 1), the N–H bond in the indole ring (Radical 2) and C–H bond in the acetyl group (Radical 3) (Fig. 2).

Mechanism (iii) is not expected to occur in melatonin because there is no functional group that can be easily deprotonated. This is also confirmed by previous investigations [22] which have found no evidence for mechanism (iii). Thus, the first two mechanisms ((i) and (ii)) need to be investigated in more detail.

The electron ionization energy is a quantitative descriptor of the ability of a compound to participate in scavenging of oxidants based on mechanism (i). With reasonable accuracy, the ionization energy from the highest occupied molecular orbital (HOMO) can be estimated by Koopmans theorem [23]. The descriptor related to mechanism (ii) is the bond-dissociation enthalpy (BDE). The BDE (at the 298 °K) was calculated according to the formula given in [24]. Apart from these two descriptors, another informative quantity, which is more closely associated with the reaction degree than with the reaction mechanism, is the spin density. The spin density serves as a quantum-chemical estimation of the above commented electron/spin delocalization and shows the stability of the final product of the scavenging reaction. The more stable the radical, the larger the shift of the reaction equilibrium to the right (see Fig. 1), i.e. the higher the antioxidant activity of the scavenging compound.

The theoretical values were obtained after geometry optimization of all the investigated structures at the unrestricted DFT level of theory with the B3LYP functional [25] using the GAUSSIAN03 program package [26]. It has been found that the B3LYP functional gives more reliable results with respect to BDE than the post Hartree–Fock methods [27]. Moreover, the utilization of post Hartree–Fock methods is usually more costly. The orbital basis set was chosen to be 6–31G(d,p) [28]. Further extension of the basis set usually does not improve the results [27,29]. The environment effect on the spin and charge distribution in Radicals 1 and 2, and in the cation radical was accounted for by means of introduction of implicit solvent (water) employing the polarizable continuum model (PCM) [30].

3. Experiment

The experiment was based on the fact that the serum levels of MDA are an excellent indicator for membrane lipid peroxidation by free radicals. This approach has been used before for patients with CRF where it has been found that high levels of MDA in the plasma and in the erythrocytes are related to decreased levels of some endogenous antioxidants [7,8]. Furthermore, it has been shown that melatonin traps a number of initiators of lipid peroxidation (hydroxyl radical, peroxynitrite, etc.) and in this way reduces the production of MDA [31,32].

MDA was assayed spectrophotometrically by its thiobarbituric acid reactivity in serum, using the method of Porter et al. [33] (the values measured were expressed in μmol/L). The method is based on the formation of a colored product between MDA and thiobarbituric acid. The effect of melatonin on the membrane lipid peroxidation was examined for a period of 3 months. The plasma MDA levels were investigated in a group of 25 CRF patients (11 females, 14 males) on maintenance HD. Their mean age was

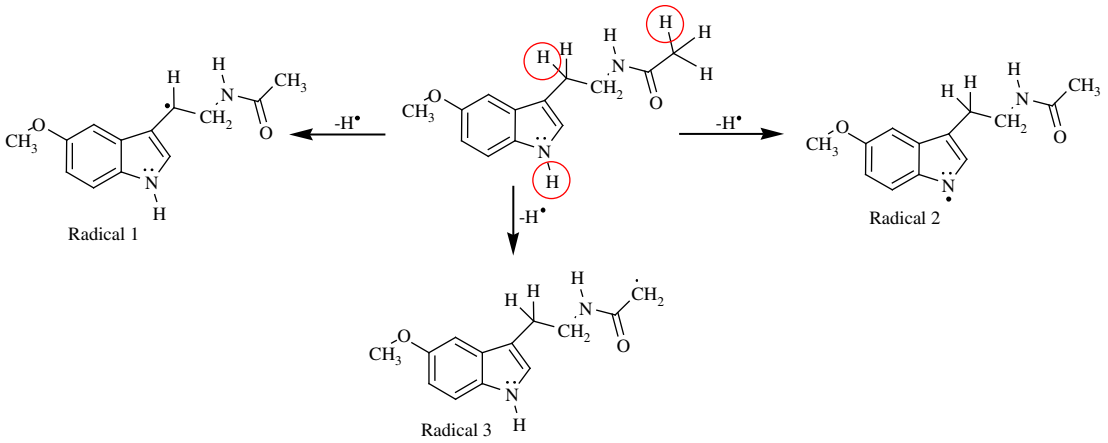

Fig. 2. The most probable melatonin radicals resulting from the scavenging reaction.

54.4 ± 11.0 years (range 32–75 years). The dialysis was performed with cellulose acetate membranes (1.5 m^2) thrice a week. The patients with clinical or biochemical history of acute infections, inflammations, and diabetes mellitus were not enrolled in the study. All patients gave informed consent for study participation.

Melatonin pills (2 × 1 mg, Adipharm, Bulgaria) were administered as a supplementary therapy once a day before night sleep for a period of 3 months. The MDA levels were examined before the melatonin treatment (the 0th month) and at the end of the 1st, 2nd and 3rd month of the supplementary melatonin therapy. Two parallel samples of venous blood were drawn from each patient before the HD session. The plasma was obtained by centrifugation of the blood at 2000 × *g* for 20 min at 20 °C, and was assayed immediately for MDA.

The results obtained were compared to the results for a reference group of 37 clinically healthy persons of average age 55.0 ± 10.0 years. The data were analyzed statistically by one-way analysis of variance (ANOVA) and expressed as mean ± SEM. A value of the statistical probability $p < 0.05$ was considered to be statistically significant.

Before the administration of melatonin (0th month), the MDA levels (5.47 ± 1.99 μmol/L) were almost 50% higher compared to the control values (2.68 ± 0.44 μmol/L) (Chart 1). The supplementary treatment with melatonin in a dose of 2 mg/day led to a considerable time-dependent reduction of the MDA levels. A month after the melatonin treatment began, a significant decrease ($p < 0.01$) in the levels of MDA was established (4.02 ± 1.78 μmol/L), but the levels still remained higher than the control values (Chart 1). Two months after the administration of melatonin an additional drop ($p < 0.001$) of the MDA levels (3.09 ± 0.49 μmol/L) was observed. At the end of the 3rd month the MDA levels reached the control values (2.79 ± 0.86 against 2.68 ± 0.44 μmol/L). The time-dependent decrease in this parameter is an evidence for the decline of the processes of lipid peroxidation after the administration of the melatonin dose used.

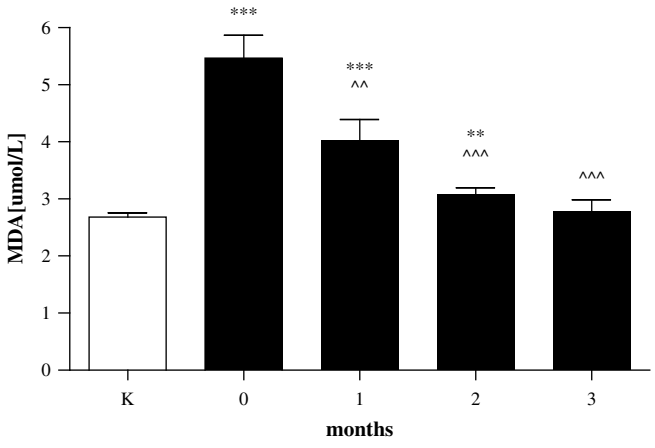

Chart 1. MDA plasma levels in HD patients treated with melatonin.

These results suggest that melatonin is an effective protector of the lipid membrane structures against free radical oxidation. Administered in a single, relatively low dose, it limits lipid peroxidation as seen from the sizeable lowering of the MDA levels in HD patients.

4. Discussion

It is more adequate to refer to the descriptors discussed above (see Section 2) in terms of scavenging rather than antioxidant activity. However, as far as the antioxidant activity could be considered as in vivo scavenging activity, these descriptors could be used successfully for evaluation of the intracellular interaction between pro- and antioxidants.

The HOMO energy is a good descriptor for mechanism (i) of the scavenging process. The higher the HOMO energy, the more active antioxidant the compound is. The HOMO energy of melatonin is −5.09 eV (−0.1877 a.u). In comparison, none of the twenty phenolic antioxidants previously investigated by us has such high HOMO energy [34] (see Table 1). The same is true for phlavonoid [35] and phenolic acids [24]. Even for α-tocopherol, the main component of vitamin E, the HOMO energy is lower: −5.23 eV (−0.1927 a.u) [34]. According to this index, melatonin should possess very high radical-trapping potential. This result shows that the most probable mechanism of radical scavenging reaction must be mechanism (i).

Further insight into mechanism (i) can be given by the NBO [36] charge distribution in the melatonin cation radical calculated in vacuum and in PCM (Fig. 3). The high positive charge of the hydrogen atoms connected to the nitrogen atoms suggests that the melatonin cation radical possesses significant N–H acidity and can easily release protons, thus facilitating the scavenging process. The NBO charges in PCM (solvent = water) reveal even higher polarity of the N–H and other polar bonds which aids additionally the proton detachment.

The descriptor relevant to the scavenging reaction following mechanism (ii) is BDE. We calculated BDE of three distinct radicals of melatonin. BDE of the C–H bond in the methylene group connected to the indole ring (Fig. 2, Radical 1) is 76.16 kcal/mol, BDE of

Table 1
Basic descriptors of scavenging/antioxidant activity for melatonin and referent antioxidants.

HOMO	Melatonin[a]	α-Tocopherol[b]	Phenolic antioxidants[b]	Phlavonoids[e]	Phenolic acids[g]
eV (kcal/mol)	−5.09 (−117.8)	−5.23 (−120.9)	−5.24 (−120.9) ÷ −6.79 (−156.6)	−7.85 (181.0) ÷ −8.83 (−203.6)	−6.06 (−140.3) ÷ −6.35 (−146.4)
BDE at 298 °K kcal/mol	Melatonin[a]	α-Tocopherol[c]	*para*-Substituted phenol[d]	Phlavonoids[f]	Phenolic acids[g]
	C–H[h]: 76.2 N–H[h]: 77.0 C–H[h]: 88.2	75.8	75.5 ÷ 94.2	72.4 ÷ 80.9	62.3 ÷ 73.1
Maximum spin density	Melatonin[a]	α-Tocopherol	Phenolic antioxidant radicals[b]	Glutathione radical[a]	Phenolic acid radicals[g]
	Radical 1[h]: 0.719 *Radical 2*[h]: 0.558 *Radical 3*[h]: 0.978	radical[b] 0.351	0.286 ÷ 0.424	0.843	0.290 ÷ 0.320

[a] Current calculations.
[b] B3LYP/6–31 + G(d,p) calculations, Ref. [30].
[c] B3LYP/6–311 + G(d,p) calculations, Ref. [33].
[d] Experiment, Refs. [37,38].
[e] HF/6–31G(d,p) calculations, Ref. [31].
[f] B3LYP/6–311 ++ G(3df,2p) calculations, Refs. [34–36].
[g] B3LYP/6–31 + G(d) calculations, Ref. [21].
[h] See the marked bonds in Fig. 2.

the N–H bond (Radical 2) is 77.00 kcal/mol, and BDE of the C–H bond in acetyl group (Radical 3) is 88.18 kcal/mol (see Table 1). It is clear that the formation of Radical 3 is much more difficult than the other two radicals. In comparison, the value of BDE of α-tocopherol is 75.8 kcal/mol [37]; the values of some phlavonoids are as follows: Fisetin – 72.35 kcal/mol; Taxifolin – 74.54 kcal/mol; Kaemferol – 74.73 kcal/mol; Epicatechin – 80.94 kcal/mol; and Myricetin – 73.72 kcal/mol [38–40]. It has been experimentally shown that different *para*-substituted phenols (X–C_6H_4–OH) have BDE (at 298 K) between 75.5 and 94.2 kcal/mol [41,42]. Unfortunately, we could not find BDE data for other indole-containing antioxidants in the literature. The comparison shows that it is not excluded that

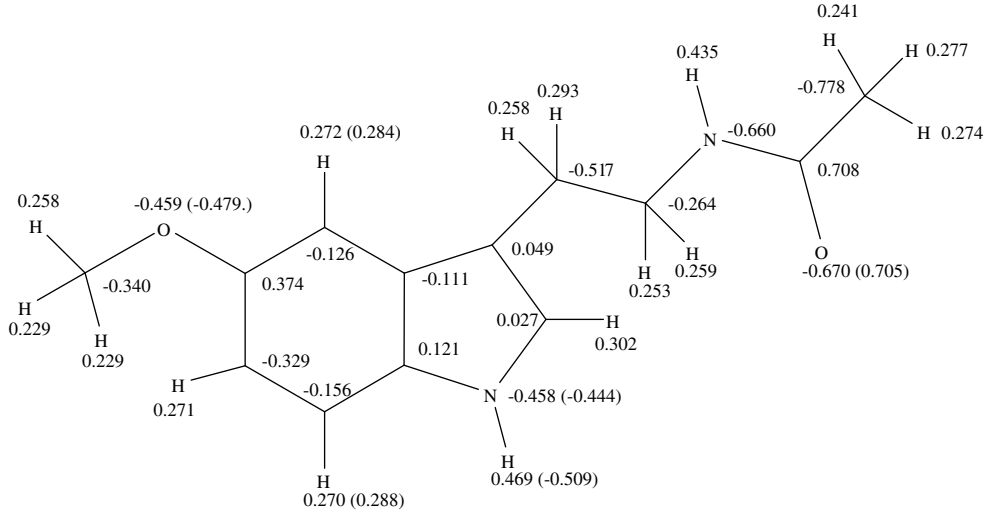

Fig. 3. NBO charge distribution in the melatonin cation radical. The values in brackets are obtained in PCM. Only the values differing by more than 0.01 in PCM are presented.

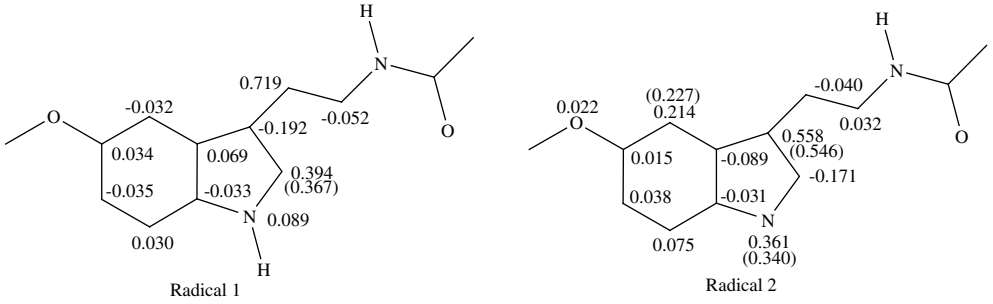

Fig. 4. Spin density distribution of the melatonin Radicals 1 and 2. Hydrogen atoms connected to carbon atoms are omitted for clarity. The values in brackets are obtained in PCM.

dissociation of the considered C–H and N–H bonds occurs according to radical scavenging mechanism (ii). Also, the formation of Radical 2 seems the most probable.

The degree of delocalization of the uncoupled electron in the radicals resulting from the scavenging reaction is a clear indicator of the reaction degree regardless of the reaction mechanism. The unpaired electron delocalization in the radicals is evident from the spin density distribution. Thus, the spin density is a descriptor of the radical-trapping activity which is widely used in the literature [21].

Our calculations showed that Radical 3 has rather low degree of delocalization. The Radical 3 spin density is localized at a single atom: the carbon atom at the end of chain (0.978). According to the above criterion, Radical 3 is unlikely to be formed in the reaction with oxidants. Fig. 4 shows the spin distributions at the most relevant atoms in Radicals 1 and 2. The values in brackets are the spin densities in PCM and reflect the polarizing effect of the (aqueous) medium. It is evident that the spin density at the carbon atom connected to the third position in the indole ring (Radical 1, Fig. 4) is rather high, 0.719 (and remains almost unchanged in PCM (0.721) whereas the value at C2 slightly decreases (0.367)). The spin density of Radical 2 is also high, 0.558. In PCM it decreases at both C3 (0.546), and the nitrogen in the heterocycle (0.340) at the expense of the C4 from the benzene ring (0.227). However, it is interesting to note that the uncoupled electron is mostly localized at the third carbon atom of the indole ring rather than at the nitrogen atom. The computed maximum values of spin density are larger than the values calculated for radicals of phenolic antioxidants [34]. According to this descriptor (Table 1), the melatonin radicals are rather active and this is not favorable for melatonin's antioxidant activity.

We mentioned above that the mechanisms considered do not take into account further transformations of the products of the scavenging reaction. However, if any of the products is directly involved in further biochemical transformations, the stability of the final radical (Ao•) is not so crucial for the reaction degree. In order to show such an example, we calculated the spin density of the thiyl radical (GS•) of glutathione (GSH). The maximum value obtained (at the sulfur atom) is 0.843, i.e. much higher than in the melatonin Radicals 1 and 2. In vitro, GSH inactivates prooxidants like OH•, HOCl, RO•, RO_2•, $^1O_2$, etc., and forms GS•. In the next step, GS• is transformed according to Scheme 1 below, forming glutathione disulphide and superoxide ($O_2^{\bullet -}$).

In vivo glutathione disulphide (GSSG) is reduced to GSH by the enzyme glutathione reductase [1]. The superoxide $O_2^{\bullet -}$ is inactivated by the enzyme superoxide dismutase [1]. The conjugation of the radical scavenging with other reactions makes the spin density descriptor irrelevant to the reaction degree in this case. There are data in the literature suggesting that the radical scavenging by melatonin might be similarly conjugated with other transformations. The reaction of radical scavenging by melatonin has been shown to be coupled with other reactions leading to stable final molecules like N^1-acetyl-N^2-formyl-5-methoxykynuramine and N^1-acetyl-5-methoxykynuramine [43,44].

Having accepted the possibility that the scavenging reaction of melatonin is conjugated with other reactions, we should also admit the possibility that the radical scavenging might occur in ways that are conceptually different from the mechanisms considered in Section 2.

$$GS^{\bullet} + GS^{-} \rightarrow GSSG^{\bullet -} + O_2 \rightarrow GSSG + O_2^{\bullet -}$$
$$2\,O_2^{\bullet -} + 2H^{+} \rightarrow H_2O_2 + O_2$$

Scheme 1. Thiyl radical transformation.

5. Conclusion

The energy of HOMO, one of the primary descriptors of radical scavenging/antioxidant activity, speaks in favor of a very high radical-trapping activity of melatonin, whereas the spin density distribution in the melatonin stable radicals gives evidence for rather low scavenging activity. On the other hand, the in vivo antioxidant activity of melatonin is unquestionable. Apparently, the interaction of melatonin with active radicals follows a non-conventional mechanism. Unlike the phenolic antioxidants, where stable radicals incapable of initiating chain radical processes are formed, here the interaction resembles the case of glutathione, which also yields an active high spin density radical but the latter is rapidly transformed into stable metabolites. This approach explains the lack of antioxidant activity in systems containing no metal ions. The high HOMO energy of the investigated melatonin manifests its electron-donating potential and thereof its coordination capacity. This means that melatonin can coordinate metal ions and thus inhibit ion-catalyzed oxidation processes. Moreover, the metal (iron) ion mediated electron separation from melatonin is not ruled out.

It is also possible that the active melatonin radical quickly oxidizes the α-tocopherol, which produces a much more stable radical. This hypothesis elucidates the reason for their synergy.

The biochemical experiment clearly showed that the melatonin significantly decreases the levels of MDA in the serum. This proves that the high spin density of the melatonin radical is not an obstacle for antioxidant processes. On the other hand, if higher melatonin doses are administrated, the conjugated reactions will not be able to transform the corresponding radical into stable metabolites. As a result, the total antioxidant activity of melatonin will decrease. The experimental verification of the last hypothesis is in progress.

The results from the quantum-chemical calculations and the biochemical experiment as well as the low toxicity of melatonin give reasons for further investigations of melatonin effect on pathological conditions in which oxidative stress plays important role.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2008.12.017.

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