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On the in vitro antioxidative properties of melatonin

Abstract: The aim of this study is to examine possible in vitro antioxidant effects of melatonin. Thus, the total in vitro antioxidant activity of melatonin was studied using a thiocyanate method. Additionally, the reducing power, the superoxide anion scavenging activity and free radical scavenging activity of melatonin were determined. Melatonin exhibited potent antioxidant activity in a linoleic acid emulsion system. The antioxidant activity increased with increasing concentrations of melatonin (50–500 μg). The 50, 100, 250 and 500 μg melatonin doses showed 41, 60, 86 and 99% inhibition of peroxidation of linoleic acid, respectively. On the other hand, a 500-μg dose of α-tocopherol showed 34% inhibition of peroxidation of linoleic acid. Like the total antioxidant activity, the reducing power of melatonin increased in a dose-dependent manner. The reducing power of melatonin was statistically significant versus control, but lower than butylated hydroxytoluene (BHT) or quercetin. Additionally, melatonin had potent superoxide radical scavenging activity and exhibited a higher superoxide radical scavenging activity than quercetin or BHT but lower than butylated hydroxyanisole (BHA). Melatonin's direct free radical scavenging actions may account, at least in part, for its ability to reduce lipid peroxidation. Melatonin may have utility in protecting stored foods from free radical-induced deterioration.

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Introduction

The deterioration of lipids in foodstuffs and oils leads to a large amount of wastage. Currently, a number synthetic and natural antioxidant are used in the prevention and retardation of lipid oxidation [1]. While synthetic antioxidants have proven effective, unpleasant side-effects have been reported [2, 3]. Reactive oxygen species (ROS), also referred to as active oxygen species, have aroused significant interest among scientists in the past decade. Their broad range of effects in biological and chemical systems have been defined in numerous reports. ROS cause lipid peroxidation in food, which leads to the deterioration of the food [4, 5]. Also, excessive generation of ROS induced by various stimuli and which exceed the antioxidant capacity of the organism leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity and cancer [6–8].

Melatonin is the chief secretory product of the pineal gland but it is also produced in other organs. Melatonin is known to influence a variety of biological processes including circadian rhythms, neuroendocrine, cardiovascular and immune functions as well as thermoregulation [9]. Additionally, however, this molecule functions in protecting cell components (nuclear DNA, membrane lipids, cytosolic proteins) from free radical damage [8, 10–13].

Because of the protective effects of antioxidants against lipid peroxidation, several natural compounds have been screened for their possible in vivo or in vitro antioxidant activities as a less harmful alternative to the synthetic products. In this study, we examined the possible in vitro antioxidant effects of melatonin using a thiocyanate and other methods. Melatonin is known to be a potent in vivo antioxidant [14, 15].

Materials and methods

Chemicals

Ammonium thiocyanate was purchased from E. Merck (Darmstadt, Germany). Melatonin, ferrous chloride, polyoxyethylenesorbitan monolaurate (Tween-20), α-tocopherol, nicotinamide adenine dinucleotide (NADH), trichloroacetic acid (TCA), quercetin, butylated hydroxytoluene (BHT) and buthylated hydroxyanisole (BHA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of analytical grade. Butylated hydroxytoluene, BHA and quercetin were dissolved in ethanol.

Determination of antioxidant activity

The total antioxidant activity of melatonin was determined according to the thiocyanate method [16]. Ten milligrams of melatonin was dissolved in 10 mL ethanol (96%). Melatonin (from 50 to 500 μg, in a 0.5-mL volume) was added to a linoleic acid emulsion in potassium phosphate buffer (2.5 mL, 0.04 M, pH 7.0). The mixed solution was incubated at 37°C in a glass flask. The peroxide value was determined

by reading the absorbance at 500 nm, after reaction with $FeCl_2$ (3.5%) and thiocyanate (30%) at intervals during incubation (JASCO V-530 spectrophotometer, Japan). An ethanol solution lacking melatonin served as blank samples. Fifty milliliters of linoleic acid emulsion included 175 mg Tween-20, 155 μL linoleic acid, and 0.04 M phosphate buffer (pH 7.0). All data are an average of duplicate analyses.

Inhibition of lipid peroxidation in percentage was calculated using the following equation:

$$\text{Inhibition}(\%) = (A_0 - A_1/A_0) \times 100$$

where A_0 is the absorbance of control reaction and A_1 is the absorbance in the presence of melatonin (17).

Reducing power

The reducing power of melatonin, BHT and quercetin were determined according to the method of Oyaizu [18]. Melatonin (1.0 mg) in 1 mL of ethanol (96%) was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of TCA (10%) was added to the mixture, which was then centrifuged at 1036 *g* (MSE Mistral 2000, UK) for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. All data are an average of triplicate analyses.

Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of melatonin was carried out based on the method described by Nishikimi [19] with slight modification. One milliliter of nitroblue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL NADH solution (468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of sample solution of melatonin in methanol were mixed. The reaction was initiated by adding 100 μL of a phenazine methosulphate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. All data are an average of triplicate analyses.

Free radical scavenging activity

The free radical scavenging activity of melatonin, BHA and quercetin were measured by using the method of Blois [20] with a slight modification. A 0.1-mM solution of 1,1-diphenyl-2-picryl-hydrazyl (DPPH˙) in ethanol was prepared and 1 mL was added to 3 mL of melatonin solution in ethanol at a range of concentrations (50–500 μg). After 30 min, a decrease in absorbance was measured at 517 nm. All data are an average of triplicate analyses.

Statistical analysis

Data were presented as mean ± standard error of mean (S.E.M.). Three parallel measurements were analyzed by Student's *t*-test or ANOVA. *P*-values < 0.05 were accepted as significant.

Results and discussion

There are numerous studies concerning the in vitro and in vivo antioxidant properties of melatonin [8, 10, 21]. Antioxidant effects of melatonin can occur by at least two mechanisms. In one case, melatonin itself exerts direct antioxidant effects via free radical scavenging and/or by inhibiting their generation [22, 23]. Additionally, melatonin alters the activities of enzymes, which improve the endogenous antioxidant defence capacity of the organisms. These enzymes include superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase [8, 24, 26].

In the current study, total antioxidant activity of melatonin was determined by the thiocyanate method. The amount of peroxides formed in emulsion during incubation was determined spectrophotometrically by measuring absorbance at 500 nm. High absorbance is the indication of high concentration of formed peroxides [27]. The effects of various amounts of melatonin on peroxidation of linoleic acid emulsion were shown in Fig. 1. The antioxidant activities of all concentrations (50–500 μg) of melatonin were greater than 500 μg α-tocopherol. The antioxidant activity of 50 μg of melatonin was almost equivalent to that of 500 μg α-tocopherol (Table 1).

For the measurements of reducing power, we investigated the Fe^{3+}–Fe^{2+} transformation in the presence of melatonin using the Oyaizu method. As with the antioxidant activity, the reducing power of melatonin increased with increasing concentrations. All concentrations of melatonin had higher activities than control and these differences were statistically significant ($P < 0.01$ versus control, using the Student's *t*-test). The reducing power of melatonin compared with BHT and quercetin as standard are shown in Fig. 2. The reductive capability of melatonin, at all concentrations, was lower than those of BHT and quercetin; differences between the effects of melatonin and BHT, and between the effects of melatonin and quercetin were statistically significant ($P < 0.001$, for each comparison using the Student's *t*-test).

In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces the NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of the superoxide anion in the reaction mixture. Fig. 3 shows the superoxide radical scavenging activity of 100 μg melatonin compared with equivalent concentrations of BHA, BHT, and quercetin. Melatonin has strong superoxide radical scavenging activity and exhibited higher superoxide radical scavenging activity than quercetin or BHT but lower than BHA. The effects of all drugs were statistically significantly different than the control values ($P < 0.001$, using the Student's *t*-test). Again, the differences in the mean values among the treatment groups were statistically significant ($P < 0.01$, using an ANOVA).

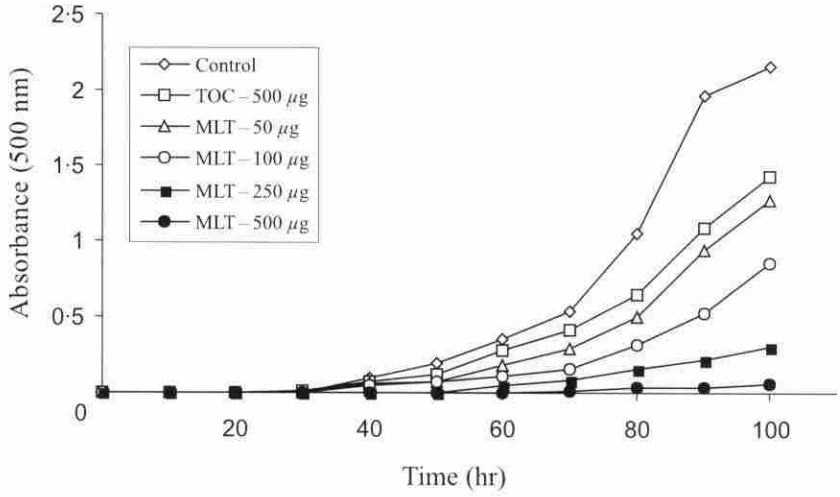

Fig. 1. Total antioxidant (antilipid peroxidative) activities of different concentrations (50–500 μg) of melatonin or α-tocopherol (500 μg) as determined using the thiocyanate method (TOC, α-tocopherol; MLT, melatonin) (n = 2).

Table 1. Percentage inhibition of lipid peroxidation by melatonin and α-tocopherol in the linoleic acid emulsion (n = 2)

Antioxidant	Percentage inhibition of lipid peroxidation
α-tocopherol (500 μg)	34
Melatonin (50 μg)	41
Melatonin (100 μg)	60
Melatonin (250 μg)	86
Melatonin (500 μg)	97

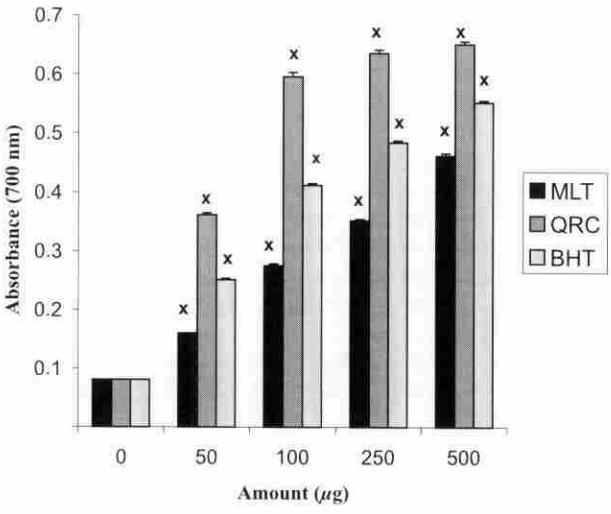

Fig. 2. Reducing power of different concentrations (50–500 μg) of melatonin, BHT and quercetin. $^{X}P < 0.001$ versus control using the Student's *t*-test (n = 3) (MLT, melatonin; QRC, quercetin; BHT, buthylated hydroxytoluene).

DPPH is a stable free radical in an aqueous or an ethanol solution. In order to evaluate the free radical scavenging activity by the test samples, a change of optical density of DPPH radicals was monitored. Fig. 4 illustrates the decrease in absorbance of DPPH caused by the

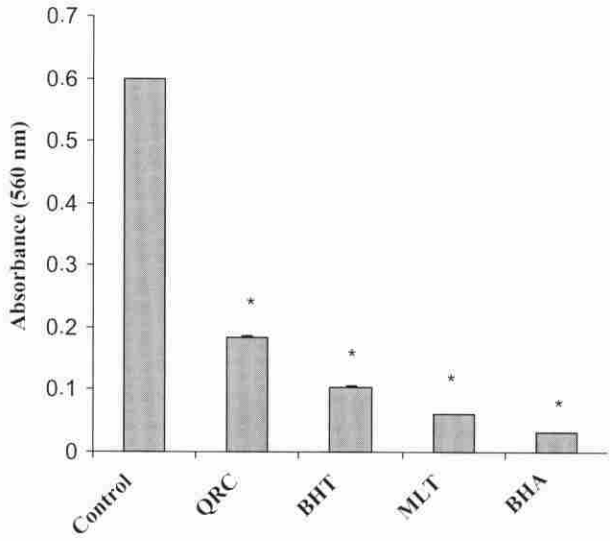

Fig. 3. Superoxide anion scavenging activity of 100 μg of melatonin and equal doses of quercetin, BHA, or BHT using the PMS/NADH-NBT method. $^{*}P < 0.001$ versus control using the Student's *t*-test (n = 3) (BHA, buthylated hydroxyanisole; MLT, melatonin; BHT, buthylated hydroxytoluene; QRC, quercetin).

scavenging ability of different concentrations of melatonin. BHA and quercetin were used as standards. In this test, the free radical scavenging activities of melatonin are statistically and significantly higher than the control ($P < 0.008$ for 50 μg, $P < 0.002$ for 100 μg, $P < 0.001$ for 250 and 500 μg, using the Student's *t*-test) and significantly lower than BHA and quercetin ($P < 0.001$ for each concentration, using the Student's *t*-test). The findings suggest that melatonin contributes to the total antioxidant capacity of solutions. Previously, melatonin levels were found to correlate with the total antioxidant capacity of rat [28] and human [29] serum.

In conclusion, results show that melatonin has highly potent antioxidant activity under in vitro conditions, and its total antioxidant activity is higher than at the same

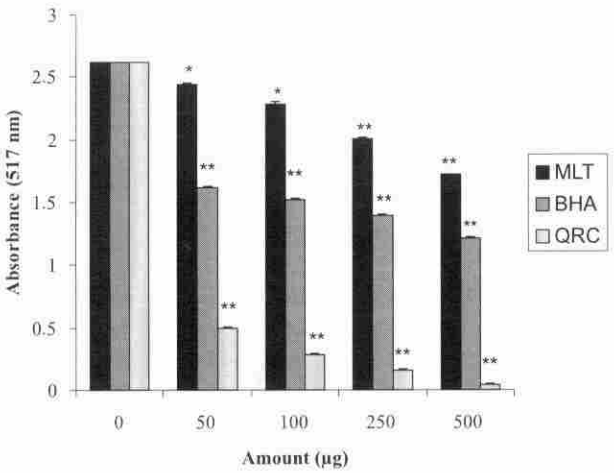

Fig. 4. Free radical scavenging activity of melatonin, BHA and quercetin of 1,1-diphenyl-2-picrylhydrazyl radicals. $^{*}P < 0.01$; $^{**}P < 0.001$ versus control using Student's *t*-test (n = 3) (MLT, melatonin; BHA, buthylated hydroxyanisole; QRC, quercetin).

concentration of α-tocopherol. From the point of view of the reducing power, its effect is lower than quercetin or BHT. Also, in terms of the superoxide radical scavenging activity, melatonin's effect is lower than BHA, but higher than BHT or quercetin. Melatonin exhibits significant free radical scavenging activity under the conditions used here. This is lower than that of BHA or quercetin. Based on these results, it is suggested that, besides functioning as an in vivo antioxidant, melatonin also has significant in vitro antioxidant activity; these findings are consistent with other published reports [8, 14, 30]. We speculate that melatonin may have potential as an in vitro antioxidant and may protect food against deterioration by oxidation. Additional studies on this aspect of melatonin's protective action may support its use for this purpose.

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