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Individual and synergistic antioxidative actions of melatonin: studies with vitamin E, vitamin C, glutathione and desferrrioxamine (desferoxamine) in rat liver homogenates

Eloisa Gitto, Dun-Xian Tan, Russel J. Reiter, Malgorzata Karbownik, Lucien C. Manchester, Salvatore Cuzzocrea, Francesco Fulia and Ignazio Barberi

Department of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA

Eloisa Gitto, Dun-Xian Tan, Russel J. Reiter, Lucien C. Manchester

Department of Thyroidology, Institute of Endocrinology, Medical University of Lódz, Poland

Malgorzata Karbownik

Institute of Medical Pediatrics, Neonatal Intensive Care Unit, University of Messina, Italy

Eloisa Gitto, Francesco Fulia, Ignazio Barberi

Institute of Pharmacology, University of Messina, Italy

Salvatore Cuzzocrea

Correspondence: R. J. Reiter, Department of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio, Mail Code 7762, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA. E-mail: reiter@uthscsa.edu

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Abstract

The pharmacological effects of melatonin, vitamin E, vitamin C, glutathione and desferrioxamine (desferoxamine) alone and in combination on iron-induced membrane lipid damage in rat liver homogenates were examined by estimating levels of malondialdehyde and 4-hydroxyalkenals (MDA + 4-HDA). Individually, melatonin (2.5–1600 μM), vitamin E (0.5–50 μM), glutathione (100–7000 μM) and desferrioxamine (1–8 μM) inhibited lipid peroxidation in a concentration-dependent manner. Vitamin C had both a pro-oxidative (25–2000 μM) and an antioxidative (2600–5000 μM) effect. The IC50 (concentration that reduces damage by 50%) values were 4, 10, 426, 2290 and 4325 μM for vitamin E, desferrioxamine, melatonin, glutathione and vitamin C, respectively. The synergistic actions of melatonin with vitamin C, vitamin E, and glutathione were systematically investigated. When melatonin was combined with vitamin E, glutathione, or vitamin C, the protective effects against iron-induced lipid peroxidation were dramatically enhanced. Even though melatonin was added at very low concentrations, it still showed synergistic effects with other antioxidants at certain concentrations. Furthermore, melatonin not only reversed the pro-oxidative effects of vitamin C, but its efficacy in reducing lipid peroxidation was improved when it was combined with pro-oxidative concentrations of vitamin C. The results provide new information in terms of the possible pharmacological use of the combination of melatonin and classical antioxidants to treat free radical-related conditions.

Introduction

The chief secretory product of the pineal gland, melatonin (Reiter 1991), has been found to be a direct free radical scavenger (Tan et al 1993; Bandyopadhyay et al 2000; Brömme et al 2000) and an indirect antioxidant (Hardeland et al 1995; Hardeland 1997). Due to these actions, melatonin has been pharmacologically tested for its ability to reduce oxidative damage in a wide variety of experimental situations (Reiter et al 1997; Reiter 1998). In each of those studies, melatonin was found generally to be effective in reducing induced oxidative damage to lipids, proteins and DNA. Besides its direct free radical scavenging action, melatonin functions as an indirect antioxidant by stimulating the activities of antioxidative

enzymes (Reiter et al 1997; Reiter 1998). In addition to protecting against lipid peroxidation (Chen et al 1995; De La Lastra et al 1997), it also reduces free radical-induced alterations in membrane fluidity (Garcia et al 1997). The ability of melatonin to reduce the oxidation of lipids, however, seems not to be related to it peroxyl radical scavenging activity, which appears limited (Antunes et al 1999).

Vitamin E (α-tocopherol) is a well-known antioxidant capable of reducing free radical-induced lipid damage in cell membranes (Goss-Sapson et al 1988; Lloyd 1990; Liebler 1993). It functions primarily as a chain breaking antioxidant (Goss-Sapson et al 1988). Once lipid peroxidation is underway the peroxyl radicals that are formed react with α-tocopherol, rather than with an adjacent fatty acid, thus terminating the peroxidative process. Besides functioning as a chain breaking antioxidant, vitamin E is a universal stabilizer of biological membranes (Liebler 1993). In contrast to vitamin E, which is confined to lipid-rich compartments of the cell, vitamin C (ascorbic acid) is water-soluble and functions as an antioxidant in the aqueous phase (Reed 1993). Vitamin C works synergistically with vitamin E by recycling the tocopheryl radical to tocopherol thereby increasing the efficiency of the antioxidative defence system (Reed 1993).

Besides direct free radical scavenging, lowering unbound (''catalytic'') iron levels is an effective means of reducing the generation of the highly toxic hydroxyl radical ($^{\bullet}OH$) (Cheeseman 1993; Farber 1994). Iron, when it encounters hydrogen peroxide (H_2O_2), induces the formation of $^{\bullet}OH$ via the Fenton reaction. This process is blocked by desferrioxamine (desferoxamine), an iron chelator, which by binding iron makes it unavailable for the Fenton reaction (Blatt et al 1989; Radi 1993). Glutathione serves as an important source of reducing equivalents during oxidative stress. This thiol, which is in high concentrations in the cytosol, is highly protective against oxidative stress in virtually every cell (Anderson 1997).

In this study, we have compared the pharmacological efficacies of melatonin, vitamin E, vitamin C, glutathione and desferrioxamine in reducing oxidatively damaged lipids. We have characterized the synergistic effects of the non-indole antioxidants with melatonin. The concentration of malondialdehyde (MDA) plus 4-hydroxyalkenals (4-HDA) in hepatic cell membranes was used as an index of the degree of lipid damage.

Materials and Methods

Chemicals

Melatonin, vitamin E, vitamin C, desferrioxamine, and glutathione were obtained from Sigma Chemical Company (St Louis, MO).

Sample preparation

The studies were performed with the approval of the Institutional Animal Care and Utilization Committee. Male Wistar rats were decapitated and their livers were removed and frozen on solid CO_2. Pooled liver samples were homogenized in ice-cold Tris HCl buffer (20 mM, pH 7.4) (1 g tissue in 10 mL buffer). Homogenate samples (1 mL) were incubated at 37°C for 60 min with H_2O_2 (100 μM) and $FeSO_4$ (1–15 μM). In the first experiment, liver homogenates were incubated with melatonin (2.5 μM–10 mM), vitamin E (0.5–50 μM), vitamin C (25–3500 μM), desferrioxamine (1–8 μM) or glutathione (100–7000 μM) in combination with 100 μM H_2O_2 and 15 μM $FeSO_4$. In the second study, to investigate potential interactions of melatonin with the respective antioxidants, melatonin was co-incubated with vitamin E, vitamin C or glutathione. As in the first experiment, H_2O_2 (100 μM) and $FeSO_4$ (15 μM) were used to induce lipid peroxidation in liver homogenates.

To stop the reactions after incubation the homogenates were placed into ice-cold water for 10 min. The homogenates were then centrifuged at 3000 *g* for 10 min at 4°C. Each experiment was repeated three times. Lipid peroxidation in the supernatant was assayed using a Bioxytech LPO-586 kit (Calbiochem, La Jolla, CA).

Measurement of lipid peroxidation

At the end of the incubation period, the levels of MDA + 4-HDA in each sample were measured. This assay, using the LPO-586 test kit, is based on the reaction of a chromogenic reagent with MDA and 4-HDA to yield a stable chromophore, with maximal absorbance at 586 nm. The tubes were mixed well, closed, and incubated for 40 min in a water bath at 45°C. The samples were then cooled on ice and the absorbance was measured with an Ultrospec-3000 spectrophotometer (Milton Roy, Philadelphia, PA) at 586 nM.

Protein assay

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard.

Calculation of synergistic effects

To examine whether melatonin had synergistic actions with the classical antioxidants in the inhibition of lipid peroxidation, the following formula (Webb 1993) was used for the calculation:

$$i_{1,2} = i_1 + i_2 - i_1\, i_2$$

where i_1 is the % inhibition of MDA + 4-HDA formation by melatonin, i_2 is the % inhibition of MDA + 4-HDA formation by other antioxidants, and $i_{1,2}$ is the % inhibition of the combination of melatonin with other antioxidants on MDA + 4-HDA formation.

If the $i_{1,2}$ is $> i_1 + i_2 - i_1\, i_2$ a synergistic effect is accepted. The rationale for using this formula is that we presumed that melatonin and the other antioxidants used reacted with the same substrates (radicals) and that the reaction rates were dependent on the concentration of radicals. Thus, the two inhibitors influenced each other's reaction rate.

Statistical analysis

The data were analysed using a one-way analysis of variance followed by the Student-Newman Keuls *t*-test. Data are expressed as means ± s.e.m.

Results

Initially, liver homogenates were incubated with increasing concentrations of $FeSO_4$ (0, 1, 2.5, 5, 10, 20, 40 or 80 μM) and 180 μM H_2O_2 for 60 min at 37°C. These concentrations of $FeSO_4$ caused a dose-response increase in lipid peroxidation products (MDA + 4-

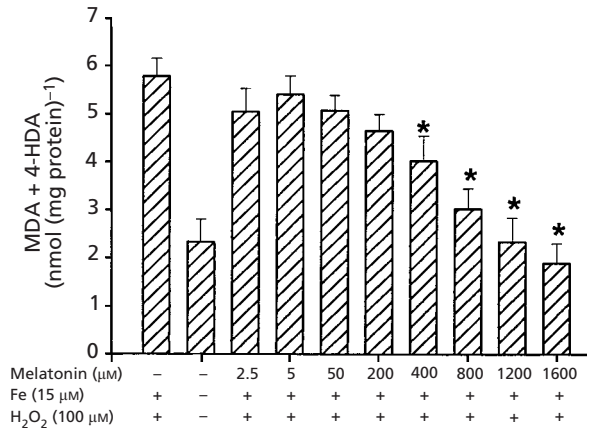

Figure 1 The effect of different concentrations of melatonin on the levels of lipid peroxidation products (MDA + 4-HDA) induced by $FeSO_4$ (15 μM) and H_2O_2 (100 μM) in liver homogenates. The incubation time was 60 min. Values are mean ± s.e.m. *$P < 0.001$.

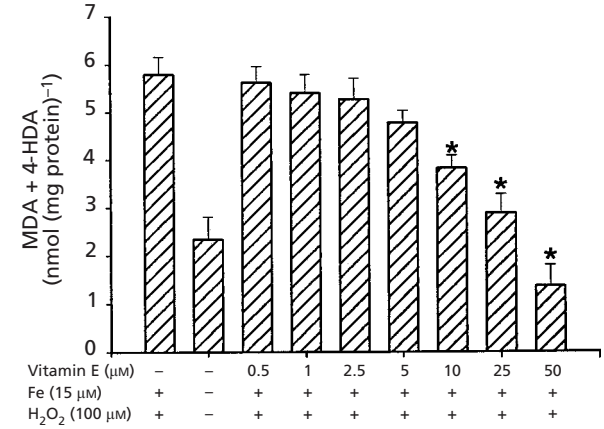

Figure 2 The effect of different concentrations of vitamin E on the levels of lipid peroxidation products (MDA + 4-HDA) induced by $FeSO_4$ (15 μM) and H_2O_2 (100 μM) in liver homogenates. The incubation time was 60 min. Values are mean ± s.e.m. *$P < 0.001$.

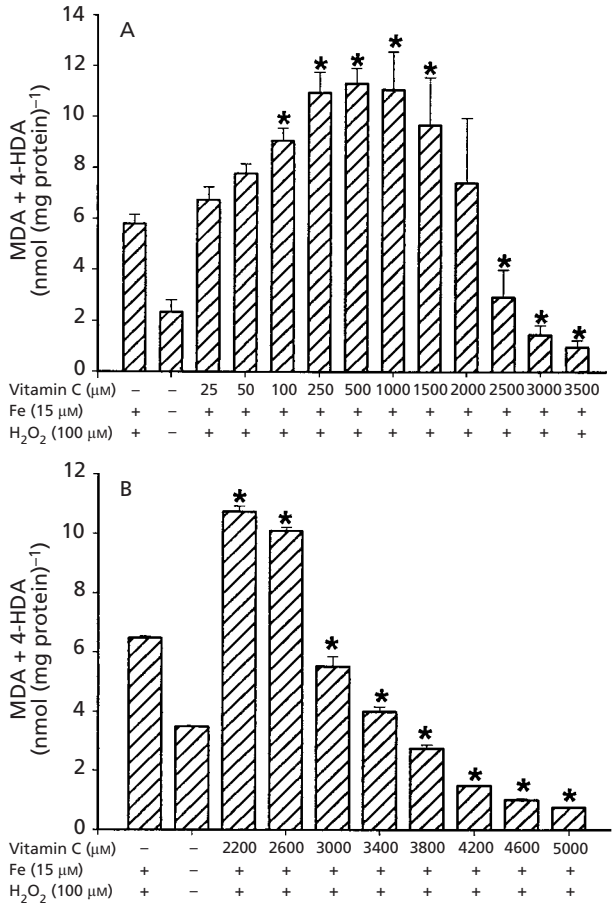

Figure 3 The effect of different concentrations of vitamin C (A, 0–3500 μM; B, 0–5000 μM) on the levels of lipid peroxidation products (MDA + 4-HDA) induced by $FeSO_4$ (15 μM) and H_2O_2 (100 μM) in liver homogenates. The incubation time was 60 min. Values are mean ± s.e.m. *$P < 0.001$.

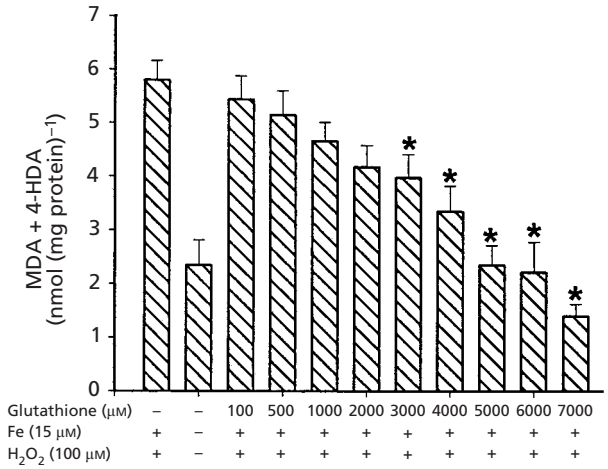

Figure 4 The effect of different concentrations of glutathione on the levels of lipid peroxidation products (MDA + 4-HDA) induced by $FeSO_4$ (15 μM) and H_2O_2 (100 μM) in liver homogenates. The incubation time was 60 min. Values are mean ± s.e.m. *$P < 0.001$.

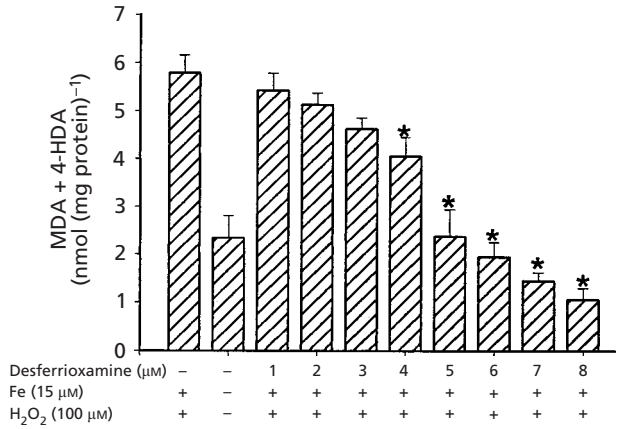

Figure 5 The effect of different concentrations of desferrioxamine on the levels of lipid peroxidation products (MDA + 4-HDA) induced by $FeSO_4$ (15 μM) and H_2O_2 (100 μM) in liver homogenates. The incubation time was 60 min. Values are means ± s.e.m. *$P < 0.001$.

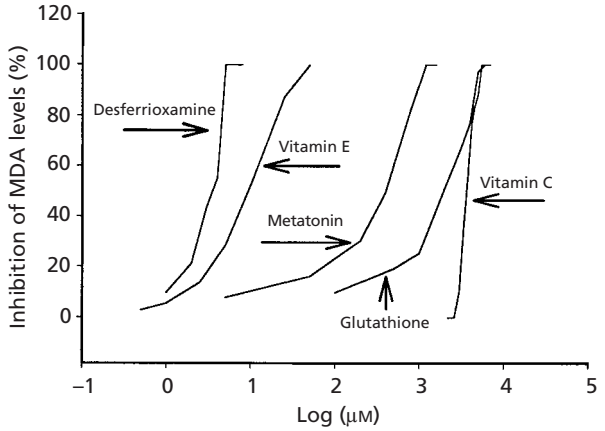

Figure 6 The dose-response functions for the inhibition of lipid peroxidation by desferrioxamine, vitamin E, melatonin, glutathione and vitamin C. The IC50 values for desferrioxamine, vitamin E, melatonin, glutathione, and vitamin C were 4, 10, 426, 2298 and 4325 μM, respectively.

HDA) in the homogenates with the 80 μM concentration of $FeSO_4$ inducing a 5.6-fold rise in MDA + 4-HDA levels (data not shown). From these data the EC50 (concentration that increases damage by 50%) was calculated and determined to be roughly 15 μM $FeSO_4$. In all subsequent studies 15 μM $FeSO_4$ in combination with 100 μM H_2O_2 was used to induce lipid peroxidation.

Figure 1 shows the levels of MDA + 4-HDA obtained after 60-min incubation of homogenate with various concentrations of melatonin and $FeSO_4 + H_2O_2$. The co-incubation of an hepatic homogenate with melatonin (5 μM–16 mM) reduced in a dose-dependent manner the levels of MDA + 4-HDA induced by $FeSO_4 + H_2O_2$. Figure 2 shows the level of lipid peroxidation products in homogenates after 60-min incubation with different concentrations of vitamin E (0.5–50 μM). As with melatonin, vitamin E, albeit at lower concentrations, dose-dependently inhibited lipid peroxidation induced by the combination of $FeSO_4$ and H_2O_2.

In contrast to melatonin and vitamin E, the induced lipid peroxidation exhibited a very different response when vitamin C was added to hepatic homogenates (Figure 3). At concentrations ranging from 100 to 1500 μM, vitamin C statistically significantly increased MDA + 4-HDA levels. Only at concentrations of 2500 μM and above did vitamin C function as an anti-oxidant to reduce lipid peroxidation.

Due to the bimodal response to vitamin C (Figure 3A), we tested the efficacy of vitamin C in a dose range of 2200 to 5000 μM (Figure 3B). Only concentrations of ascorbate 3000 μM or higher reduced the levels of MDA + 4-HDA induced by 15 μM $FeSO_4$ and 100 μM H_2O_2 (Figure 3A). At concentrations of 2200 or 2600 μM, vitamin C functioned as a pro-oxidant. Figures 4 and 5 summarize the dose-response inhibition of lipid peroxidation by glutathione and desferrioxamine, respectively. The doses of glutathione ranged from 100 to 7000 μM and showed that at concentrations of 3000 μM and above the thiol reduced the breakdown of lipid that was induced by $FeSO_4 + H_2O_2$.

Figure 6 summarizes the dose-response functions for the inhibition of MDA + 4 HDA levels by desferrioxamine, vitamin E, melatonin, glutathione and vitamin C. The IC50 (concentration that reduces damage by

Table 1 Mean MDA + 4-HDA levels (± s.e.m.) (nmol (mg protein)$^{-1}$) in homogenates after 60-min incubation with H_2O_2 (100 μM) and $FeSO_4$ (15 μM) at 37°C, without the addition of antioxidant or with melatonin alone, vitamin E alone, or with combinations of melatonin plus vitamin E. The mean value of samples without $FeSO_4$ and H_2O_2 was 0.338.

Melatonin (μM)	Vitamin E (μM)					
	0	1	2	4	8	16
0	3.71 ± 0.32	3.34 ± 0.51	2.93 ± 0.46	2.61 ± 0.44	2.07 ± 0.48	1.61 ± 0.41
1	3.38 ± 0.45	2.92 ± 0.48	2.66 ± 0.51°	2.00 ± 0.57°	1.62 ± 0.64°	1.09 ± 0.44° s
30	2.99 ± 0.49	2.68 ± 0.45	2.13 ± 0.44	1.85 ± 0.53*	1.31 ± 0.44*	1.08 ± 0.55* s
200	2.57 ± 0.43	2.34 ± 0.36	1.89 ± 0.48	1.61 ± 0.43	1.43 ± 0.44#	0.88 ± 0.45# s
400	2.11 ± 0.36	1.90 ± 0.41	1.52 ± 0.47	1.18 ± 0.60♣ s	0.81 ± 0.39♣ s	0.67 ± 0.30♣ s
800	1.29 ± 0.32	1.18 ± 0.17◆	0.99 ± 0.23◆	0.73 ± 0.31◆ s	0.49 ± 0.26◆ s	0.32 ± 0.13◆ s

The values represent the average MDA + 4-HDA levels of three determinations. Each determination was run in duplicate. All mean MDA + 4-HDA levels in homogenates treated with an antioxidant differed significantly ($P < 0.05$) from the control ($FeSO_4 + H_2O_2$). °$P < 0.05$ compared with 1 μM melatonin only; *$P < 0.05$ compared with 30 μM melatonin only; #$P < 0.05$ compared with 200 μM melatonin only; ♣$P < 0.05$ compared with 400 μM melatonin only; ◆$P < 0.05$ compared with 800 μM melatonin only. s indicates synergistic effects as calculated according to Webb (1963).

Table 2 Mean MDA + 4-HDA levels (± s.e.m.) (nmol (mg protein)$^{-1}$) in homogenates after 60-min incubation with H_2O_2 (100 μM) and $FeSO_4$ (15 μM) at 37°C, without the addition of antioxidant or with melatonin alone, vitamin C alone, or with combinations of melatonin plus vitamin C. The mean value of samples without $FeSO_4$ and H_2O_2 was 0.655.

Melatonin (μM)	Vitamin C (μM)			
	0	1000	2000	2500
0	4.59 ± 0.18	7.48 ± 0.20	2.03 ± 0.05	1.43 ± 0.03
1	4.16 ± 0.04	8.06 ± 0.18	1.96 ± 0.07	1.41 ± 0.01
30	3.98 ± 0.05	4.40 ± 0.07	1.78 ± 0.06	0.99 ± 0.08
200	2.49 ± 0.10	2.20 ± 0.05	0.95 ± 0.01°	0.81 ± 0.39° s
400	2.04 ± 0.01	1.52 ± 0.09* s	0.82 ± 0.02*	0.59 ± 0.01* s
800	2.08 ± 0.05	1.05 ± 0.06#	0.67 ± 0.01#	0.45 ± 0.05# s

The values represent the average MDA + 4-HDA levels of three determinations. Each sample was run in duplicate. All mean MDA + 4-HDA levels in homogenates treated with an antioxidant differed significantly ($P < 0.05$) from the control ($FeSO_4 + H_2O_2$). °$P < 0.05$ compared with 200 μM melatonin only; *$P < 0.05$ compared with 400 μM melatonin only; #$P < 0.05$ compared with 800 μM melatonin only. s indicates synergistic effects as calculated according to Webb (1963).

50%) values were 4, 10, 325, 426 and 2290 μM for desferrioxamine, vitamin E, melatonin, glutathione and vitamin C, respectively.

Table 1 summarizes the levels of MDA + 4-HDA in liver homogenates incubated with 15 μM $FeSO_4$ and 100 μM H_2O_2 with or without the addition of melatonin or vitamin E alone or in combination. Vitamin E and melatonin alone, in increasing concentrations, reduced the level of lipid peroxidation products. When both antioxidants were added to the incubation medium, their efficacy in reducing the breakdown in lipids was increased. All combinations of melatonin and vitamin E significantly reduced the levels of MDA + 4-HDA.

Melatonin in combination with vitamin C was also highly effective in reducing the peroxidation of lipids in hepatic homogenates treated with 15 μM $FeSO_4$ and 100 μM H_2O_2 (Table 2). This was particularly significant since vitamin C by itself at a concentration of 1000 μM stimulated MDA + 4-HDA levels. This pro-oxidant effect of vitamin C was inhibited when it was given in

Table 3 Mean MDA + 4-HDA levels (± s.e.m.) (nmol (mg protein)$^{-1}$) in homogenates after 60-min incubation with H_2O_2 (100 μM) and $FeSO_4$ (15 μM) at 37°C, without the addition of antioxidant or with melatonin alone, glutathione alone, or with combinations of melatonin plus glutathione. The mean value of samples without $FeSO_4$ and H_2O_2 was 0.493.

Melatonin (μM)	Glutathione (μM)					
	0	50	200	1000	2000	4000
0	5.33 ± 0.02	5.08 ± 0.06	4.57 ± 0.03	3.46 ± 0.16	3.30 ± 0.06	1.97 ± 0.10
1	4.82 ± 0.14	4.82 ± 0.05	4.37 ± 0.24°	3.75 ± 0.06°	2.70 ± 0.06°	1.59 ± 0.02°
30	4.54 ± 0.02	4.58 ± 0.04	4.17 ± 0.07*	3.73 ± 0.02*	2.53 ± 0.07*	1.68 ± 0.03*
200	3.64 ± 0.04	3.56 ± 0.02	3.24 ± 0.07#	2.18 ± 0.07#s	1.87 ± 0.01# s	1.40 ± 0.05#
400	2.96 ± 0.05	2.95 ± 0.08	2.69 ± 0.04	1.60 ± 0.03♣ s	0.96 ± 0.02♣ s	0.81 ± 0.02♣ s
800	2.08 ± 0.05	1.56 ± 0.03◆	1.24 ± 0.01◆ s	0.8 ± 0.009◆ s	0.59 ± 0.02◆ s	0.26 ± 0.01◆ s

The values represent the average MDA + 4-HDA levels of three determinations. Each sample was run in duplicate. All mean MDA + 4-HDA levels in homogenates treated with an antioxidant differed significantly ($P < 0.05$) from the control ($FeSO_4 + H_2O_2$). °$P < 0.05$ compared with 1 μM melatonin only; *$P < 0.05$ compared with 30 μM melatonin only; #$P < 0.05$ compared with 200 μM melatonin only; ♣$P < 0.05$ compared with 400 μM melatonin only; ◆$P < 0.05$ compared with 800 μM melatonin only. s indicates synergistic effects as calculated according to Webb (1963).

combination with melatonin at concentrations of 30 μM or higher.

Table 3 shows the efficacy of melatonin and glutathione acting independently and in combination on the reduction of lipid peroxidation. When used in combination, melatonin exhibited significant synergistic effects with the classic antioxidants in reducing lipid peroxidation in rat liver homogenates (Tables 1–3).

Discussion

The toxicity of free radicals can be mitigated with the use of direct free radical scavengers or indirect antioxidants (Cao et al 1993). Examples of well-known molecules that function as antioxidants include α-tocopherol (vitamin E), melatonin, ascorbic acid (vitamin C), β-carotene, and glutathione (Stahl & Sies 1997; Reiter 1998). A number of enzymes act as indirect antioxidants since they metabolize free radicals or their reactive intermediates to harmless products. Enzymes that function in this capacity include superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GRd) and catalase (CAT) (Radi 1993). Other substances such as desferrioxamine, an iron chelator, possess antioxidant effects because they reduce the formation of $^{\bullet}OH$ (Blatt et al 1988, 1989; Richardson 1997).

In this study, we have examined the levels of lipid peroxidation induced by H_2O_2 and $FeSO_4$. We have compared the antioxidant capacity of melatonin, vitamin E, vitamin C, glutathione and desferrioxamine in terms of their ability to reduce $H_2O_2 + FeSO_4$-induced lipid peroxidation in rat liver homogenates. The tissue concentrations of MDA + 4-HDA were used as indices of damaged lipid products. Clearly, H_2O_2 and $FeSO_4$ increased MDA + 4-HDA levels in liver homogenates in a concentration-dependent manner.

Using liver homogenates the antioxidant effects of desferrioxamine, vitamin E, melatonin, glutathione and vitamin C independently were compared following the exposure of the homogenates to H_2O_2 and $FeSO_4$. Desferrioxamine, vitamin E, melatonin and glutathione significantly decreased the levels of lipid peroxidation with the degree of inhibition being proportional to the concentration of the antioxidant; conversely, vitamin C was a pro-oxidant as well as an antioxidant depending on its concentration.

Of the antioxidants tested, desferrioxamine caused the greatest reduction in the levels of induced lipid peroxidation. This was not unexpected since in the system used Fe^{2+} was the initiator of the oxidative reaction, and desferrioxamine, a chelator of Fe^{2+}, would be expected to bind the iron ions and reduce oxidative damage (Farber 1994). In-vivo it is known that the administration of ferrous salts exacerbates lipid peroxidation (Farber 1994). Also, chelation therapy in animals decreases lipid peroxidation and brain oedema and enhances survival (Komara et al 1986; Kompala et al 1986).

When the antioxidant effects of melatonin and vitamin E were compared, both decreased the levels of lipid peroxidation products in a dose-dependent manner; however, melatonin was found to be less effective than vitamin E in protecting against this destruction in-vitro. When the IC50 values for melatonin and vitamin E were calculated, vitamin E was found to be roughly 100-times more efficient than melatonin in reducing lipid deterioration under the experimental conditions used. This is consistent with other observations wherein, under in-vitro conditions, vitamin E was more effective than melatonin in inhibiting the oxidative breakdown of lipids (Reiter et al 2000).

In reference to the efficacy of melatonin as a chain-breaking antioxidant, the reports are controversial. Pieri et al (1994) compared the peroxyl radical scavenging ability of melatonin to that of vitamin E using β-phycoerythrin as a fluorescent indicator protein. On the basis of their findings, they claimed that melatonin was twice as effective as vitamin E in its scavenging ability of the peroxyl radical. However, the findings of Pieri et al (1994) are brought into question by the work of Antunes et al (1999) who found that melatonin had limited peroxyl radical scavenging activity. Siu et al (1998) compared the efficacy of vitamin E and melatonin as antioxidants against lipid peroxidation in rat retinal homogenates and showed that while vitamin E and melatonin protected the retina against lipid breakdown, the IC50 value for melatonin was approximately 7.2-times greater than that of vitamin E. Likewise, Escames et al (1997) compared the protective effects of melatonin and vitamin E against nitric oxide (NO)-induced lipid peroxidation in rat brain homogenates. Again, the results in-vitro showed that vitamin E was more efficient in preventing NO-induced lipid peroxidation. The current findings show the lower efficacy of melatonin, relative to that of vitamin E, in reducing lipid breakdown in an in-vitro system. In contrast to their relative effects in protecting against free radical damage to lipids in-vitro, melatonin is superior to vitamin E in reducing oxidative damage to purified DNA under in-vitro conditions (Qi et al 2000).

Glutathione is an important intracellular reductive source and an effective antioxidant (Anderson 1997). In this study, it significantly decreased lipid breakdown due to H_2O_2 plus iron, although it was found to be less effective than melatonin. Calculating the IC50 values for melatonin and glutathione indicated that glutathione was 5.3-times less efficient than melatonin in reducing lipid damage under the experimental conditions used.

Vitamin C is the widely used classical antioxidant (Aruoma 1997; Stahl & Sies 1997). However, vitamin C exhibits pro-oxidative and antioxidative properties depending on the experimental conditions. Consistent with this, in this study we found that vitamin C (25–2000 μM) increased significantly MDA + 4-HDA levels, that is, it exhibited obvious pro-oxidant activity. At concentrations of 2000–3500 μM vitamin C behaved as an antioxidant and thereby decreased lipid peroxidation significantly. Since vitamin C, like other antioxidants (vitamin E or glutathione) is an electron donor and exhibits redox reactions, it can cause oxidation of macromolecules under certain circumstances. This promotes pro-oxidative reactions and ultimately increases molecular damage (Herbert 1993). Actually, several classical antioxidants are known pro-oxidants (Aruoma 1997). They are what is referred to as ''double-edged swords'', important for health yet harmful in some situations (Stahl & Sies 1997). In the presence of transition metals, vitamin C reduces Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^{1+}. Fe^{2+}or Cu^{+} catalyse the Fenton reaction resulting in the formation of the highly toxic $^{\bullet}OH$ which initiates destructive radical chain reactions. From this perspective, antioxidants with potential for pro-oxidative activity are considered opportunistic antioxidants (Aruoma 1997).

Melatonin is a highly effective $^{\bullet}OH$ scavenger (Tan et al 1993, 2000a; Mahal et al 1999; Bandyopadhyay et al 2000; Brömme et al 2000; Reiter et al 2000). Indeed, each molecule of melatonin scavenges two $^{\bullet}OH$ with the formation of a stable end product, cyclic-3-hydroxy-melatonin, which is excreted in the urine (Tan et al 1998). Melatonin also scavenges H_2O_2, during which it is metabolized to *N*-acetyl-N^2-formyl-5-methoxy-kynuramine (AFMK) (Tan et al 2000b). Since the melatonin is sacrificed and does not participate in redox cycling after scavenging free radicals, not only does melatonin not consume the basic cellular reductive force, glutathione, but it also preserves or even increases the content of glutathione in tissues (Sewerynek et al 1995a, b; Hara et al 1997; Urata et al 1999). Melatonin has been shown to be devoid of pro-oxidative activity in a variety of experimental models (Hardeland et al 1995; Reiter et al 1997; Reiter 1998; Tan et al 2000b). Thus, melatonin is a suicidal or terminal antioxidant (Tan et al 2000a).

A second aim of this study was to compare the synergistic effects of melatonin with vitamin E, vitamin C and glutathione in preventing lipid peroxidation induced by the combination of H_2O_2 (100 μM) and $FeSO_4$ (15 μM). Poeggeler et al (1995) proposed that in purely chemical systems melatonin acts synergistically with the antioxidants, ascorbate, vitamin E and glutathione, in the scavenging of ABTS (2,2-azino-bis-3-ethylbenz-

thiazoline-6-sulfonic acid) radical. Other than this report, few systematic studies have been carried out to test the synergistic actions of melatonin with the classical antioxidants in biological samples. Here we observed that the combination of melatonin with other antioxidants resulted in a marked reduction in lipid peroxidation induced by H_2O_2 and $FeSO_4$ in the rat liver homogenates (Tables 1–3). For several of the antioxidants tested, the results indicated that melatonin acted synergistically to reduce oxidative damage. For example, when melatonin was combined with vitamin E, vitamin C or glutathione, the effects in reducing lipid breakdown were greater than the sum of the two antioxidants given individually (Tables 1–3).

As already noted, vitamin C can function as a prooxidant under certain situations (Stahl & Sies 1997). In this study, vitamin C in the dose range of 25–2000 μM promoted lipid peroxidation in rat liver homogenates. Interestingly, when melatonin, even at a concentration below its effective dose, was combined with a prooxidant concentration of vitamin C, potent antioxidative actions were observed. This synergistic action increased as the melatonin concentration increased. It has been proposed that vitamin C may recycle melatonin (Mahal et al 1999), if so, this could explain the synergistic effects of melatonin with vitamin C.

The synergism of melatonin with the classical antioxidants implies that melatonin may interact in a yet unknown manner with these molecules to increase their efficacy and that the mechanisms of the antioxidative action of melatonin may differ from that of other antioxidants (Aruoma 1997).

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