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Comparison of melatonin versus vitamin C on oxidative stress and antioxidant enzyme activity in Alzheimer's disease induced by okadaic acid in neuroblastoma cells

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Abstract

We demonstrated that exposure of cells to 50 nM okadaic acid for 2 h induced a reduction in cellular glutathione transferase, glutathione reductase and catalase activity. Likewise, this acid prompted an increase in lipid peroxidation. Treatment of cells with 10^{-5} M melatonin or 0.5 μg/ml vitamin C prevented the effects of okadaic acid. These results indicate that okadaic acid induces an oxidative stress imbalance, while melatonin and vitamin C prevent the oxidative stress induced by okadaic acid. Likewise, these data indicate the great importance of oxidative stress in both this experimental model and in the development and course of neurodegenerative disease, especially Alzheimer's disease. They show that melatonin is much more efficient than vitamin C in reducing the extent of oxidative stress. This phenomenon was demonstrated by the smaller dose of melatonin needed to obtain effects similar to those obtained with vitamin C on lipid peroxidation and by the protective effect of melatonin on antioxidant enzyme activity.

Keywords: Melatonin; Vitamin C; N1E-115 cell; Okadaic acid; Oxidative stress; Antioxidant enzyme

1. Introduction

In recent years, research has clearly pointed to the importance of oxidative stress in Alzheimer's disease. Under normal conditions, damage by oxygen radicals is kept in check by an efficient array of antioxidant systems that display extensive redundancy. However, during pathological conditions, the oxidant versus antioxidant balance is necessarily altered, either primarily or secondarily. The oxidative damage in Alzheimer's disease involves advanced glycation end products, nitration, lipid peroxidation adduction products, carbonyl-modified neurofilament protein and free carbonyls (Smith et al., 2000; Sayre et al., 2001).

In Alzheimer's disease, β-amyloid peptide has been implicated in oxidative stress and free radical production (Pappolla et al., 1997a). Furthermore, oxidative stress appears to mediate β-amyloid peptide toxicity by free radical production, suggesting a pathophysiological link between β-amyloid peptide and an imbalance between reactive oxygen production and protective systems. Recent findings suggest that lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidosis (Pratico et al., 2001). There are contradictory reports about the activity of antioxidant enzymes in Alzheimer's disease (Lovell et al., 1995; Marcus et al., 1998; Karelson et al., 2001). In some cases, these activities were lower in Alzheimer's disease groups than in control groups (Marcus et al., 1998; Karelson et al., 2001), but other studies showed a significant elevation of the activity of antioxidant enzymes in the brain in Alzheimer's disease (Lovell et al., 1995).

Pappolla et al. (1997a,b) demonstrated that incubation of these neuroblastoma with either β-amyloid peptide or

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melatonin greatly reduced cell death compared to that seen in cells incubated with either peptide alone. Furthermore, the extent of β-amyloid peptide-induced lipid peroxidation in the cells was reduced in the presence of melatonin.

Melatonin is synthesized in the pineal gland of mammals but its production is not limited to this organ (Conti et al., 2000). Many studies have shown that melatonin is a powerful antioxidant (Tan et al., 2000a). In addition to its ability to scavenge directly peroxyl radicals, hydroxyl radicals and other reactive oxygen species, melatonin also stimulates enzymes related to the antioxidative defence system (Tan et al., 2000b). Numerous studies support that melatonin may prevent oxidative damage in the brain and other tissue (Montilla et al., 1997a, 1998; Cruz et al., 2001; Clapp-Lilly et al., 2002).

Many studies suggest that supplementation with antioxidants may delay the development of Alzheimer's disease. A large clinical trial demonstrated a beneficial effect of α-tocopherol and selegiline by slowing progression of the disease (Sano et al., 1997). The antioxidative efficacy of α-tocopherol can be considerably increased by co-supplementation with vitamin C (Stocker, 1994; Kontush et al., 2001). Vitamin C is well known as a hydrophilic antioxidant in human cerebrospinal fluid (Schippling et al., 2000). It is the antioxidant first consumed during the oxidation of plasma (Frei et al., 1989) and cerebrospinal fluid (Borghini et al, 1993) and forms the first line of antioxidative defence.

Okadaic acid induces biochemical, physiological and morphological changes similar to those seen in neurodegenerative diseases (Arias et al., 1993, 1998; Tapia et al., 1999; Traore et al., 2000). Studies carried out in recent years show that okadaic acid induces an oxidative state characterized by increased lipid peroxides (Chen et al., 2000; Traore et al., 2000). Likewise, Perez et al. (2002) showed that okadaic acid increased levels of hydroxynonenal (a product of oxidative stress) in cultured neural cells.

In this work we studied the effects of melatonin or vitamin C on the lipid peroxidation and on the decrease in the activity of antioxidant enzymes induced by okadaic acid (50 nM). Results suggest a beneficial effect of melatonin and vitamin C and their possible use in the treatment of neurodegenerative diseases.

2. Materials and methods

2.1. Materials

Murine neuroblastoma N1E-115 cells were generous gift from Dra. Muñoz (School of Medicine). Culture media, bovine serum, horse serum, antibiotics and okadaic acid were purchased from Gibco BRL (Gaithersburg, MD). Melatonin, vitamin C and all other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

N1E-115 cells (passage Nos. 66–77) were seeded into 35-mm Petri dishes at a seeding density of 200,000 cells mm^2. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a 5% CO_2 atmosphere for 96 h.

2.2.1. Experimental design

Ninety-six hours after passage of cells into 35-mm culture dishes, they were incubated with a final concentration of 50 nM of okadaic acid (in saline solution) for 2 h. In some experiments, cells were treated with a final concentration of 10^{-5} M of melatonin (in ethanol/Tris–HCl) or the vehicle. Melatonin was added 2 h before incubation with okadaic acid and was present until the end of the experiment (2 h later added okadaic acid). Some groups were treated

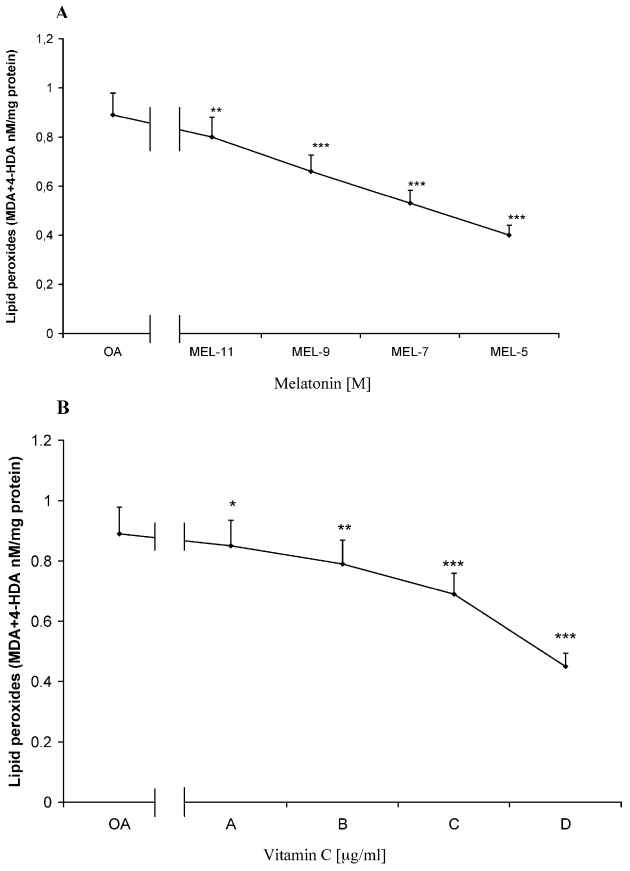

Fig. 1. (A) The effect of increasing concentrations of melatonin on lipid peroxides formation induced by okadaic acid. Concentrations were 10^{-11} M (MEL-11), 10^{-9} M (MEL-9), 10^{-7} M (MEL-7) and 10^{-5} M (MEL-5). (B) Dose-response curve of vitamin C. Concentrations were: 0.00005 μg/ml (A), 0.0005 μg/ml (B), 0.005 μg/ml (C) or 0.5 μg/ml (D). Data represent the means ± S.E.M. of six individual experiments and are expressed as nanomoles per milligram of protein. * $P<0.05$ versus okadaic acid group; ** $P<0.01$ versus okadaic acid group; *** $P<0.001$ versus okadaic acid group.

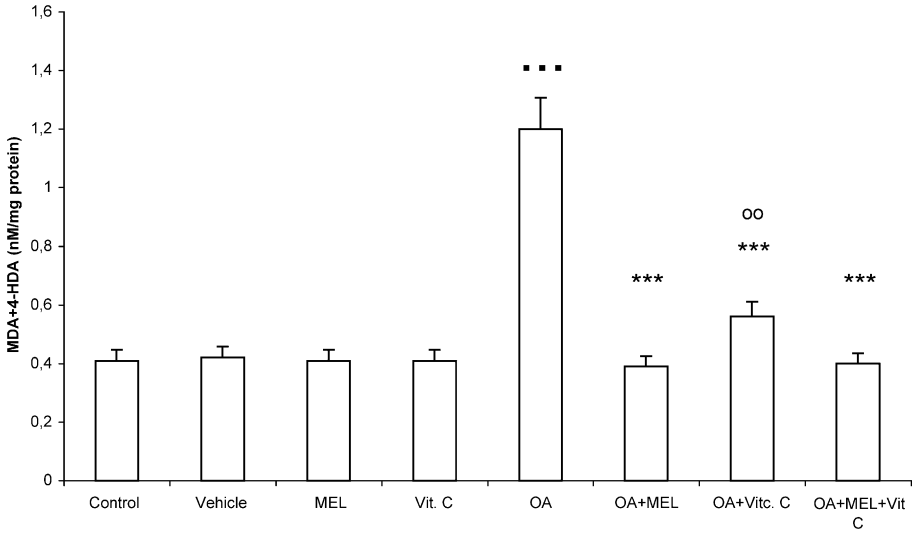

Fig. 2. Effect of melatonin and vitamin C on lipid peroxidation induced by okadaic acid. Melatonin and vitamin C decreased lipid peroxidation. Groups used were: (1) control, (2) vehicle, (3) melatonin (MEL), (4) vitamin C (Vit. C), (5) okadaic acid (OA), (6) okadaic acid plus melatonin (OA + MEL), (7) okadaic acid plus vitamin C (OA + Vit. C), and (8) okadaic acid plus melatonin and vitamin C (OA + MEL + Vit. C). Data represent the means ± S.E.M. of six individual experiments and are expressed as nanomoles per milligram of protein. ▪▪▪ $P < 0.001$ versus control group; *** $P < 0.001$ versus okadaic acid group; ○○ $P < 0.01$ versus okadaic plus melatonin group.

with a final concentration of 0.5 μg/ml of vitamin C (in saline solution) 2 h before okadaic acid was added. These experiments were performed to select an effective dose of melatonin and vitamin C. We made dose-response curves for both antioxidants, determining their effects on oxidative stress induced by okadaic acid in neuroblastoma cells.

2.2.2. Lipid peroxidation

Cells (2×10^{10}) were homogenized with a Virtis stirrer in ice-cold 20 mM Tris–HCl, buffer, pH 7.4, to produce a homogenate. The homogenates were then centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was collected and immediately tested for lipid peroxidation using the Bioxytech LPO-586 kit (OXIS International, Portland, USA). The kit uses a chromatogenic reagent which reacts with the lipid peroxidation products malondialdehyde and 4-hydroxynonenales at 45 ± 1 °C, yielding a stable chromophore with maximum absorbance at 586 nm.

Proteins were detected by the Bradford method using bovine serum albumin as a standard.

2.2.3. Antioxidant enzymes

The activity of glutathione transferase and glutathione reductase was measured according to the method of Flohé and Gunzler (1984). Cells (2×10^{10}) were homogenized with a Virtis stirrer in ice-cold buffer (0.1 M KH_2PO_4/K_2HPO_4, pH 7.0, plus 29.2 mg ethylenediaminetetraacetic acid (EDTA) in 100 ml of distilled water and 10.0 mg digitonin in 100 ml of

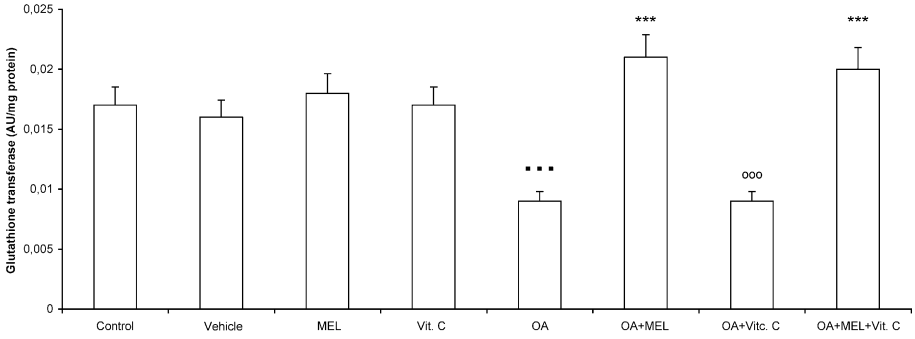

Fig. 3. Effect of melatonin and vitamin C on the changes induced by okadaic acid in glutathione transferase activity. Groups used were: (1) control, (2) vehicle, (3) melatonin (MEL), (4) vitamin C (Vit. C), (5) okadaic acid (OA), (6) okadaic acid plus melatonin (OA + MEL), (7) okadaic acid plus vitamin C (OA + Vit. C), and (8) okadaic acid plus melatonin and vitamin C (OA + MEL + Vit. C). Data represent the means ± S.E.M. of six individual experiments and are expressed as activity units per milligram of protein. ▪▪▪ $P < 0.001$ versus control group; *** $P < 0.001$ versus okadaic acid group; ○○○ $P < 0.001$ versus okadaic plus melatonin group.

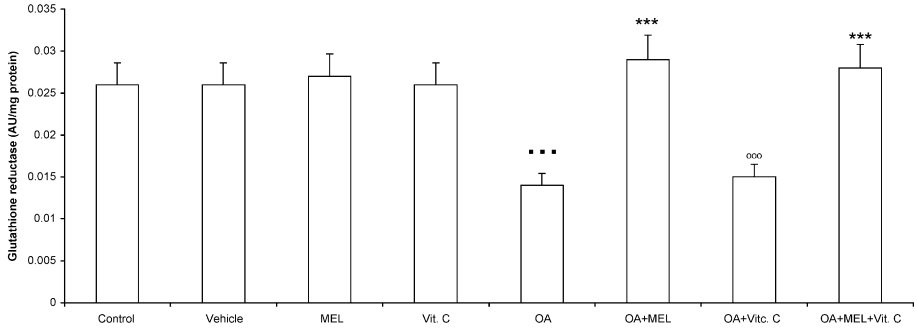

Fig. 4. Effect of melatonin and vitamin C on the changes induced by okadaic acid in glutathione reductase activity. Groups used were: (1) control, (2) vehicle, (3) melatonin (MEL), (4) vitamin C (Vit. C), (5) okadaic acid (OA), (6) okadaic acid plus melatonin (OA + MEL), (7) okadaic acid plus vitamin C (OA + Vit. C), and (8) okadaic acid plus melatonin and vitamin C (OA + MEL + Vit. C). Data represent the means ± S.E.M. of six individual experiments and are expressed as activity units per milligram of protein. ■■■ $P < 0.001$ versus control group; *** $P < 0.001$ versus okadaic acid group; ○○○ $P < 0.001$ versus okadaic plus melatonin group.

distilled water, final volume, 2000 ml) to produce a homogenate. The homogenates were then centrifuged at 10,000 × *g* for 10 min at 4 °C. The glutathione transferase assay is based upon the glutathione transferase-catalyzed reaction between CDNB and reduced glutathione, with maximum absorbance at 340 nm, while the glutathione reductase assay is based on the oxidation of NADPH to $NADP^+$, catalyzed by a limiting concentration of glutathione reductase, with maximum absorbance at 340 nm.

Catalase was measured in the cell cytosol fraction. Catalase was assayed following Aebi (1984), by the rate of decomposition of H_2O_2 at 240 nm. Cells (2×10^{10}) were homogenized with Virtis stirrer in ice-cold phosphate buffer (6.81 g KH_2PO_4 in water, made up to 1000 ml plus 8.90 g $NA_2HPO_4 \cdot 2H_2O$ in water, made up to 1000 ml). The homogenates were then centrifuged at 1000 × *g* for 10 min at room temperature. H_2O_2 (10 mM) was used as reagent, with the rate of dismutation of H_2O_2 to water and molecular oxygen being proportional to the concentration of catalase and with maximum absorbance at 240 nm.

Proteins were detected by the Bradford method using bovine serum albumin as a standard.

2.2.4. Statistical analysis

All results are expressed means ± S.E.M. Comparisons between groups were made using an analysis variance

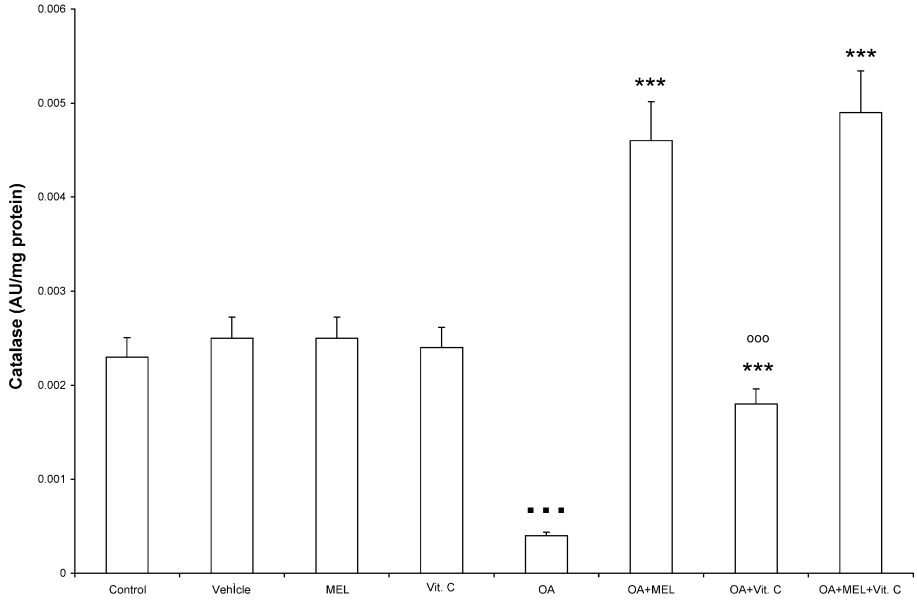

Fig. 5. Effect of melatonin and vitamin C on the changes induced by okadaic acid in catalase activity. Groups used were: (1) control, (2) vehicle, (3) melatonin (MEL), (4) vitamin C (Vit. C), (5) okadaic acid (OA), (6) okadaic acid plus melatonin (OA + MEL), (7) okadaic acid plus vitamin C (OA + Vit. C), and (8) okadaic acid plus melatonin and vitamin C (OA + MEL + Vit. C). Data represent the means ± S.E.M. of six individual experiments and are expressed as activity units per milligram of protein. ■■■ $P < 0.001$ versus control group; *** $P < 0.001$ versus okadaic acid group; ○○○ $P < 0.001$ versus okadaic plus melatonin group.

(ANOVA). The differences were considered significant for $P < 0.05$.

3. Results

3.1. Dose-response curves

Fig. 1 shows the dose-response curve of melatonin and vitamin C on lipid peroxidation induced by okadaic acid. Melatonin 10^{-5} M and vitamin C 0.5 μg/ml were the most effective doses against the oxidative stress induced by okadaic acid.

3.1.1. Baseline profile

Incubation of neuroblastoma cells with okadaic acid resulted in oxidative stress, characterized by a significant increase in levels of malondialdehyde + 4-hydroxynonenal in N1E-115 cells (0.45 ± 0.001 nM/mg protein in the control group versus 1.20 ± 0.003 nM/mg protein in the okadaic group, $P < 0.001$, Fig. 2).

The activity of all scavenger enzymes measured in this study (glutathione transferase, glutathione reductase and catalase) was significantly lower in the group treated with okadaic acid than in the control or vehicle group. Glutathione transferase activity was: 0.016 ± 0.0002 AU/mg protein in the control group versus 0.007 ± 0.0004 AU/mg protein in the okadaic acid group, $P < 0.001$ (Fig. 3) and glutathione reductase activity was: 0.028 ± 0.0001 AU/mg protein in the control group versus 0.018 ± 0.0003 AU/mg protein in the okadaic acid group, $P < 0.001$ (Fig. 4). Catalase activity was: 0.0026 ± 0.00006 AU/mg protein in the control group versus 0.0014 ± 0.00007 AU/mg protein in the okadaic acid group, $P < 0.001$ (Fig. 5).

3.1.2. Effects of melatonin or vitamin c incubation

When the cells were protected with melatonin, both malondialdehyde and 4-hydroxynonenal levels returned to normal values, $P < 0.001$ (Fig. 2).

Melatonin also had an important effect on the antioxidant enzyme system. In fact, N1E-115 cells protected with the antioxidant drug showed an increase in the activity of all scavenger enzymes studied, $P < 0.001$.

Although vitamin C treatment caused a reduction in the parameters of oxidative stress, the changes were significantly smaller than those induced by melatonin (Fig. 2). Also, melatonin had a more powerful effect in restoring antioxidative enzyme activity (Figs. 3–5).

4. Discussion

The experiments presented in this paper showed that melatonin and vitamin C had a dose-dependent protective effect against the oxidative stress induced by okadaic acid, melatonin 10^{-5} M and vitamin C 0.5 μg/ml, being the most effective doses. Likewise, this study demonstrated an increase in lipid peroxide levels and a decrease in antioxidant enzymes activity in a model of Alzheimer's disease induced by okadaic acid, while melatonin and vitamin C prevented the effects of okadaic acid in N1E-115 cells.

This protective action of melatonin confirms previous findings. Melatonin is known to prevent oxidative damage to the cell membrane, cytosol organelles and nuclear and mitochondrial DNA by donating electrons (Reiter et al., 2000). In addition, melatonin stimulates the synthesis of antioxidant enzymes (Pablos et al., 1998; Albarran et al., 2001), suggesting that melatonin may act not only directly against free radicals, but also indirectly as an enzyme stimulator, although the specific induction mechanism is unknown (Albarran et al., 2001). In many studies, this research group has demonstrated an improvement not only in oxidative stress parameters measured in plasma, serum, liver, kidney and brain, but also in scavenger enzyme activity in other experimental models (Montilla et al., 1997a,b, 1998; Cruz et al., 2001). Also, patients with Alzheimer's disease have decreased levels of melatonin in the periphery. The administration of this indole induces improvement in circadian rhythms and insomnia in these patients (Monti and Cardinali, 2000).

Likewise, different studies show that there is a decrease in vitamin C levels in Alzheimer's disease (Riviere et al., 1998). The results presented by different authors suggested that the use of high-dose vitamin C supplements might lower the risk of Alzheimer's disease (Morris et al., 1998) and protect against vascular dementia and improve cognitive function in late life (Masaki et al., 2000). Our results are consistent with these data and support the hypothesis that oxygen free radicals and oxidative stress may be the main cause of damage in Alzheimer's disease. In these studies, vitamin C provided effective protection against oxidative stress. However, there are no comprehensive studies comparing the effect of vitamin C and melatonin.

In the present study, we show that both vitamin C and melatonin reduce free radical damage, although the reduction was significantly greater with melatonin. Both vitamin C and melatonin treatments reduced significantly the extent of oxidative stress. However, vitamin C failed to increase the activity of glutathione transferase and glutathione reductase, and only had a weak effect on catalase activity. The increase in enzyme activity was significantly greater in the melatonin group. The differences between vitamin C and melatonin suggest that the effect of melatonin can be related to a direct antioxidant activity of the indole and to the stimulation of antioxidant enzymes.

In summary, our study proves that melatonin decreases, more than vitamin C, levels of lipid peroxides and increases the activity of antioxidant enzymes. According to these data, melatonin is much more efficient than vitamin C in

reducing the extent of oxidative stress (decrease lipid peroxidation, with a significant recovery of antioxidative enzyme activity). This phenomenon was demonstrated by the smaller dose of melatonin needed to obtain effects similar to those obtained with vitamin C on lipid peroxidation and by the protective effects of melatonin on antioxidant enzyme activity. Nevertheless, more investigations are required to evaluate the antioxidant-neuroprotective effect of melatonin in clinical and experimental models.

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