Neurotoxicity Research, 2007, VOL. 11(2). pp. 93-99

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Melatonin Decreases The Oxidative Stress Produced by 2,4-Dichlorophenoxyacetic Acid in Rat Cerebellar Granule Cells

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(Submitted 30 May 2006; Revised 7 August 2006; In final form 7 August 2006)

2,4-Dichlorophenoxyacetic acid (2,4-D) is one of the most widely used herbicides due to its relatively moderate toxicity and to its biodegradability in the soil. In toxic concentrations, 2,4-D displays strong neurotoxicity, partly due to generation of free radicals. Since melatonin has remarkable antioxidant properties, the objective of this study was to assess to what extent it was effective in preventing the 2,4-D effect on redox balance of rat cerebellar granule cells (CGC) *in vitro*. Cellular viability, generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), reduced glutathione (GSH) levels, and the activities of the antioxidant enzymes Cu/Zn-superoxide dismutase (Cu/Zn-SOD), Mn-SOD, selenium-glutathione peroxidase (Se-GPx) and catalase (CAT) were measured in CGC exposed to 2,4-D and/or melatonin for 48 h. In CGC cultures exposed to 2,4-D, cell viability, GSH levels and CAT activity decreased significantly whereas ROS generation and Se-GPx activities were augmented. Except for Se-GPx activity, all these changes were counteracted by the concomitant addition of 0.1 or 0.5 mM melatonin. In addition, incubation of CGC with melatonin alone resulted in augmentation of cell viability, GSH levels and Se-GPx activity. RNS generation and SOD activity remained unaffected by either treatment. Since melatonin was able to counteract most of redox changes produced by 2,4-D in CGC in culture, the experimental evidence reported further support the efficacy of melatonin to act as a neuroprotector.

Keywords: 2,4-Dichlorophenoxyacetic acid; Herbicides; Melatonin; Free radicals; Cerebellar granule cells

Abbreviations

2,4-D: 2,4-Dichlorophenoxyacetic acid
CAT: catalase
CGC: Cerebellar granule cells
CNS: Central nervous system
Cu/Zn-SOD: Cu/Zn-superoxide dismutase
DCF: 2',7'-dichlorofluorescein
DCFH: 2'7'-dichlorofluorescin
GSH: reduced glutathione
Mn-SOD: Mn-superoxide dismutase
MTT: 3-(4,5-dimethylthiazol-2-eyl)-2,5-diphenyl tetrazolium bromide
NMDA: *N*-methyl-D-aspartate
NO: nitric oxide
RNS, reactive nitrogen species
ROS, reactive oxygen species
Se-GePx: selenium-glutathione peroxidase

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ISSN 1029 8428 print/ ISSN 1476-3524 online.

The central nervous system (CNS) is especially sensitive to oxidative stress. One reason is its high O_2 consumption. The mitochondrial respiratory chain is responsible for generation of most reactive oxygen species (ROS). Under physiological O_2 level, 1-2% of the O_2 consumed is converted to ROS (Emerit *et al.*, 2004). Another cause of oxidative stress is nitric oxide (NO) production, a reactive nitrogen species (RNS). This gaseous free radical is an important and highly diffusible biological messenger that plays a major role in the physiology of the CNS.

The detoxification of ROS in brain cells involves the cooperative action of the intracellular antioxidant enzymes Cu/Zn-superoxide dismutase (Cu/Zn-SOD) that is cytosolic, Mn-superoxide dismutase (Mn-SOD) that is mitochondrial, and selenium-glutathione peroxidase (Se-GPx) and catalase (CAT) that are present in cytosol and peroxisomes, respectively. Furthermore, reduced glutathione (GSH) contributes critically to the homeostasis of oxidant metabolism by scavenging ROS and by reducing erroneous disulfide linkages of proteins (Park *et al.*, 1999).

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) has been widely used in agricultural and forestry since the 1940s. Previous studies from our laboratory strongly supported that the CNS is a target organ for the 2,4-D effects. Exposure of newborn rats to 2,4-D resulted in modification of astroglial cytoarchitecture and of neuronal function (Brusco *et al.*, 1997). Both a deficit in myelin lipid deposition and behavioral alterations have also been observed (see Bortolozzi *et al.*, 2001). Recently, we observed that 2,4-D induced selective alterations of enzymatic activities and increased ROS levels in cerebellar granule cells (CGC) in culture (Bongiovanni *et al.* submitted). The CGC are glutamatergic neurons, which express all glutamate receptor subtypes and have been used as a model to study excitotoxicity caused by neurotoxins (Savill *et al.*, 2005).

In view of the fact that the pineal product melatonin has remarkable antioxidant properties (see Pandi-Perumal *et al.*, 2006), the objective of the present study was to assess to what extent melatonin was effective in preventing the 2,4-D effect on redox balance of CGC *in vitro*. Melatonin and several of its oxidation products scavenge hydroxyl, carbonate and various organic radicals, peroxynitrite and other reactive nitrogen. Melatonyl radicals formed by melatonin scavenging, combine with and, thereby, detoxify superoxide anions in processes terminating the radical reaction chains. Melatonin also enhances the antioxidant potential of the cell by stimulating the synthesis of antioxidant enzymes such as Mn-SOD, Se-GPx and glutathione reductase, and by augmenting GSH levels (Pandi-Perumal *et al.*, 2006). Melatonin has been successfully used to reduce the toxic action of kainic acid on non-NMDA receptors in CGC in culture (Giusti *et al.*, 1995). Cellular viability, ROS and RNS generation, GSH content and SOD, Se-GPx, and CAT activities in CGC exposed to 2,4-D and/or melatonin were assessed.

Rat CGC cultures were made as described by Gallo *et al.* (1987). Briefly, cells were obtained from 8 day old rat cerebella aseptically dissected in Hank's balanced salt solution and treated with trypsin followed by trituration. The dissociated cells were plated in multiwells previously coated with poly-L-lysine (100 µg/ml in 0.1 M borate buffer, pH 8.3) at a high density (3×10^5 cells/cm^2). Cells were cultured in the presence of 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin in a humidified 37ºC incubator with 5% CO_2 and were exposed for 48 h to 2,4-D (1 mM) and/or melatonin (0.1 or 0.5 mM). Drugs were added every 24 h. Cell viability assays were performed by using the dye 3-(4,5-dimethylthiazol-2-eyl)-2,5-diphenyltetrazolium bromide (MTT) which is metabolized by viable mitochondria to a colored product that can be detected photometrically. Thus, the extent of MTT metabolism is an indicator of mitochondrial function. After removal of the media, 100 µl MTT (0.5 mg/ml of medium) was added to each well, and the plate was incubated for 4 h at 37ºC. The MTT solution was then removed, and 100 µl of dimethyl sulfoxide was added; the color intensity was assessed with a plate reader at 590 nm.

ROS generation was measured by a modification of the procedure described by Myhre *et al.* (2000). The method is based on the incubation of cells with the nonfluorescent probe DCFH-DA, which diffuses passively through the cellular membrane. Intracellular esterase activity results in the formation of 2',7'-dichlorofluorescin (DCFH), a nonfluorescent compound that will be oxidized to the fluorescent compound 2',7'-dichlorofluores-

cein (DCF) by ROS. CGC were loaded with 5 µM DCFH-DA (DCFH-DA stock solution 5 mM in methanol, stored at -20ºC) directly in the cell culture medium for 20 min (at 37ºC with 5% CO_2 and constant humidity). The medium with DCFH-DA was replaced by 1.5 ml incubation medium (HBSS with 20 mM HEPES and 10 mM glucose) and cells were harvested with a cell scraper. Each cell culture was placed into a glass tube and fluorescence was recorded in a luminescence spectrometer (excitation wavelength 485 nm, emission wavelength 530 nm). A standard curve was prepared for commercially obtained DCF.

Nitrite production was determined as described by Miranda *et al.* (2001). The principle of this assay is the reduction of nitrate by vanadium (III) combined with detection by the acidic Griess reaction. Briefly, nitrite and nitrate were determined by mixing 100 µl of sample with 100 µl of VCl_3 and 100 µl of Griess reagent (1.5% sulfanilamide in 1M HCl plus 0.15% *N*-(1-naphthyl) ethylenediamine dihydrochloride in distilled water, v/v). After 10 min incubation at room temperature, the absorbance at 540 nm was determined and nitrite concentration was calculated from a sodium nitrite standard curve.

For the enzyme assays, the cells were harvested with a cell scraper and homogenized in 200 µl of cold distilled water. The homogenates were sonicated for 15 sec and centrifuged for 2 min at 10,000*g*. Samples for the determination of CAT activity were used immediately while those for the determination of SOD and Se-GPx activities were stored at -20ºC. CAT activity was assayed by the method described by Beers and Sizer (1951) in which the disappearance of peroxide is followed spectrophotometrically at 240 nm. The incubation mixture contained 50 mM potassium phosphate, pH 7.0, 20 mM hydrogen peroxide (H_2O_2), and the sample (50 µl) in a final volume of 1 ml. The decrease in absorbance was recorded at 240 nm for 2 min and the rate of decrease in absorbance per min was calculated from the initial linear portion of the curve (up to 45 sec). The value of 0.0394 cm^2/µmol proposed was used as the extinction coefficient of H_2O_2. One unit of catalase was defined as the amount of enzyme that decomposed 1 µmol of H_2O_2/min at 25ºC and pH 7.0 under the specified conditions.

SODs were determined by the method of McCord and Fridovich (1969) based on the inhibition of the reduction of ferricytochrome C in presence of O_2. The assay consisted in adding to 900 µl of solution A (0.01 mM ferricytochrome C and 0.05 mM xanthine in 50 mM potassium phosphate buffer at pH 7.8 containing 0.1 mM EDTA), and 15 µl of xanthine oxidase (solution B). One unit of activity was defined as that producing a rate of reduction of ferricytochrome C of 50%, i.*e.*, 0.0125 absorbance unit per min at 25ºC. Differentiation between the different types of SODs (Cu/Zn-SOD and Mn-SOD) was performed by the addition of potassium cyanide (0.5 mM) to the incubation medium. Cu/Zn-SOD was defined as the enzyme fraction that was inhibited by potassium cyanide, while Mn-SOD was not affected.

Se-GPx activity was measured by a modification of the procedure by Paglia and Valentine (1967). The standard assay mixture (500 µl) contained 0.1 M buffer phosphate pH 7, 1 mM GSH, 0.2 mM NADPH, 1.4 U GSH-reductase, 0.25 mM H_2O_2 and a sample (80 µl) of supernatant fluid. H_2O_2 was used as a substrate, and 1 mM sodium azide was added to the reaction mixture in order to inhibit possible residual CAT activity after freezing the samples. Blank values obtained without the addition of sample were subtracted from the assay values. Conversion of NADPH was monitored continuously in a spectrophotometer at 340 nm for 3 min at 25ºC ($\varepsilon_{(NADPH)}$=6220 $mmol^{-1}cm^{-1}$). One unit of Se-GPx was defined as the amount of enzyme which metabolized 1 nmol NADPH/min at 25ºC and pH 7.0.

GSH content was measured by the method described by Thomas *et al.* (2000), based on a fluorimetric method using o-phthaldialdehyde condensation reaction in samples rendered protein-free by precipitation with ice-cold 0.1 M H_3PO_4. Briefly, 200 µl of sample were buffered with 0.1 M sodium phosphate buffer; pH 8.0 and 0.1% *o*-phthaldialdehyde (in ethanol) was added. Following 20 min of incubation at room temperature the fluorescence was read at activation/emission wavelengths 337/423 nm. A standard curve was prepared for commercially obtained GSH.

Statistical analysis of results was performed by an analysis of variance (ANOVA) following a factorial model of two factors, *i.e.*, with or without 2,4-D and in the presence of 0, 0.1 or 0.5 mM melatonin. A *post-hoc* Tukey´s multiple comparison test was applied when one or the two factors, or their interaction, was significant. *P* values less than 0.05 were

considered evidence for statistical significance. Validity of linear and non-linear models was assessed by residual analysis and Shapiro Wilk´s and Bartlett´s tests. Since results from cell viability analysis did not fit normality or homogeneity of variance a logarithmic transformation of data was used.

Figure 1 shows the changes in cell viability, ROS and RNS generation and GSH levels in rat CGC cultures exposed to 1 mM 2,4-D in the presence or absence of 0.1 or 0.5 mM melatonin for 48 h. Mean cell viability was decreased significantly by 2,4-D ($F_{1,42}$=1046, p <0.00001) and was increased significantly by 0.1 or 0.5 mM melatonin ($F_{2,42}$=312, p <0.00001) as shown by a factorial ANOVA taking each treatment as a main factor (FIG.1, upper left panel). A significant interaction was found in the factorial ANOVA ($F_{2,42}$= 21.3, p <0.00001), the protective effect of melatonin being significantly greater at the 0.1 mM concentration level (p <0.0001, Tukey test).

ROS generation augmented by 84% after 2,4-

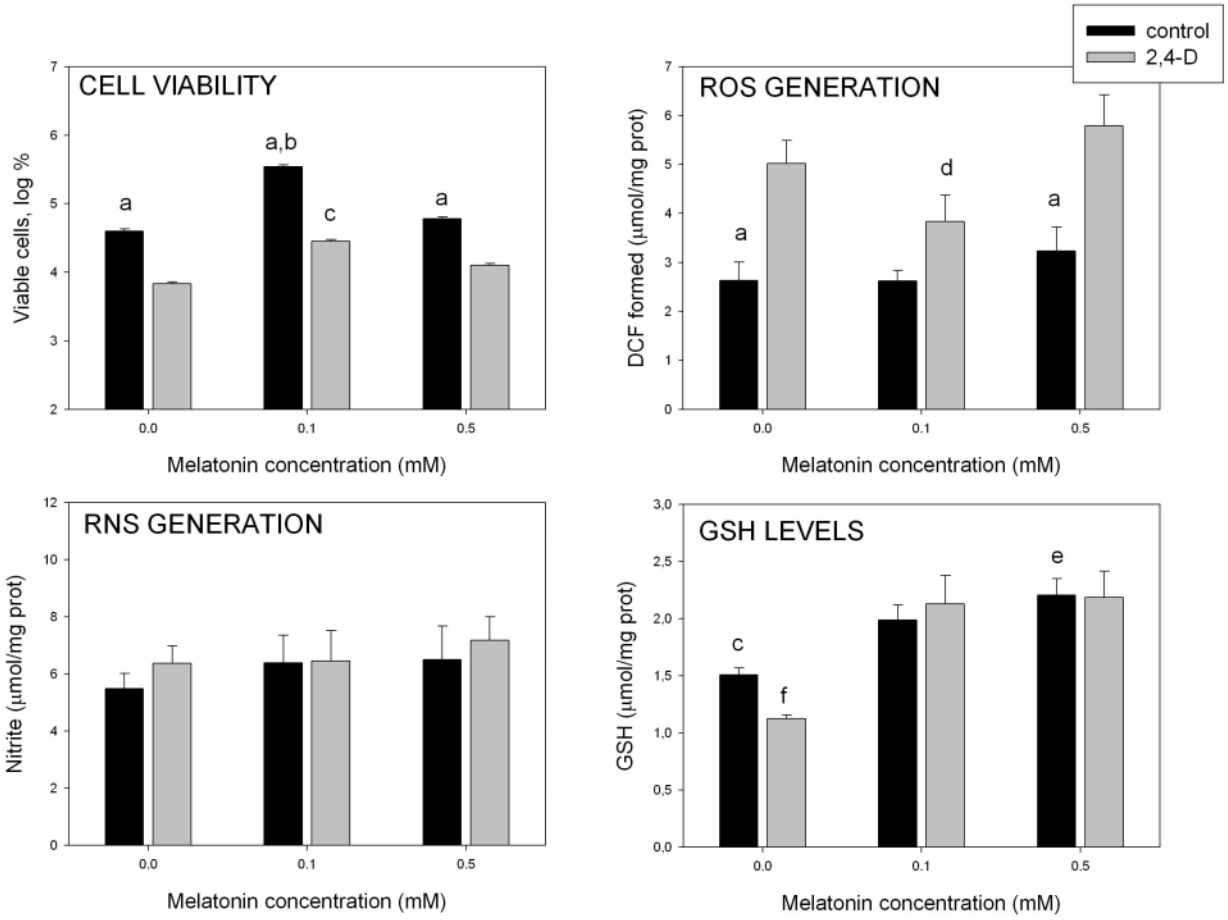

FIGURE 1 Cell viability, ROS and RNS generation, and GSH levels in rat cerebellar granule cell cultures exposed to 1 mM 2,4-D in the presence or absence of 0.1 or 0.5 mM melatonin for 48 h. Shown are the means ± SEM (n= 8/group). A factorial ANOVA followed by a Tukey test indicated that: (i) mean cell viability decreased significantly after 2,4-D (p< 0.00001) and increased after 0.1 or 0.5 mM melatonin (p< 0.00001) with the protective effect of 0.1 mM melatonin being greater (p< 0.0001); (ii) ROS generation augmented after 2,4-D exposure (p< 0.00001), 0.1 mM melatonin decreasing 2,4-D effect (p< 0.00001); (iii) RNS generation remained unaffected; (iv) GSH levels decreased after 2,4-D exposure an effect counteracted by 0.1 or 0.5 mM melatonin (p< 0.007). Melatonin augmented GSH levels both in the presence or absence of 2,4-D (p< 0.00001). [a]p< 0.05 *vs* 2,4-D. [b]p< 0.05 *vs* control incubated in the absence or with 0.5 mM melatonin. [c]p< 0.05 *vs* 2,4-D, incubated in the absence or with 0.5 mM melatonin. [d]p< 0.05 *vs* 2,4-D incubated with 0.5 mM melatonin. [e]p< 0.05 *vs* control, incubated in the absence of melatonin. [f]p< 0.05 *vs* 2,4-D, incubated with 0.1 or 0.5 mM melatonin.

D exposure ($F_{1,42}$= 513, p <0.00001, factorial ANOVA) (FIG. 1, upper right panel). Melatonin did not modify ROS generation in the absence of 2,4-D but was effective at a 0.1 mM concentration, to decrease pesticide effect significantly ($F_{2,42}$=16.3, p <0.00001 for factor interaction). RNS generation remained unaffected by any treatment (FIG. 1, lower left panel).

GSH levels decreased by 31% after 2,4-D exposure, an effect totally reverted by 0.1 or 0.5 mM melatonin ($F_{2,42}$=5.7 for factor interaction, p <0.007) (FIG. 1, lower right panel). Melatonin was very effective in augmenting GSH levels both in the presence or absence of 2,4-D ($F_{2,24}$=48.2, p <0.00001).

Figure 2 depicts the changes in CAT, SOD and Se-GPx activities in the same cell cultures. While neither Mn-SOD nor Cu/Zn SOD changed after treatment (FIG. 2, upper right and lower left panels), CAT activity decreased by 37% in the presence of 2,4-D ($F_{1,54}$=106, p <0.00001) (FIG. 2, upper left panel), Melatonin did not modify CAT activity in the absence of 2,4-D but was effective in counteracting pesticide activity at either dose ($F_{2,54}$=6.1, p <0.01 for factor interaction). Both 2,4-D and melatonin significantly augmented Se-GPx activity ($F_{1,52}$=135, p <0.00001 and $F_{2,52}$=8.7, p <0.0005, respectively), the interaction of both factors being significant ($F_{2,52}$=15.2, p <0.00001) (FIG. 2, lower right panel).

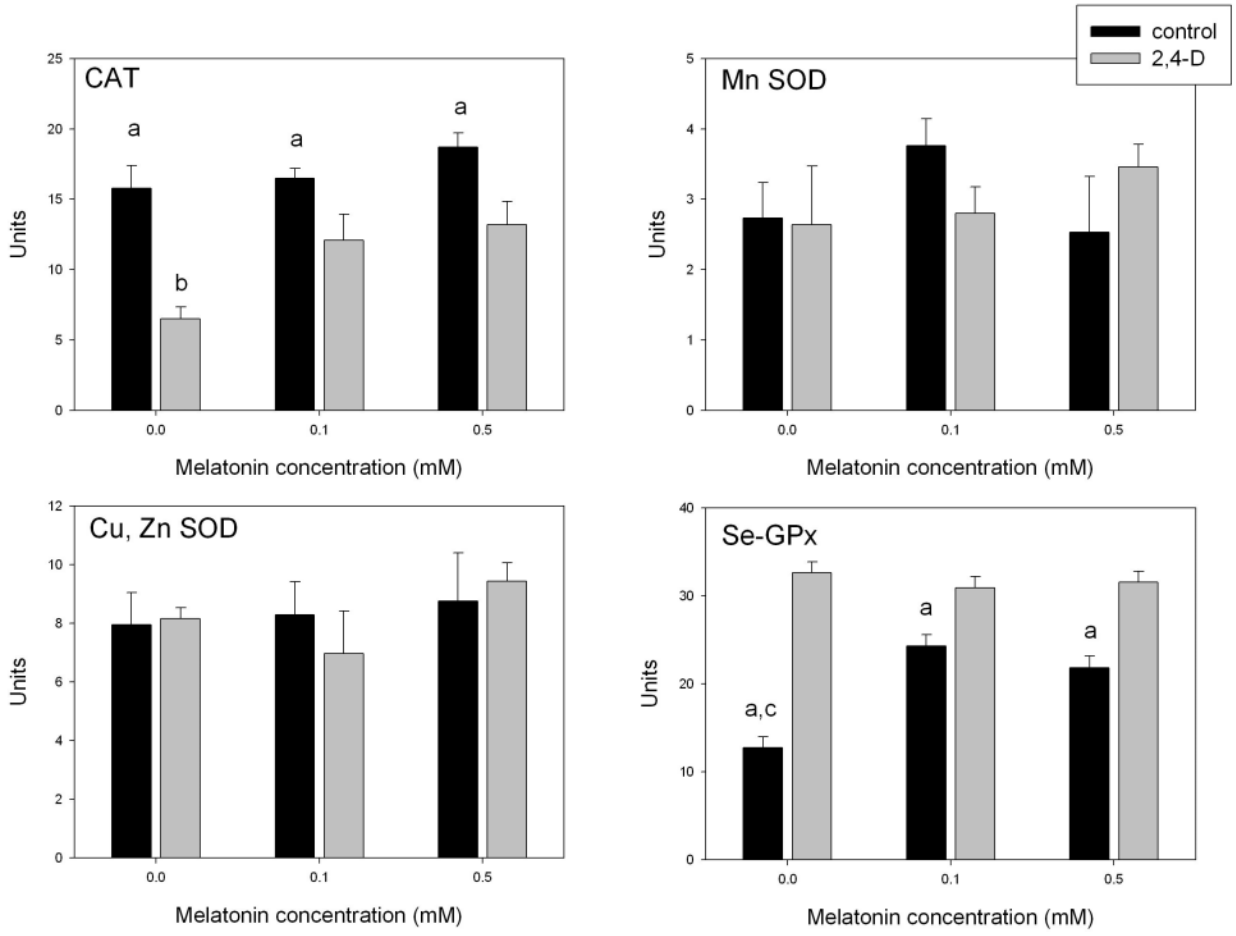

FIGURE 2 CAT, SOD and Se-GPx activities in rat cerebellar granule cell cultures exposed to 1 mM 2,4-D in the presence or absence of 0.1 or 0.5 mM melatonin for 48 h. Shown are the means ± SEM (n= 8/group; n= 10/group for CAT). A factorial ANOVA followed by a Tukey test indicated that: (i) CAT activity was decreased by 2,4-D (p<0.00001), an effect prevented by melatonin (p<0.01); (ii) neither Mn SOD nor Cu/Zn SOD changed after treatment; (iii) Se-GPx activity increased after 2,4-D exposure (p< 0.00001) or melatonin (p<0.0005). [a]p<0.05 *vs* 2,4-D. [b]p<0.05 *vs* 2,4-D incubated with 0.1 or 0.5 mM melatonin. [c]p<0.05 *vs* control incubated with 0.1 or 0.5 mM melatonin.

A remarkable body of evidence indicates that melatonin exerts antioxidative protection in numerous cell culture and *in vivo* systems (Pandi-Perumal *et al.*, 2006). A special but important aspect is melatonin's role in neuroprotection (Srinivasan *et al.*, 2005). Protection takes place at different levels, including the attenuation of radical formation by antiexcitatory and antiinflammatory effects. This is not restricted to scavenging, although melatonin efficiently interacts with various reactive oxygen and nitrogen species as well as organic radicals, but includes up-regulation of antioxidant enzymes (Se-GPx, GSH reductase, γ-glutamylcysteine synthase, glucose 6-phosphate dehydrogenase, sometimes Cu/Zn- and Mn-SOD and CAT) and down-regulation of prooxidant enzymes (NO synthases, lipoxygenases) (Srinivasan *et al.*, 2005; Pandi-Perumal *et al.*, 2006). Other antioxidant effects may be mediated by binding to quinone reductase 2, which had previously been assumed to represent a new melatonin receptor. Recently, mitochondrial effects of melatonin have come into the focus of interest, which comprise safeguarding of respiratory electron flux, reduction of oxidant formation by lowering electron leakage) and inhibition of opening of the mitochondrial permeability transition pore (Pandi-Perumal *et al.*, 2006).

The foregoing results indicate that, in rat CGC cultures exposed to 2,4-D for 48 h, cell viability, GSH levels and CAT activity decreased whereas ROS generation and Se-GPx activity was augmented. Except for Se-GPx activity, all these changes were counteracted by the concomitant addition of 0.1 or 0.5 mM melatonin. Moreover, incubation of CGC with melatonin alone resulted in augmentation of cell viability, GSH levels and Se-GPx activity. RNS generation and SOD activity remained unaffected by either treatment. Although the doses used here are clearly pharmacological versus circulating melatonin levels. Studies on cerebrospinal fluid (Tricoire *et al.*, 2003) and hypothalamic (Cardinali *et al.*, 1991) levels of melatonin indicated a concentration 0.1 μM, about two orders of magnitude higher than in blood.

2,4-D is one of the most widely used herbicides due to its relatively moderate toxicity, when in concentrations resulting from adequate use in agriculture, and to its biodegradability in the soil. However, toxic concentrations of this herbicide lead to apoptosis of human lymphocytes (Kaioumova *et al.*, 2001), HepG2 cells (Tuschl and Schwab, 2003) and CGC (De Moliner *et al.*, 2002) and are associated with non-Hodgkin's lymphoma and other cancers (*e.g.*, see Bradberry *et al.*, 2000). By *in vivo* spin-trap electron paramagnetic resonance spectroscopy it was demonstrated that sudden exposure of a yeast cell population to concentrations of 2,4-D leads to the generation of free-radicals, as well as several antioxidant enzymes, *e.g.*, CAT, SOD (Teixeira *et al.*, 2004). In the present study on CGC after 48-h of culture, cells exposed to 2,4-D showed a decrease of CAT, an increase of Se-GPx and no changes in SOD. Except for the increase of SE-GPx, melatonin was able to counteract the redox changes produced by 2,4-D in CGC in culture. Why both 2,4-D and melatonin augmented Se-GPx is not presently understood. Further studies are needed to analyze the time-course of the effects observed.

Summarizing, the experimental evidence reported in the present study supports the conclusion that melatonin is an effective neuroprotector for CGC *in vitro*. To what extent it could be also a neuroprotector *in vivo* deserves to be further explored.

Acknowledgements

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, to R.D. We thank Prof. Mercedes Leiva and Hebe Bottai for assistance in the statistical analysis. A.M.D., D.P.C. and R.D. are Research Career Awardees from CONICET.

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