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Melatonin, lipid peroxidation, and age in heterophils from the ring dove (*Streptopelia risoria*)

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Abstract

Numerous recent studies have shown the ability of physiological as well as all pharmacological concentrations of melatonin to prevent oxidative stress. We have found that incubating avian heterophils from young birds with a pharmacological concentration of 100 μM (23×10^6 pg/ml) melatonin reduced superoxide anion levels by modulating the activity of superoxide dismutase while also enhancing phagocytosis. There was also a decline in lipid peroxidation levels with both physiological and pharmacological concentrations of this indolamine.

In the present work, we evaluated malonaldehyde (MDA) levels as an indicator of lipid peroxidation (both basal and antigen-induced) in young and old animals (ring doves) at different times of day (16:00 and 00:00) and with two incubation times (15 and 60 min). The lipid peroxidation was also measured in heterophils from old animals, incubated with the physiological concentrations of melatonin measured in young animals (50 and 300 pg/ml, diurnal and nocturnal, respectively). The results, expressed as nmol MDA/mg protein, show that MDA levels were higher in heterophils of old animals than in the young birds in all the experimental groups studied at both 16:00 and 00:00 (00:00 is the time at which the lowest peroxidation levels were obtained). Incubation with melatonin was found to reduce MDA levels, with the maximum reduction being after the 60 min incubation time and the nocturnal melatonin concentration. At both concentrations (diurnal and nocturnal), melatonin also counteracted the enhancement of MDA levels caused by latex beads, with the effect being greater at the longer incubation time. In conclusion, the results are further evidence of the antioxidant effect of melatonin even at physiological concentrations, and suggest its utility as a therapeutic agent in some pathological processes associated with age.

Keywords: *Melatonin, lipid peroxidation, heterophils, doves, age*

Introduction

Melatonin has been identified as a powerful direct free radical scavenger [1] and general antioxidant [2,3], reducing oxidative damage at both physiological and pharmacological concentrations [4]. What seems particularly unusual is the high efficacy of melatonin as a protector against reactive oxygen and reactive nitrogen species [4].

Surgical removal of the pineal gland, a procedure which lowers endogenous melatonin levels in the blood, exaggerates molecular damage due to free radicals during an oxidative challenge. Likewise, providing supplemental melatonin during periods of massive free radical production greatly lowers the resulting tissue damage and dysfunction. These findings are relevant to neurodegenerative diseases, cancer, ischaemia/reperfusion injury, and aging. Besides being a highly effective direct free radical scavenger and indirect antioxidant, melatonin has several features that makes it of clinical interest [5]. Thus, melatonin's efficacy has been confirmed in reducing biomolecular damage under many experimental conditions where free radicals are believed to be involved. Melatonin's beneficial effects are likely to

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be related to its direct detoxification of free radicals, its indirect antioxidant actions, its ability to preserve efficient oxygen metabolism in mitochondria, and possibly other actions. These processes are assisted by the ready absorbability of the indolamine, allowing it to cross all morpho-physiological borders and to enter all compartments of all cells. What is lacking is extensive clinical trials of its potential beneficial effects, studies that are entirely feasible considering the low toxicity of this substance [5].

In 1991 Harman [6] summarized the evidence, and has since been a firm advocate of what is referred to as the free radical theory of aging. This theory suggests that longevity is determined by the amount of accumulated free radical damage that animals sustain throughout their lifetime, i.e. the more oxidative damage the higher the incidence of age-associated free radical-based diseases, and the shorter the lifespan. More recently, a number of theories have proposed that melatonin modifies some processes of aging [7]. Some of these ideas arose because endogenous melatonin production diminishes in advanced age in all species in which it has been investigated, including humans [8].

Since melatonin production in mammals diminishes with age, interest has been generated in the possibility that the functional and morphological deterioration that accompanies aging is in part related to the loss of this antioxidant and free radical scavenger [8]. This idea is supported by several observations that the degree of oxidative damage which rats accumulate is greater in advanced age when they had to live their life in a relatively melatonin-deficient state because they had been pinealectomized [9], although some other changes induced by pinealectomy (e.g. circadian rhythm disruption) may have accounted for the reduction in survival and the augmented oxidative damage [10].

The process of phagocytosis is accompanied by a series of oxygen-dependent biochemical events, leading ultimately to the production of highly reactive oxidants which play a key role in the microbicidal activity of phagocytes. Oxidative stress generated during phagocytosis provides protection against micro-organisms, but may cause tissue damage if it occurs to an excessive degree and/or the antioxidant mechanisms do not function properly [11]. The exposure of biological membranes to oxidative stress results in a progressive degeneration of membrane structure and loss of integrity. Lipid peroxidation is a process that leads to the destruction of membrane lipids and production of lipid peroxides and their by-products, such as aldehydes [12,13]. In the present work, we evaluated malonaldehyde (MDA) levels as an indicator of lipid peroxidation (both basal and antigen-induced) in young and old animals (ring doves), as well as in heterophils of old animals, incubated with physiological concentrations of melatonin that had been observed in the blood of young animals.

Materials and methods

Animals

Male and female ring dove (*Streptopelia risoria*) at 2–3 years (young) or 8–10 years (old) of age were used in the study, the maximum lifespan of these animals being 12 years. The birds were housed isolated in cages measuring $40 \times 40 \times 45\,cm^3$, with an outside window, natural lighting, and direct ventilation, and fed *ad libitum* (food and water). The study was conducted during April/June when the nocturnal photoperiod was approximately 14 h light and 10 h dark (dark period from 21:30 ± 30 min to 07:30 ± 30 min). The temperature was maintained at 22 ± 2°C. The trials were conducted under dim red light which is perceived as darkness.

The study was approved by the Ethical Committee of the University of Extremadura (Badajoz, Spain) in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Isolation of heterophil leukocytes

Heterophil leukocytes were obtained immediately after the extraction of 1 ml of blood (at 00:00 and 16:00) from the brachial vein, to which 0.5 ml phosphate buffered saline solution (PBS) and 0.5 ml of lithium heparin were added, followed by centrifugation at 600*g* for 15 min in a gradient using Histopaque (1 ml of 1119, 1 ml of 1077; Sigma, St. Louis, MO). The heterophils were then washed in PBS and adjusted to 5×10^6 cells/ml of medium.

Melatonin

N-acetyl-5-methoxytryptamine (Sigma) was prepared in PBS solution, starting from a base solution of 10 mg/ml which was dissolved by heating and stirring, followed by dilution to the working solutions (50 and 300 pg/ml). The choice of these concentrations was based on our previous studies [14], where we had found that these doses observed in both young and mature ring doves affect some phagocytic biochemical processes. All determinations were accompanied by a control sample free of melatonin.

Experimental design

Experiment 1. Evaluation of lipid peroxidation, both basal and that induced by an inert antigen (latex beads), in heterophils from young and old animals after different incubation times (15 and 60 min) and at different times of day (at 16:00 during the light period, and at 00:00 some hours after the onset of darkness).

Experiment 2. Evaluation of lipid peroxidation levels (basal at 0 min and induced by incubation with latex for 15 or 60 min) in heterophils from old ring doves, incubated with the physiological doses of melatonin that had been measured in young and mature animals (50 and 300 pg/ml, diurnal and nocturnal levels, respectively) [14]. All determinations were performed at 10:00.

Measurement of lipid peroxidation

Lipid peroxidation (LPO) is a well-established mechanism of cell injury in both plants and animals. The LPO-586 assay (Bioxytech PLO-586) is based on the reaction of a chromogenic reagent.

The cell precipitate (5×10^6 cells/ml) was separated into aliquots of 300 μl per tube. In experiment 1, one tube contained only cells (negative control) and a second tube contained cells with latex beads (1.091 μm diameter, at 1% in PBS, Sigma), constituting the positive control. In experiment 2, one tube contained only cells (negative control); a second contained cells with latex beads (1.091 μm diameter, at 1% in PBS, Sigma), constituting the positive control; a third and fourth contained cells with melatonin at diurnal and nocturnal concentrations (50 and 300 pg/ml, respectively); and the fifth and sixth contained cells with a combination of melatonin (50 or 300 pg/ml) and latex beads. All the tubes (samples from young or old animals and at 16:00 or 00:00) were incubated in a thermal bath at 37°C for different times (15 or 60 min).

Melatonin, latex, and a combination of these substances were tested with the standard of the Bioxytech kit to evaluate any potential interference between these chemicals and the kit reagents.

Total protein levels

The levels of cell lysate total protein were measured using the Bradford protein assay (Bradford, 1976) (Sigma). The concentration of total protein was calculated by means of a standard curve with bovine albumin (0.05–0.8 mg/ml, 2 mg/ml; Sigma) and the results expressed as mg of protein/ml.

Calculation of concentrations

The following equation gives the concentration (M) of MDA in a sample:

$$[\text{MDA}] = (A - A_0) \times 5/\varepsilon$$

where A is the absorbance in the presence of sample, A_0 is the absorbance in the absence of the sample, 5 is the sample dilution factor in the cuvette (200 μl of sample in a total volume of 1 ml), and ε is the apparent molar extinction coefficient obtained from the standard curve using a standard (solution of 10 mM 1,1,3,3-tetramethoxypropane in 20 mM *tris*–HCl buffer, pH 7.4, 0–20 μM; Bioxytech) diluted 100 times with PBS. Results are expressed as nmol MDA/mg protein.

Statistical analysis

All data are expressed as means ± standard error of the mean for the number of samples assayed. This number (N) is indicated for each case. Results were analyzed by using the non-parametric ANOVA-Scheffe F-test to compare differences between groups, followed by a Student's t-test. Only values with $p < 0.05$ were accepted as significant.

Results

Figure 1 shows the results of the comparative study of lipid peroxidation (nmol MDA/mg protein) in ring dove heterophils in different situations (with and without latex beads), after different incubation times

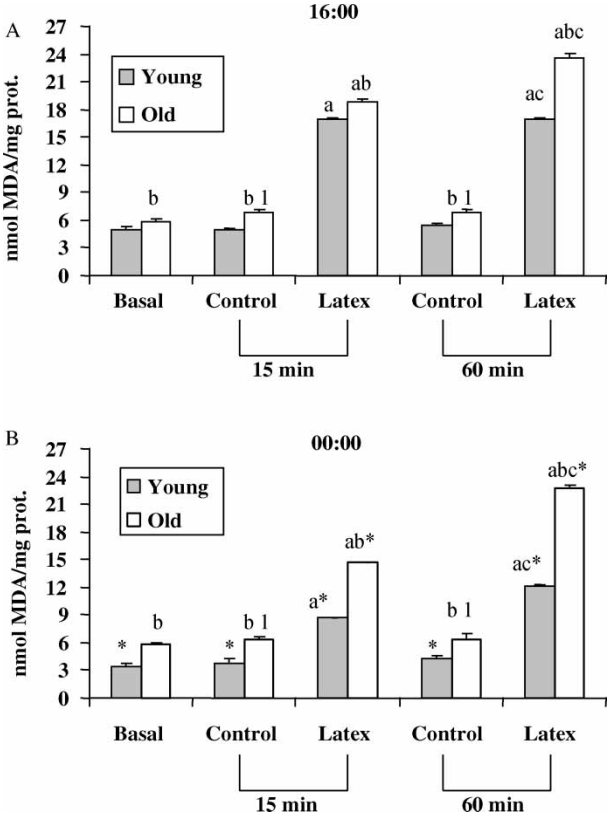

Figure 1. Comparative study of the lipid peroxidation levels (both basal and latex induced) in ring dove heterophils from young and old animals, alter different incubation times. Each value represents the mean ± SE of eight determinations performed in duplicate. $p < 0.05$ 1, a, b, c, *: (1) with respect to their basal value; (a) with respect to their basal and control after the same incubation time; (b) with respect to their values in young animals at the same time of day; (c) with respect to their value at 15 min; (*) with respect to their value at 16:00.

(15 or 60 min), at different times of the day (16:00 and 00:00), and in different age groups (young and old). Only in the old animals there was a significant increase ($p < 0.05$) in the MDA concentration of the control group with respect to the basal value after both incubation times and at both hours of the day. When the heterophils were incubated with latex beads, there were significant increases ($p < 0.05$) in MDA concentrations in both young and old animals with respect to the basal and the control values for both incubation times, and at both times of day (16:00 and 00:00). Also, the MDA levels in heterophils were significantly higher ($p < 0.05$) in the old animals than in the young, again for both incubation times and at both times of day, in all the experimental groups. Lipid peroxidation was greater ($p < 0.05$) following 60 min incubation with latex beads than the corresponding value for 15 min incubation at both times of day and in both old and young animals. Finally, for both the incubation times studied, the values obtained at 00:00 were less ($p < 0.05$) than at 16:00 in the latex bead group (in young and old animals) and in the control group (in young animals only). In the basal group, the values obtained at 00:00 were less ($p < 0.05$) than at 16:00 in young animals only.

Table I is a summary of the MDA levels found in the heterophils of old animals, for different times of incubation (15 and 60 min) in the presence or the absence of latex beads and of melatonin. There was an increase ($p < 0.05$) in MDA concentrations with respect to the basal and control values at both incubation times and in the presence of latex beads, and after 60 min incubation time in the presence of latex beads plus melatonin (both the diurnal and the nocturnal concentrations). Incubation with melatonin alone (both the diurnal and the nocturnal concentrations) reduced ($p < 0.05$) MDA levels, reaching the basal value after 15 min of incubation, and declining markedly after 60 min of incubation. Also, incubation with melatonin (both the diurnal and the nocturnal concentrations) alone or in combination with latex led to a reduction ($p < 0.05$) with respect to the values obtained in the presence of latex beads alone for both incubation times. After 60 min of incubation, the values obtained with melatonin plus latex beads were greater ($p < 0.05$) than those with melatonin alone (both the diurnal and the nocturnal concentrations). The lowest values of lipid peroxidation were observed in the presence of the nocturnal concentration of melatonin after the longer of the two incubation times.

Finally, Figure 2 shows the changes with incubation time (15 and 60 min) in the lipid peroxidation of heterophils from old ring dove, incubated with the physiological concentrations observed in young animals (50 and 300 pg/ml, diurnal and nocturnal concentrations, respectively). In all groups except the controls, there were significant ($p < 0.05$) changes in lipid peroxidation with incubation time. Thus, in the presence of latex (either alone or in combination with diurnal or nocturnal concentrations of melatonin), the highest levels of peroxidation were observed after 60 min of incubation, and the values were significantly ($p < 0.05$) less after 60 min of incubation in the presence of melatonin alone (both the diurnal and the nocturnal concentrations).

In sum: (i) the highest values of lipid peroxidation were obtained when the heterophils were incubated in the presence of latex beads, and the lowest values after incubation with the antioxidant melatonin; (ii) heterophils from old ring doves presented higher

Table I. Levels of lipid peroxidation in ring dove (*Streptopelia risoria*) heterophils incubated with melatonin doses at physiological concentrations observed in young animals.

Basal (t=0)	Incubation time	
	15 min	60 min
5.47 ± 0.06	(nmol MDA/ mg protein)	
Control	6.07 ± 0.05*	6.17 ± 0.05*
Latex	13.55 ± 0.03†	27.26 ± 0.07†
Melatonin (diurnal)	5.66 ± 0.09‡,¶	4.18 ± 0.16‡,¶
Melatonin (nocturnal)	5.47 ± 0.03‡,¶	3.88 ± 0.03$^{\dagger,\S,\|\|}$
Melatonin (diurnal) + Latex	7.11 ± 0.02¶	11.89 ± 0.02$^{\dagger,\P,\#}$
Melatonin (nocturnal) + Latex	6.52 ± 0.12¶	10.25 ± 0.13†,¶,**,§

Each value represents the mean ± SE of eight determinations performed in duplicate.
$^{\star}p < 0.05$ with respect to the basal.
$^{\star\star}p < 0.05$ with respect to nocturnal melatonin (300 pg/ml).
$^{\dagger}p < 0.05$ with respect to the basal and their control.
$^{\ddagger}p < 0.05$ with respect to their control.
$^{\P}p < 0.05$ with respect to latex.
$^{\S}p < 0.05$ with respect to their corresponding value with diurnal melatonin.
$^{||}p < 0.05$ with respect to the rest of the groups studied.
$^{\#}p < 0.05$ with respect to diurnal melatonin (50 pg/ml).

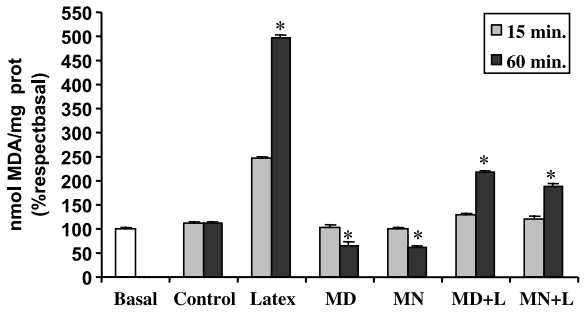

Figure 2. Changes with incubation time in lipids peroxidation (nmol MDA/mg protein) in heterophils from old ring dove (*Streptopelia risoria*) incubated with the physiological concentrations of melatonin found in young animals (50 and 300 pg/ml as diurnal and nocturnal concentrations, respectively). Each value represents the mean ± SE of eight determinations performed in duplicate. The results are expressed as % with respect to the basal value (0 min). $*p < 0.05$ with respect to 15 min; MD: Diurnal melatonin (50 pg/ml); MN: Nocturnal melatonin (300 pg/ml); L: Latex.

concentrations of MDA (both basal and antigen-induced) than did those from young animals; and (iii) in heterophils from old ring doves, incubation with the physiological concentrations of melatonin found in young animals diminished the latex-induced enhancement of MDA production, the effect being dependent on the dose and on the time of incubation with melatonin.

Discussion

In roughly the last five years, numerous *in vitro* and *in vivo* studies have documented the ability of both physiological and pharmacological concentrations of melatonin to protect against free radical damage [5]. Melatonin seems to function via a number of means to reduce oxidative stress. Thus, as an antioxidant its functions include: (a) direct free radical scavenging [15], (b) stimulation of antioxidative enzymes [3], (c) protecting antioxidative enzymes from oxidative damage [16,17], and (d) augmenting the efficiency of other antioxidants [4,18,19]. Melatonin's protective action against a wide variety of processes and toxins that generate oxidizing agents have been extensively documented. A major unanswered question, however, relates to the precise relationship of melatonin to senescent decline, a process widely believed to be, in part, a result of the persistent bludgeoning of macromolecules by free radicals [8]. Indeed, the impetus for the proposed relationship between melatonin and senescence derives, in part, from melatonin's ability to function as an antioxidant and free radical scavenger [8]. It is known that melatonin provides benefits for increasing survival and/or reducing the severity of debilitating diseases, and that the effect seems to be related to its ability to reduce oxidative stress, to strengthen the immune system, to improve mitochondrial function, and to synchronize circadian rhythms [20].

In previous studies on ring dove heterophils, we found that 100 μM melatonin controls superoxide anion levels and modulates superoxide dismutase activity, in both the presence and the absence of a particulate antigen, and that the effect is probably due to the action of this molecule as a scavenger of superoxide anion [21]. Later, with the same pharmacological concentration of the indolamine and in the same experimental model, we observed a suppression of both basal and antigen-induced lipid peroxidation [22], and an enhancement of the phagocytic function at the same time as a neutralization of the oxidative stress deriving from this immune function [23]. Other workers have reported similar findings concerning the protective effect of the antioxidant against both basal and stress-related lipid peroxidation in other *in vitro* models using bacterial lipopolysaccharide or H_2O_2 to induce toxicity [24,25]. And we have found in heterophils from young doves that physiological concentrations of melatonin also reduce lipid peroxidation [11].

Our recent observations too [26,27] have shown there to be higher superoxide anion concentrations in heterophils from old animals relative to young animals. Also, the oral administration of melatonin to old ring dove increases plasma melatonin levels, and in parallel enhances the phagocytosis of heterophils and reduces their superoxide anion levels. In view of these results, we have approached the study of the levels of MDA as an indicator of lipid peroxidation in heterophils from young and old ring dove, and in heterophils from old ring dove following incubation with the physiological concentrations of melatonin found in young animals.

The present results demonstrate that, in general, and independent of the age of the animal, the values of lipid peroxidation are highest when the heterophils are incubated in the presence of latex beads, and the lowest after incubation with melatonin. Melatonin thus effectively protects against both basal and latex-bead-induced toxicity. The heterophils from old ring dove presented higher MDA concentrations (both basal and antigen-induced) than did those from young animals, and the incubation of heterophils from old animals with the physiological concentrations of melatonin found in young animals (50 and 300 pg/ml, diurnal and nocturnal, respectively) reduced MDA levels after the longer of the two incubation times tested, with the greatest reduction being in the presence of the nocturnal concentration of melatonin. In addition, melatonin at both the diurnal and the nocturnal concentrations depressed the enhanced MDA levels caused by latex beads. This effect was time and dose dependent.

The protection provided by melatonin against lipid peroxidation may be related to its capacity to act as an antioxidant, and to stabilize cell membranes thereby making their intrinsic lipids more resistant to peroxidation [22,28,29].

Particularly worthy of note were the results of incubating heterophils from old animals in the presence of melatonin. After the shorter of the two incubation times, the MDA levels which had been raised by latex beads returned to the control values even in the presence of the beads. After the longer of the incubation times, MDA levels in the melatonin alone groups were lower than in the controls, and in the melatonin + latex group were significantly lower than in the presence of latex alone. As we have indicated in previous work [22], this could be because with prolonged incubation times there is an additive effect of melatonin together with several enzyme systems and cell components that protect against activated forms of oxygen following the respiratory burst, such as superoxide dismutase, myeloperoxidase, catalase, glutathione peroxidase, vitamins C and E etc.

Most *in vivo* and *in vitro* studies [22,30] have used pharmacological doses of melatonin that exceed the low physiological concentrations that are normally found in plasma, and are much higher than the peak nocturnal concentrations in the serum of mammals (including humans) and birds [31,32]. The present results therefore are of major relevance in view of the augmented oxidative stress observed in the old animals (with MDA levels significantly greater than in the young animals), and how melatonin, at the physiological concentrations reached in the plasma of young animals, was able to reduce the lipid peroxidation in heterophils from animals of advanced age. Furthermore, judgements of melatonin's efficacy (or lack thereof) as a physiological antioxidant are also often based on the low levels (10–200 pg/ml) of melatonin in the blood, and therefore the presumably low intracellular concentrations of the indolamine [5].

Age-related decline in melatonin secretion may have various consequences, including sleep inefficiency, circadian rhythm dysregulation, reduced antioxidant protection, depressed immune function, and possibly others [33]. Besides aging, there are many age-related diseases that have as their basis, at least in part, free radical damage (Alzheimer's and Parkinson's diseases are examples). Thus, in agreement with other workers [4], we suggest that melatonin's use in disease states and processes where free radical damage is involved should be increased.

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