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Effect of melatonin and tryptophan on humoral immunity in young and old ringdoves (*Streptopelia risoria*)

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ABSTRACT

Melatonin is involved in the regulation of both cellular and humoral immunity. In the present study we have evaluated the effect of the oral administration of melatonin and its precursor, the amino acid tryptophan, on humoral immune response in ringdoves (*Streptopelia risoria*) from different age groups. Male and female ringdoves of 4–5 years of age (young) and 12–14 years of age (old) were used in this study. The animals received a single capsule of 300 mg/kg b.w. of tryptophan (old animals) for 3 consecutive days 1 h after lights on or a single oral dose (0.25 or 2.5 mg/kg body weight/0.1 ml per animal/day, young and old animals, respectively) of melatonin, for 3 consecutive days 1 h before lights off. Blood samples were taken before beginning the treatment (basal values) and at the end of the treatment. Immunoglobulins, bactericidal and haemolytic activity were measured. Our results show that in old animals the humoral immune response was reduced with respect to the young. Both melatonin and tryptophan treatment increased the immunoglobulin concentration, with the nocturnal values being significantly higher than diurnal values and with a major effect in old animals. The bactericidal activity of the *S. risoria* serum against *Staphylococcus aureus*, after the treatment with melatonin or tryptophan, was increased at night with a greater effect in old animals. No significant differences were observed in the haemolytic activity of the serum in young animals, but there was an increase in old animals, with higher values at night after treatment with melatonin. In general, the oral administration of melatonin or tryptophan produced a stimulation of humoral immune response with greater effects in old ringdoves.

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1. Introduction

The mechanisms employed by the immune system for defending the organism against harmful microorganisms and foreign molecules are coordinated by the environmental 24-h light/dark period in order to achieve optimal efficacy (Skwarlo-Sonta, 2002). Aging is associated with a decline in the immune function (immunosenescence), a situation known to correlate with increased incidence of cancer, infectious and degenerative diseases (Fontana, 2009). Some immunological parameters are commonly significantly reduced in the elderly. Innate, cellular and humoral immunity all exhibit increased deterioration with age (Sansoni et al., 2008). Aging is also associated with a number of changes in the morphology, physiology and biochemistry of a variety of organs including the pineal gland. Alterations in this organ results in a significant reduction of the nocturnal synthesis of its main secretory product melatonin (Reiter et al., 1980, 1981; Pandi-Perumal et al., 2002).

A link between pineal melatonin and immune responses has been established in birds (Moore and Siopes, 2000; Moore et al., 2002; Terrón et al., 2005; Singh and Haldar, 2005). Although there are some contrary reports in the literature, most have established an immune enhancing effect of melatonin, especially under conditions when system is compromised. Removal of the primary source of melatonin due to pinealectomy reduces immune responses in the ring dove (Rodríguez and Lea, 1994) and Japanese quail (Moore and Siopes, 2002), suggesting that immune functions are at least partially regulated by physiological levels of melatonin. Moreover, the treatment of these species with exogenous melatonin restores immune function (Rodríguez et al., 2001; Moore and Siopes, 2002, 2003; Terrón et al., 2003, 2004; Paredes et al., 2007a; Siopes and Underwood, 2008).

Circulating melatonin decreases with age and in recent years much interest has been focused on the immunomodulatory consequences of this reduction (Olakowska et al., 2005). Melatonin is a natural antioxidant with significant anti-aging properties (Reiter et al., 2008). It is a direct free-radical scavenger, an indirect antioxidant via stimulation of antioxidative enzymes and an immuno-

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modulatory agent. Because enhanced oxidative stress plays a crucial role in the aging process and chronic diseases associated with senescence, the administration of a potent amphiphilic antioxidant agent with high bioavailability such as melatonin may become a promising, safe and effective intervention strategy to slow some processes of aging and the initiation and progression of age-related disorders (Poeggeler, 2005).

Melatonin has significant immunomodulatory actions in immune compromised states. Maestroni et al. (1986) was the first to show that inhibition of melatonin synthesis causes a reduction of cellular and humoral responses in mice. Numerous in vivo and in vitro studies have documented that melatonin enhances both natural and acquired immunity in animals (Carrillo-Vico et al., 2005). In our animal model (*Streptopelia risoria*), both the indolamine melatonin and its amino acid precursor, tryptophan, influence activity–rest rhythms, modulate circulating levels of melatonin and serotonin, increase cell viability and resistance to induced oxidative stress in blood heterophils, and likewise enhance the phagocytic function and neutralize superoxide anion radicals generated by heterophils (Paredes et al., 2007b). Also, in the old birds, treatment with melatonin or tryptophan at concentrations and times of administration considered suitable improved nocturnal rest and reversed the immunosuppressive and oxidative effects accompanying phagocytosis at these advanced ages (Terrón et al., 2002; Paredes et al., 2007a,c,d).

Herein, we compared whether exogenous melatonin or tryptophan treatments would have equivalent immune enhancing actions in young and old ring doves.

2. Materials and methods

2.1. Animals

Isolated, male and female ringdoves (*S. risoria*) of 4–5 years of age (young) or 12–14 years of age (old) were used in this study ($n = 10$ per age group). The birds were bred in our department, maintained on food and water ad libitum in cages measuring $40 \times 40 \times 45$ cm and exposed to 12 h light and 12 h darkness (darkness from 20:00 to 08:00 h) per day. The temperature was maintained at 22 ± 2 °C (70% humidity).

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.

2.2. Melatonin or tryptophan treatments

Young animals were treated for 3 consecutive days with a single daily oral dose (0.25 mg/0.1 ml phosphate-buffered saline [PBS] per animal) of melatonin (Sigma, St. Louis, MO, USA) at 19:00 h (1 h before the onset of the dark period). Old animals were treated for 3 consecutive days with either a single daily oral dose (2.5 mg/0.1 ml PBS per animal) of melatonin 1 h before lights off or a single oral capsule of 300 mg/kg of body weight (b.w.) of L-tryptophan (Sigma, St. Louis, MO, USA) at 09:00 h (1 h after the onset of the light period). The choice of these concentrations and of the time of administration, were based on previous studies, where it was observed that they restored the amplitude of the serum melatonin rhythm in old doves to that of the young birds, also improving their nocturnal rest, enhanced phagocytosis and free-radical scavenging in both groups of age as well as correcting age-related altered secretion of cytokines and thermoregulatory responses (Paredes et al., 2007c,d,e, 2009). Control animals received only capsules containing the excipient – methylcellulose (Sigma, St. Louis, MO, USA) – with the same schedule as for the tryptophan-treated animals or 0.1 ml of PBS with the same schedule as in the melatonin-treated animals. Basal values were obtained before treatment from animals that had not been given either methylcellulose, tryptophan or melatonin. As no significant variations were observed in these values with respect to the controls, they are not reported herein.

2.3. Serum collection

Blood samples were drawn from all ringdoves at the melatonin acrophase (times at which the variable reached their maxima) and nadirs (times at which the variable reach their minima) allowing at least 1 week between consecutive extractions. The acrophases of the melatonin rhythm in the basal groups were established at 02:00 h and 01:00 h, and nadirs at 14:00 h and 13:00 h, in young and old ringdoves, respectively (Paredes et al., 2006). In the melatonin treatment birds, the acrophase and nadirs were, respectively, established at 02:30 h and 14:30 h and 03:00 h and 15:00 h in young and old animals (Paredes et al., 2007d). In the tryptophan-treated animals, the acrophase and nadir were established at 03:30 h and 15:30 h, respectively (Paredes et al., 2007e). Blood was extracted (1 ml per animal per week) using a 25-gauge needle and a 1 ml syringe from the brachial vein and transferred unheparinized to a pre-prepared tube containing serum-separating gel. The samples were centrifuged at room temperature for 30 min at 300*g*. The serum was then divided into aliquots in Eppendorf vials, and kept frozen at −30 °C until the time of assay. Nocturnal collections were performed under dim red light which is perceived as darkness. The extractions were performed before beginning the treatment (basal values) and after the 3 days of treatment.

2.4. Bacteria

The bacteria used were *Staphylococcus aureus* (ATCC 9144) obtained from the Department of Microbiology, Faculty of Medicine, Badajoz.

2.5. Serum immunoglobulin determinations

Serum immunoglobulin (Ig) levels (isotypes A, M, and G) were measured following administration of a 0.1 ml intravenous injection of sheep erythrocytes suspension (Akbulut et al., 2001) into the brachial vein at the end of the study period and before the last melatonin injection or tryptophan capsule, in order to evoke humoral immune responses; the birds in the control groups were given 0.1 ml of phosphate buffered saline (PBS). Total serum immunoglobulin were measured with a chicken serum IgA, IgG or IgM enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX, USA) following the manufacturer's protocol.

2.6. Serum bactericidal activity

The technique used was previously described by Barriga et al. (1994). The serum was diluted in Mueller Hinton Broth (MHB) to a titre of 1:16 with a final tube volume of 100 µl. Then, 10 µl of 1×10^5 CFU (Colony-forming units) of *S. aureus* per ml were added. In addition, there was a tube with MHB and bacteria inoculum but without serum. The content of the tubes was seeded onto Mueller–Hinton agar plates and incubated at 37 °C, 100% humidity, and 5% CO_2. After 24 h, CFUs were determined and results expressed as a growth percentage (CFU%) by means of the expression:

$$\mathrm{CFU}(\%) = \frac{\text{CFU samples with serum}}{\text{CFU samples without serum}} \times 100 \qquad (1)$$

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