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ORIGINAL ARTICLE

A cherry nutraceutical modulates melatonin, serotonin, corticosterone, and total antioxidant capacity levels: effect on ageing and chronotype

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Summary
Impaired daily rhythms in vertebrate physiology occur with age. Particularly, age-related changes in melatonin and serotonin rhythms and hypercortisolemia have been reported to be linked to age-related disorders. This study was aimed at assessing the effect of a Jerte Valley cherry-based nutraceutical product (patent no ES 2342141 B1), which contains high levels of tryptophan, serotonin, and melatonin, on the serum melatonin, serotonin, corticosterone, and total antioxidant capacity (TAC) levels in young and old ring doves (*Streptopelia risoria*) and rats (*Rattus norvegicus*) as representatives of animals with diurnal and nocturnal habits, respectively. The animals consumed the cherry product for 10 days. Serum melatonin, serotonin, corticosterone, and TAC were measured with commercial ELISA kits. The consumption of the cherry product induced a significant increase in the circulating levels of melatonin and serotonin, as well as in the serum TAC and a significant decrease in the circulating levels of corticosterone in both species and groups of age as compared to their respective values in the control groups. The consumption of a Jerte Valley cherry-based nutraceutical product may help to counteract the decrease in melatonin and serotonin and the increase in oxidative stress, suggesting a potential health benefit especially in aged populations where these parameters have been found to be altered.

Key words: cherry; corticosterone; melatonin; serotonin; total antioxidant capacity

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INTRODUCTION

Serotonin is a neurotransmitter involved in many functions throughout the brain. It participates in the synchronization of the circadian clock located in the suprachiasmatic nucleus, and in the regulation of the sleep/wake cycle, as well as exerting a fundamental role in the biosynthetic pathway of melatonin, a pineal indole that also carries out regulatory functions on the sleep-wake rhythm (Zhdanova et al. 2001, Berger 2008, Paredes et al. 2009a). In addition,

melatonin is a potent free radical scavenger and antioxidant (Paredes et al. 2007a, Reiter et al. 2008a, 2010) that not only scavenges especially highly toxic hydroxyl radicals, but also performs indirect antioxidant actions via its ability to stimulate antioxidant enzymes (Gitto et al. 2001, Paredes et al. 2009b), diminishing free radical formation at the mitochondrial level by reducing the leakage of electrons from the electron transport chain (Reiter et al. 2008b). Moreover, melatonin is a lipophilic-hydrophilic molecule that diffuses widely into cellular compartments, thus providing on-site protection against free radical-mediated damage to biomolecules (Reiter et al. 2008b, Paredes and Reiter 2010).

The production of melatonin wanes with increasing age leading some to speculate that its loss contributes to the ageing process (Reiter et al. 2002, 2008a). This phenomenon seems to be universal and includes birds and mammals. It has been suggested that the age-related melatonin decrease could be produced by serotonin deficiencies. In fact, the synthesis, metabolism, and circulating levels of this neurotransmitter are strongly reduced in old ring doves (Garau et al. 2006, Paredes et al. 2006, 2007b). In mammals, age related reductions in the binding of serotonin receptors have been found in the brain of humans (Wang et al. 1995, Rosier et al. 1996) and, importantly, in the suprachiasmatic nucleus of rodents (Duncan et al. 2000).

The loss of melatonin in advanced age leads to disturbances in the circadian pacemarker, which causes internal temporal desynchronization, inducing a variety of chronopathologies, and leads to a generalized deterioration of health (Paredes and Reiter 2010, Reiter et al. 2010). The intake of tryptophan, the precursor of both serotonin and melatonin, appears to reverse, at least in part, some of the effects of ageing on the circadian impairment, as it increases the availability of brain tryptophan and consequently the brain and blood serotonin and melatonin levels in mammals and birds, as it is the case of ringdoves and rats (Fernstrom and Wurtman 1971, Esteban et al. 2004, Garau et al. 2006, Paredes et al. 2009a, b).

We recently reported high levels of tryptophan (Cubero et al. 2010), serotonin, and/or melatonin (González-Gómez et al. 2009) in Jerte Valley cherries, and that the consumption of fresh cherries had positive effects on nocturnal rest as well as elevating the levels of 6-sulfatoxymelatonin and antioxidants in the urine of middle-aged and elderly subjects (Garrido et al. 2010a). Hence, the aim of the present work was to evaluate whether the consumption of a Jerte Valley cherry-based nutraceutical product (patent no ES 2342141 B1) which contains high levels of tryptophan, serotonin, and melatonin may restore the age-related changes in melatonin and serotonin rhythms and improve the serum antioxidant status in ring doves *(Streptopelia risoria*) and rats (*Rattus norvegicus*) as representatives of animals with diurnal and nocturnal habits, respectively. Since the basal secretion of corticosterone (and cortisol) increases with ageing, the effect of the Jerte cherry-based nutraceutical on circulating corticosterone levels was also evaluated.

MATERIALS AND METHODS

Animals

Male Wistar rats (*Rattus norvegicus*) aged 6–7 months (young) and 18–20 months (old) (n=16 per age group), and male and female ring doves (*Streptopelia risoria*) of 4–5 years of age (young) and 12–14 years of age (old) (n=16 per age group) were individually housed under controlled environmental conditions (20±2 °C; 70% humidity), maintained under a 12/12 h light/dark photoperiod (darkness from 20:00 to 08:00 h) and fed *ad libitum*. All handling during lights-off was done under dim red light (<2 lux).

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, and the European Community's Council Directives (86/609/EEC).

Animal treatment

Each age group was divided into two subgroups: Control and treated. Control animals consumed tap water *ad libitum*. In the treated animals, tap water was replaced with a 27.85 g powdered freeze-dried nutraceutical product mix (patent no ES 2342141 B1) diluted in 250 ml of water. The product mix was freshly prepared every day and consisted of 18.85 g pitted freeze-dried cherries (equivalent to 141 g fresh cherries) in equal parts of 4 Jerte Valley cherry cultivars (Bourlat, Navalinda, Pico Negro, and Pico Colorado), plus 7.5 g maltodextrin, and 1.5 g ascorbic acid (Garrido et al. 2009). The treatment with the nutraceutical product mix was administered for 10 consecutive days.

Serum collection

Blood samples were drawn from all animals (control and treated) at the end of the treatment (day 10). The extractions (1 ml) were done by syringe from the lateral tail vein (rats) or the brachial vein (birds) and

then 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. The extractions were performed one hour after lights on for the determination of serum corticosterone and total antioxidant capacity, one hour before lights off for the determination of serum serotonin, and at the acrophases of the melatonin rhythm in each species and group of age, as previously reported (Mateos et al. 2009, Paredes et al. 2007c, 2009a). At least one week was allowed between consecutive extractions until all selected points of serum collection had been covered for each animal.

Measurement of corticosterone, melatonin, and serotonin in serum

Serum corticosterone, melatonin, and serotonin levels were determined by means of commercial ELISA kits (IBL, Hamburg, Germany), according to the manufacturer's instructions. Determinations were made in duplicate. Results are expressed in ng/ml for corticosterone and serotonin, and in pg/ml in the case of melatonin.

Measurement of antioxidant capacity in serum

Total antioxidant capacity was evaluated by means of a colorimetric assay kit (Cayman, MI, USA), according to the manufacturer's instructions. This assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS® (2,2'-azino-di-[ethylbenzthiazoline sulfonate]) to $ABTS^{®\bullet+}$ by metmyoglobin. The capacity of the antioxidants in the sample to prevent ABTS® oxidation was compared with that of Trolox, a water-soluble tocopherol analogue, and quantified as millimolar Trolox equivalents.

Statistical analysis

Each value represents the mean ± S.E.M. (Standard Error of the Mean) of the number of determinations. The results were analysed by using a non-parametric one-way ANOVA followed by Tukey's multiple comparison test. A significance level of $2\alpha=0.05$ was used. All analyses were performed using GraphPad Prism (version 5.0, 2007; GraphPad Software, Inc; San Diego, CA).

RESULTS

The serum melatonin levels at acrophases in young and old rats and ringdoves in control conditions and after the administration of a 10-day cherry-based nutraceutical product treatment are shown in Figs 1A and 1B, respectively. In both species, the melatonin levels in the control young animals were significantly higher than in the control old animals. Cherry product consumption increased significantly the circulating levels of melatonin in both species and age groups, as compared with the results obtained in their respective control groups. However, the levels of the indoleamine reached in both old rodents and birds after the intake of the product were still significantly lower than those obtained in the young treated animals.

The serum serotonin concentrations reached 1-hr before lights off in control conditions and after the administration of a 10-day cherry-based nutraceutical product treatment are shown in Fig. 2 for both rats (Fig. 2A) and ringdoves (Fig. 2B). Again in both species, the serotonin levels in the control young animals were significantly higher than in the control old animals. Cherry product administration significantly increased the circulating levels of serotonin in both young and old animals of both species, with the young values being still greater than those quantified in their respective counterparts from the old group.

The serum corticosterone levels measured 1-hr after lights on in young and old rats and ringdoves in control conditions and after the administration of a 10-day cherry-based nutraceutical product treatment are shown in Figs 3A and 3B, respectively. Here, in contrast with the case of melatonin and serotonin, the values of this hormone in the control conditions were higher in old animals than in young individuals, although the difference was not significant for rats. A 10-day treatment with the nutraceutical product from Jerte Valley cherries induced a significant decrease in the circulating corticosterone levels in both young and old rats and ringdoves as compared to their respective values in the control conditions, with the levels of the old animals being, however, significantly greater than in the young individuals.

The variations in the total antioxidant capacity (quantified as millimolar Trolox equivalents) from the serum collected 1-hr after lights on are shown in Fig. 4. In the control conditions, no significant changes were observed in total antioxidant capacity levels between the age groups in both rats and ringdoves. However, in relation to the values obtained after cherry product consumption, a significant rise was found in both species and age groups with respect to the control values, with the levels of the young animals being higher than those measured in the old groups (Figs 4A and 4B).

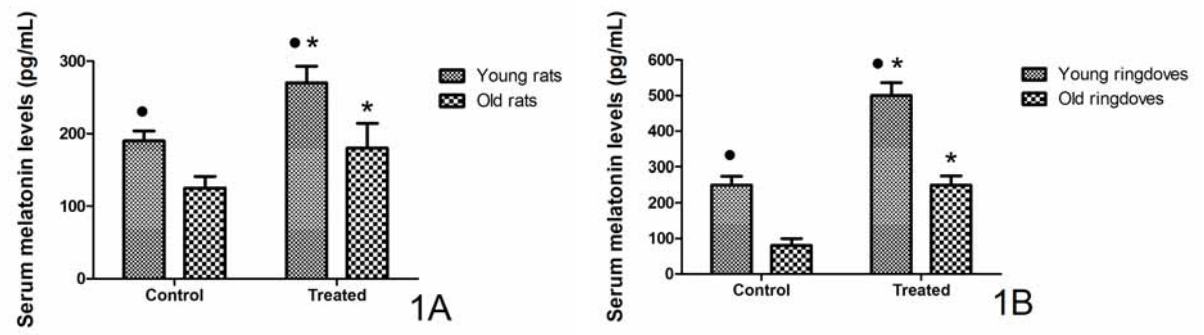

Fig. 1. **Serum levels of melatonin at their corresponding hours of acrophase in control conditions and after 10 days of treatment with a nutraceutical product based on Jerte Valley cherries to young and old rats** (Fig. 1A) **and ringdoves** (Fig. 1B). Each value represents the mean ± S.E.M. (Standard Error of the Mean) of 10 determinations performed in duplicate.
* Statistically significant as compared with their corresponding values in the control group.
• Statistically significant as compared with their corresponding values in the old animals.

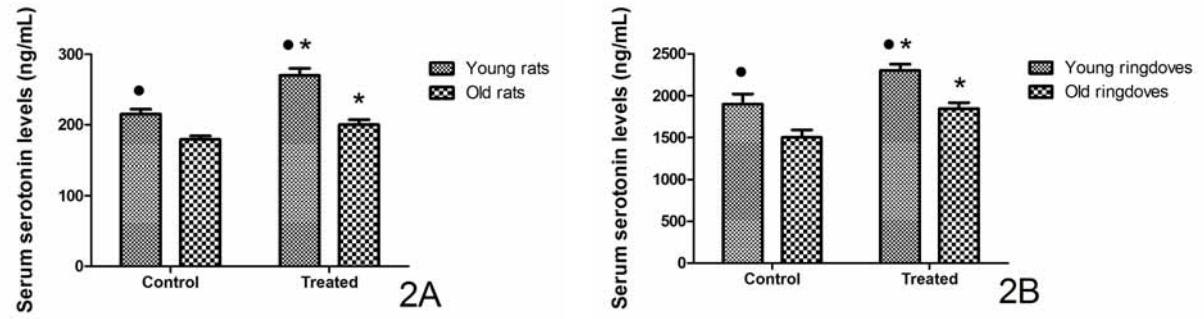

Fig 2. **Serum levels of serotonin obtained 1-hr before lights off in control conditions and after a 10-day administration with a nutraceutical product based on Jerte Valley cherries to young and old rats** (Fig. 2A) **and ringdoves** (Fig. 2B). Each value represents the mean ± S.E.M. (Standard Error of the Mean) of 10 determinations performed in duplicate.
Symbols as in Fig. 1.

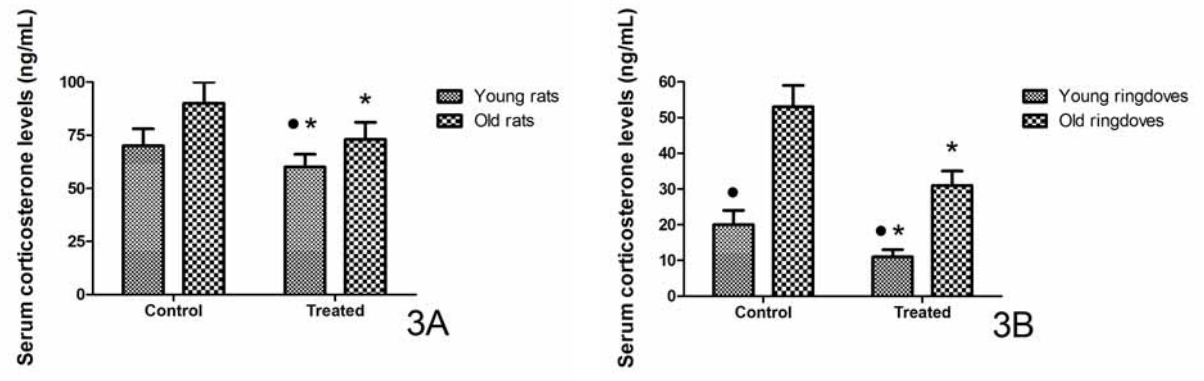

Fig. 3. **Serum levels of corticosterone obtained 1-hr after lights on in control conditions and after 10 days of treatment with a nutraceutical product based on Jerte Valley cherries to young and old rats** (Fig. 3A) **and ringdoves** (Fig. 3B). Each value represents the mean ± S.E.M. (Standard Error of the Mean) of 10 determinations performed in duplicate.
Symbols as in Fig. 1.

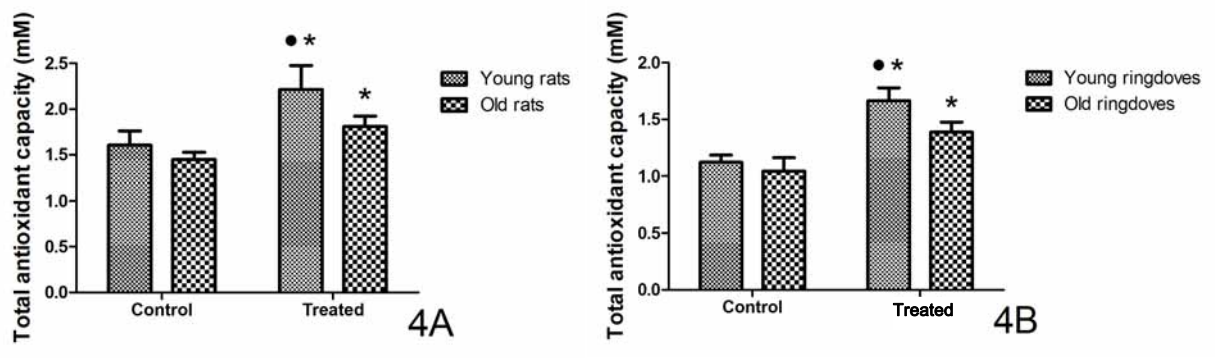

Fig. 4. **Serum levels of total antioxidant capacity (mM) measured 1-hr after lights on in control conditions and after a 10-day administration of a nutraceutical product based on Jerte Valley cherries to young and old rats** (Fig. 4A) **and ringdoves** (Fig. 4B). Each value represents the mean ± S.E.M. (Standard Error of the Mean) of 10 determinations performed in duplicate.
Symbols as in Fig. 1.

DISCUSSION

In the present work, it was observed that the consumption of the cherry product induced a significant increase in the circulating levels of melatonin and serotonin, as well as in the serum TAC, and a significant decrease in the circulating levels of corticosterone in both species and age groups as compared to their respective values in the control groups.

Older individuals appear to be more prone to internal desynchronization than younger subjects, suggesting a weakening of the internal coupling among various rhythms. Among the rhythms that appear to change considerably with age are the rhythms in the production and secretion of serotonin, melatonin and cortisol (Zisapel et al. 2005). In previous studies we have noted a significant decline in circulating melatonin levels in old ringdoves compared with the concentration observed in both mature and young animals (Terrón et al. 2002, 2004) as well as a significant decline in the amplitude and mean levels of melatonin (Paredes et al. 2006). The amplitude of the rhythm of serum serotonin was also much reduced in the old relative to the young birds (Paredes et al. 2006). Other workers have reported the absence of a circadian rhythm in brain serotonin synthesis and metabolism in old ringdoves (Garau et al. 2006). Age-related decreases in melatonin levels are also evident in gerbils, hamsters, and rats (Myers and Badia 1995). Here, the age-related decrease in the circulating levels of both melatonin and serotonin in the two animal chronotypes analysed, diurnal and nocturnal was confirmed. Also, old rats presented higher but not significant corticosterone circulating levels than their respective values found in the young group. Previous studies have shown no significant differences between the plasma corticosterone levels of young and aged rats (Bodnoff et al. 1995, Bowman et al. 2006, Garrido et al. 2010b), although basal increased brain levels of the hormone due to ageing have been reported (Garrido et al. 2010b). In humans, a basal secretion of cortisol seems to occur (Van Cauter et al. 2000). This may be related to the increased basal corticosterone levels found in control old ringdoves.

The relationship between diet and health has led to intense research into bioactive compounds in foods. As for cherries, several studies indicate that the consumption of these fruits is health promoting, particularly in reducing the effects of some diseases (Kang et al. 2003, Kim et al. 2005, Kelley et al. 2006). Previously, we reported that the intake of the cherry-based nutraceutical product tested in the present work exerted beneficial physiological effects in humans (Garrido et al. 2009). Here, we showed that the intake of this nutraceutical increased significantly the circulating levels of melatonin and serotonin in rats and ringdoves, species of nocturnal and diurnal habits respectively. A significant rise was observed in both young and old animals as compared to the values obtained in the control groups. Although there is only a modicum of evidence indicating what ingredients may be responsible for the alleged beneficial properties, the recent discovery of melatonin (González-Gómez et al. 2009, Paredes et

al. 2009c), serotonin, or tryptophan (González-Gómez et al. 2009, Cubero et al. 2010) in Jerte Valley cherries points to the fact that these molecules may be involved in the afore-mentioned restoration of the circulating levels of serotonin and melatonin. In fact, the consumption of this fruit has been shown to improve sleep as well as increasing TAC and 6-sulfatoxymelatonin levels, a metabolite that is considered to reflect the nocturnal melatonin concentration, in first-void urines of middle-aged and elderly humans (Garrido et al. 2010a).

Similar findings have been reported by other workers, where associations have been established between the consumption of vegetables that are high in melatonin content and elevated melatonin in both blood and urine (Nagata et al. 2005, Reiter et al. 2005). In particular, Reiter et al. (2005) showed that the consumption of walnuts, which are rich in melatonin, provoked a threefold increase in circulating melatonin levels and also improved serum antioxidant capacity measured in trolox equivalents. This is consistent with the results shown in the present study. In fact, both species and age groups experienced a rise in the levels of serum TAC after cherry treatment, the effect being greater in the young individuals. Heretofore it has been shown that in birds and mammals, including humans, fluctuations in blood melatonin concentrations strongly correlate with the ability of the blood to detoxify toxic free radicals and related reactants (Paredes et al. 2007a, b, Terrón et al. 2005, 2009). In addition, orally administered tryptophan (and melatonin) also enhances the phagocytic response and detoxification of superoxide anion radicals derived from this immune function in both species, as has been documented elsewhere (Paredes et al. 2007d, 2009a, b, Sánchez et al. 2004, 2008a). This has also been linked to the rise in the circulating levels of melatonin induced by both the indoleamine and its precursor (Paredes et al. 2007c, e, Sánchez et al. 2008b). However, cherries contain other important antioxidants, e.g., anthocyanins and polyphenols that are also absorbed from the gut and influence the total antioxidant capacity of serum (Garrido et al. 2009, 2010a).

That tryptophan, serotonin, and melatonin may be involved in the observed increase in the circulating levels of serotonin, melatonin and serum TAC observed in both rats and ringdoves, is reinforced by previous reports showing that dietary-rich tryptophan supplementation increases the brain and blood levels of serotonin, as well as modulating the circulating levels of melatonin (Garau et al. 2006, Paredes et al. 2007e). The increase of serum serotonin observed after the 10-day consumption of the cherry-based nutraceutical product would indicate a higher availability of tryptophan which, after passing through the blood-brain barrier, would be converted into serotonin, thus increasing the production of this neurotransmitter in the brain as has been observed in the ringdove (Garau et al. 2006) and other animal species (Fernstrom and Wurtman 1971, Huether et al. 1993, Esteban et al. 2004). This serotonin in the brain would be the substrate for melatonin synthesis, increasing the circulating levels of this neurohormone. It has been shown, using *in vivo* microdialysis and voltammetry, that an elevated dietary intake of tryptophan results in an increased functional release of serotonin also in mammals (Boadle-Biber 1993).

Treatment with the cherry product also provoked a decrease in the circulating levels of corticosterone in young and old rats and ringdoves. Again, melatonin contained in the product or its precursors, once converted into the indoleamine after being assimilated by the organism, may be involved in this effect. In fact, melatonin treatment has been repeatedly reported to decrease blood corticosterone levels in mammals and birds (Saito et al. 2005, Zisapel et al. 2005, Detanico et al. 2009).

In summary, the consumption of the Jerte Valley cherry-based nutraceutical product induced a restorative effect in the circulating levels of melatonin and serotonin in old rats and ringdoves, animals with nocturnal and diurnal habits, respectively. It also enhanced the blood levels of the neuroindole and the neurotransmitter in the young individuals as well as decreasing the circulating levels of corticosterone in both species and age groups as compared to their respective values in the control groups. The increase in melatonin levels correlated with an increased blood antioxidative capacity as reflected by augmentation of the trolox equivalent antioxidant capacity of serum values. These results suggest that the consumption of the cherry nutraceutical may help to counteract the decrease in melatonin and serotonin and the increase in oxidative stress that normally occurs in aged animals. In fact, the close relationship between age-related disorders, circadian disruption as well as oxidative stress suggests that the intake of the cherry nutraceutical may be viewed as a beneficial tool to improve human and animal physiology.

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