

ORIGINAL ARTICLE

Jerte Valley cherry-based product modulates serum inflammatory markers in rats and ringdoves

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Summary

Ageing is commonly accompanied by a chronic subclinical inflammatory status that coexists with immune dysfunction. Consumption of foods rich in antioxidants is associated with a lower incidence of chronic diseases. The aim of this study was to evaluate the effect of the consumption of a Jerte Valley cherry-based beverage on the inflammatory load in two different animal species: rats and ringdoves (*Streptopelia risoria*); each divided into two age groups. To this purpose, circulating levels of both pro-inflammatory (IL-1 β and TNF- α) and anti-inflammatory (IL-4 and IL-2) cytokines were measured before and after a 10-day treatment with the Jerte Valley cherry-based beverage. Our results suggest that the 10-day treatment with the Jerte Valley cherry-based beverage modulated the balance of pro- and anti-inflammatory cytokines in both rats and ringdoves by down-regulating the levels of pro-inflammatory (IL-1 β and TNF- α) cytokines and up-regulating the levels of anti-inflammatory (IL-4 and IL-2) cytokines. Moreover, old animals showed imbalanced levels of inflammatory markers towards a pro-inflammatory status, thereby underlining the fact that ageing is usually accompanied by systemic inflammation and inflammation-related chronic diseases. In conclusion, since an increased dietary intake of vegetable-derived bioactive compounds may retard age-related immune dysfunctions and prolong life-span, supplementing diets with the cherry-based beverage may reduce the inflammatory load by modulating the serum concentrations of some markers of inflammation.

Key words: sweet cherry; inflammation; cytokine; melatonin; rat; ringdove

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INTRODUCTION

Both chronic systemic inflammation and low grade inflammation may contribute to the reduction of the human life span (Finch and Crimmins 2004). Although there is limited information on markers of systemic inflammation in healthy adults, circulating cytokines may act as biomarkers for disease progression (Emery et al. 2008). Thus, it has been reported that there is an association between chronic

inflammation and many of the prevalent diseases found in the developed world, including obesity (Engström et al. 2004), diabetes (Hanley et al. 2004) and cancer (Hofseth and Ying 2006). Moreover, the secretion by lymphocytes of pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor- α (TNF- α) is responsible for initiating inflammation in the pathogenesis of chronic diseases, such as rheumatoid arthritis (Horwood 2008).

The ability of fruits and vegetables to protect against diseases prevalent in the developed world is well documented (Heber 2004). Although the mechanisms of protection are not fully understood, it is known that there are hundreds of potentially beneficial ingredients in plant foods, and there is relatively consistent evidence, in both humans and animals (Heber 2004, Mayorga et al. 2004), that their antioxidant and anti-inflammatory properties play a role in this protection. Cherries are an important source of phytochemicals and reportedly have important health-promoting qualities, including anti-inflammatory effects (McCune et al. 2011). It has been demonstrated that they inhibit the cyclooxygenase (COX) enzymes responsible for the inflammatory response (Seeram et al. 2001), and a pilot study investigating the effects of sweet cherry consumption on inflammatory markers in humans has revealed that cherry consumption can reduce serum C-reactive protein (hsCRP) levels (Kelley et al. 2006).

Although the anti-inflammatory effects of cherries have been attributed to anthocyanins, it has been proposed that the beneficial properties of fruits and vegetables come from the additive and synergistic effects of their phytonutrients and that isolated dietary supplements do not exhibit these same benefits (Milde et al. 2007). In this regard, sweet-cherries from Jerte Valley (Extremadura, Spain) contain not only high concentrations of anthocyanin pigments and other phenolic compounds (González-Gómez et al. 2009), but also substantial amounts of melatonin, serotonin (González-Gómez et al. 2010) and tryptophan (Cubero et al. 2010), as recently reported in seven different cultivars of these fruits: Bourlat, Navalinda, Van, Ambrunés, Pico Limón, Pico Negro, and Pico Colorado.

The amino acid tryptophan, the neurotransmitter serotonin, and the indole melatonin are present in various fruits and vegetables (Paredes et al. 2009a). These bioactive compounds participate in the physiological regulation of sleep as well as in the improvement of antioxidant defences (Paredes et al. 2007a, b, c). Moreover, melatonin possesses both immunomodulatory (Carrillo-Vico et al. 2005a) and anti-inflammatory (Carrillo-Vico et al. 2005b)

properties, and is also a potent free radical scavenger (Tan et al. 1993). In this respect, it has been reported that both a Jerte Valley cherry-enriched diet (Garrido et al. 2010) and the intake of a Jerte Valley cherry-based nutraceutical product (Garrido et al. 2009) exhibit sleep-promoting actions, and increase urinary 6-sulfatoxymelatonin (aMT6-s), a metabolite that is considered to reflect the nocturnal melatonin concentration, as well as antioxidant status in young, middle-aged, and elderly subjects. Taking into account the potential health-promoting actions of Jerte Valley sweet-cherries, the purpose of this work was to evaluate the effect of the consumption of a Jerte Valley cherry-based product on inflammatory load in both rats (a nocturnal animal) and ringdoves (a diurnal animal; *Streptopelia risoria*) from two different age groups: young and old. To this end, circulating levels of both pro-inflammatory (IL-1 β and TNF- α) and anti-inflammatory (IL-4 and IL-2) cytokines were measured before and after a 10-day treatment with the Jerte Valley cherry-based beverage.

MATERIAL AND METHODS

Animals

Male Wistar rats of 6–7 months of age (young) and 18–20 months of age (old, given an average life span of 22–24 months) were used in the study (n=8 per age group). Rats were individually housed under controlled environmental conditions (20 °C; 70% humidity), maintained under a 12/12 h light/dark photoperiod (darkness from 19:00 to 07:00 h) and fed *ad libitum* (food and water). All handling during lights-off was done under dim red light (<2 lux).

Similarly, both male and female ringdoves (*Streptopelia risoria*) of 4–5 years of age (young) and 12–14 years of age (old, given an average life span of 15 years) were used in the study (n=8 per age group) and individually housed under the same conditions as described above.

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

Both young and old animals (rats and birds) were watered with a Jerte Valley cherry-based beverage (Spanish patent no. ES 2342141 B1) for a 10-day period. The beverage was made of 27.85 g powdered

freeze-dried product mix diluted in 250 ml of water. This product mix 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.

The cherry-based beverage was freshly prepared every day. Feeding bottles containing the cherry based beverage were wrapped in tinfoil to avoid oxidization and/or destruction of light sensitive compounds, e.g. melatonin. Basal parameters were obtained from animals that had not been watered with the cherry-based beverage, but with drinking water.

Serum collection

Blood samples were drawn from all rats (n=16) and birds (n=16) at 08:00 h, 18:00 h, and the time corresponding to each group's acrophase of the melatonin rhythm, allowing at least 1 week between consecutive samplings. Based on previous research, the acrophases of the melatonin rhythm (times at which the variables reach their maximums) in basal conditions, were established at 02:00 h and 01:00 h in young and old ringdoves, respectively (Paredes et al. 2006), and at 02:00 h in both groups of rats (Sánchez et al. 2008). The collections (1 ml) were made with a 25-gauge needle and a syringe, taking blood from the lateral tail vein (rats) or brachial vein (birds) and then transferring it 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 aliquotted into Eppendorf vials and kept frozen at -30 °C until assay. Nocturnal collections were performed under dim red light, which the animals perceive as darkness. The extractions were performed before initiating the treatment (basal values) and at the end of the treatment.

Cytokines determination in serum

Serum cytokines (IL-1 β , IL-4, IL-2 and TNF- α) levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions. All ELISA kits were purchased from DRG (Marburg, Germany), except for a rat IL-4 ELISA kit that was acquired from Invitrogen (Barcelona, Spain). Determinations were made in duplicate, and cytokine results are expressed in picograms per millilitre. As aforementioned, cytokine basal levels were blood values from animals studied before treatment.

Statistical analysis

Data are expressed as mean \pm S.E.M. of the numbers of determinations carried out in duplicate. To

compare the different treatments, statistical significance was calculated by one-way analysis of variance followed by a *post hoc* Tukey test. The significance level was set at $\alpha=0.05$.

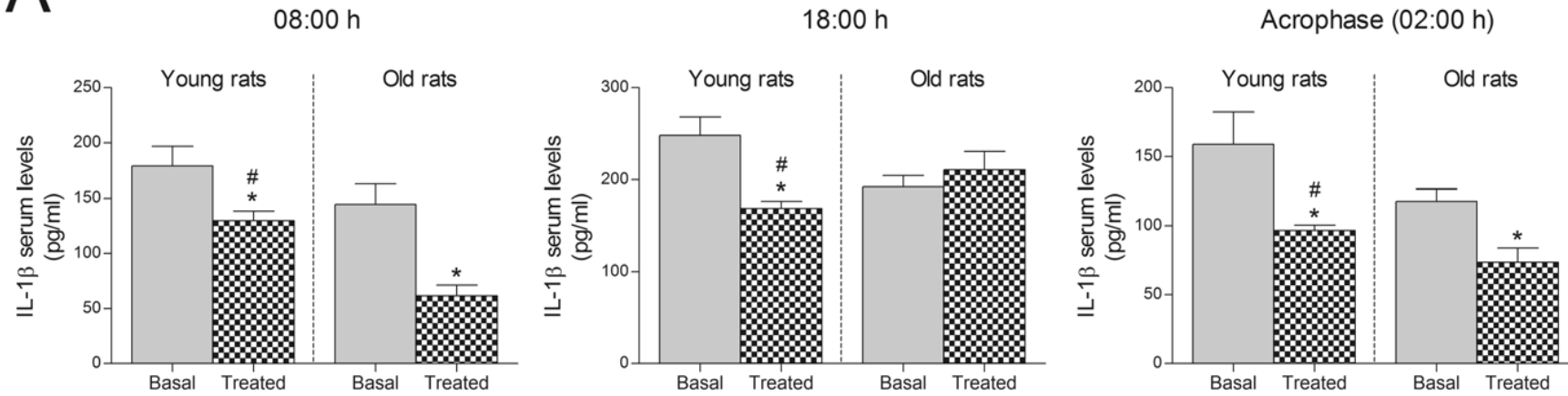
RESULTS

Several pro- and anti-inflammatory cytokines were analysed at three different time points. Thus, serum concentrations of pro-inflammatory IL-1 β in treated young rats (Fig. 1A) were substantially lowered at all hours compared to young rats in basal conditions, whereas, in old rats administered with the cherry-based beverage, serum IL-1 β levels only diminished at dawn (08:00 h) and in the acrophase of the melatonin rhythm (02:00 h). Moreover, old rats showed decreased IL-1 β levels after treatment at dawn (08:00 h) compared to young rats (Fig. 1A). Similar results were obtained in ringdoves. In fact, after the 10-day treatment with the cherry-based beverage, serum IL-1 β levels significantly decreased both at dawn and at 18:00 h in young ringdoves, as well as at 18:00 h and at 01:00 h (acrophase of the melatonin rhythm) in old ringdoves (Fig. 1B). However, in this case, old birds seemed to show substantially higher IL-1 β basal levels at all hours than young birds (Fig. 1B).

Serum levels of TNF- α were also analysed as pro-inflammatory markers. In this regard, the 10-day treatment with the cherry-based beverage only modified the serum levels of TNF- α at dawn (08:00 h) in old rats, since they dropped remarkably after the treatment (Fig. 2A). Old rats showed higher TNF- α basal levels than young rats (Fig. 2A), these differences being statistically significant at 18:00 h and 02:00 h. Likewise, the consumption of the Jerte Valley cherry-based beverage caused a significant decrease in TNF- α serum levels at dawn (08:00 h) in young ringdoves, as well as at 18:00 h and at 01:00 h (acrophase of the melatonin rhythm) in old ringdoves (Fig. 2B). Like old rats, old ringdoves exhibited greater basal levels of TNF- α at the different hours studied than young ringdoves (Fig. 2B).

Serum levels of IL-4 and IL-2 were subsequently measured as anti-inflammatory markers. Interestingly, the 10-day treatment with the Jerte Valley cherry-based beverage led to increased IL-4 concentrations at all hours tested in both young and old rats (Fig. 3A). Moreover, it is worth noting that old rats presented lower IL-4 basal levels than young rats at all hours (Fig. 3A). Similarly, the consumption of the cherry-based beverage largely enhanced the serum levels of IL-4 at all hours tested in both young

A



B

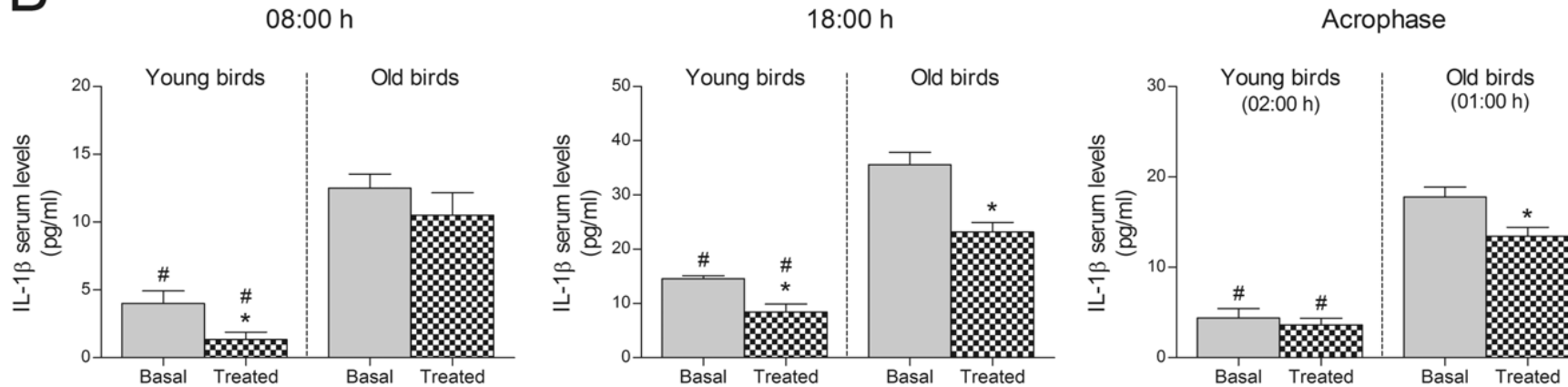


Fig. 1. **Effect of consuming a Jerte Valley cherry-based beverage on circulating concentrations of IL-1 β in rats and ringdoves.** Serum IL-1 β levels in rats (A) and ringdoves (B) before (basal) and after (treated) the consumption of the cherry-based beverage. Samples were analysed at different times, as indicated in Material and methods. Values are expressed as pg/ml, n=8.

* statistically significant regarding basal values, # statistically significant regarding its corresponding value in old animals

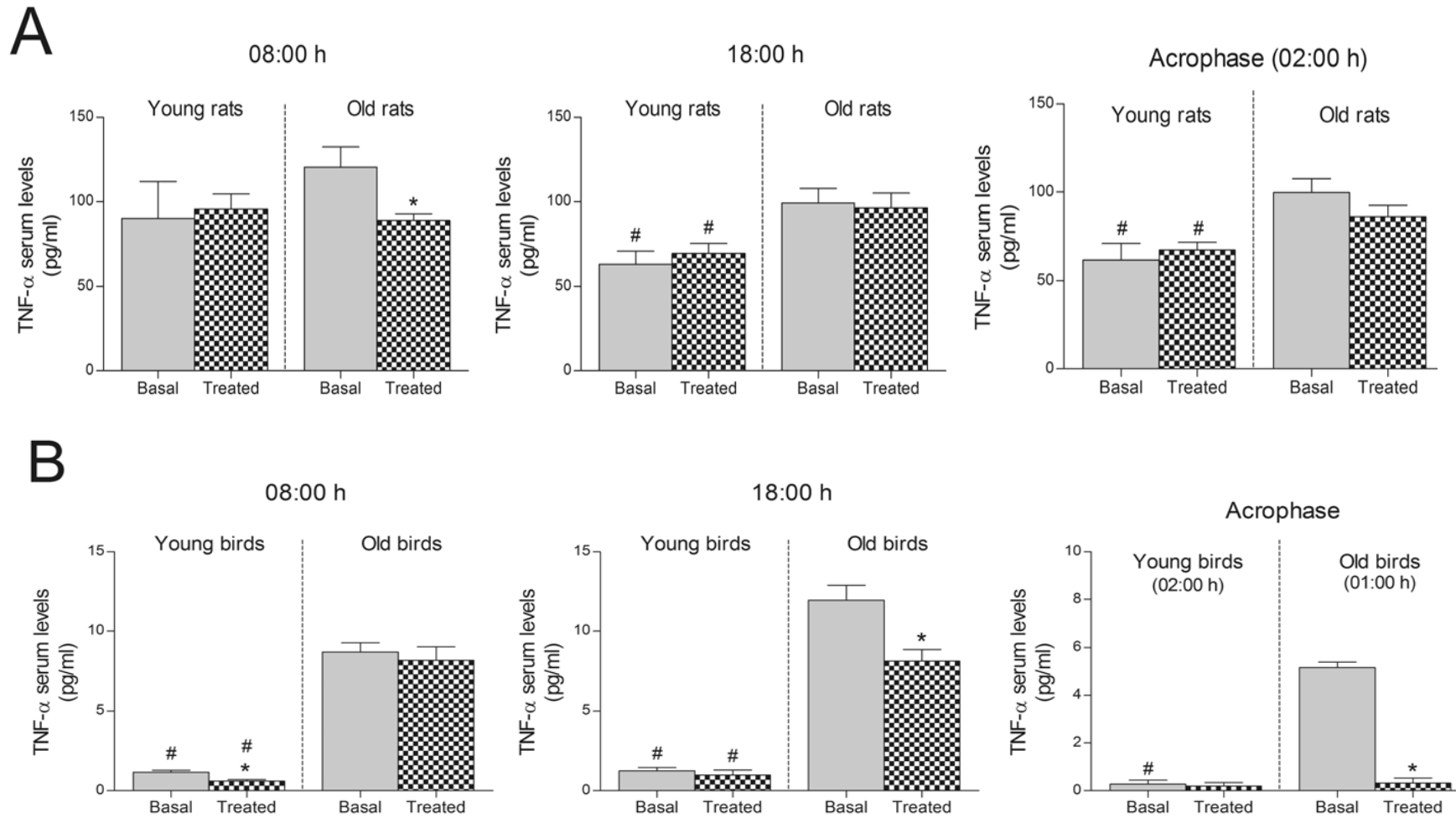
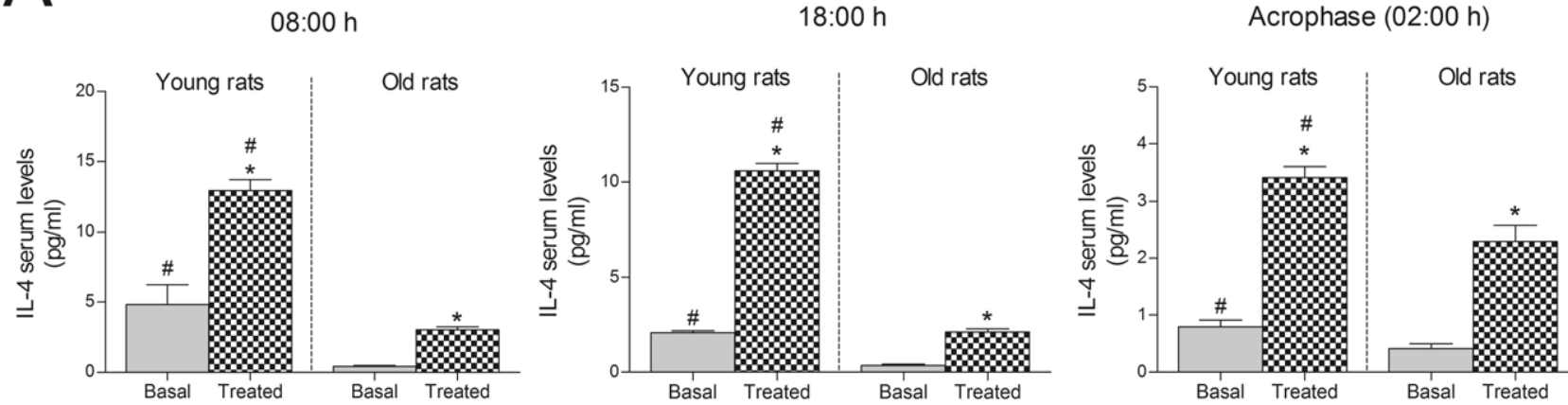


Fig. 2. **TNF- α serum levels after the consumption of a Jerte Valley cherry-based beverage in rats and ringdoves.** Serum TNF- α levels in rats (A) and ringdoves (B) before (basal) and after (treated) the consumption of the cherry-based beverage. Samples were analysed at different times, as indicated in Material and methods. Values are expressed as pg/ml, n=8.

* statistically significant regarding basal values, # statistically significant regarding its corresponding value in old animals

A



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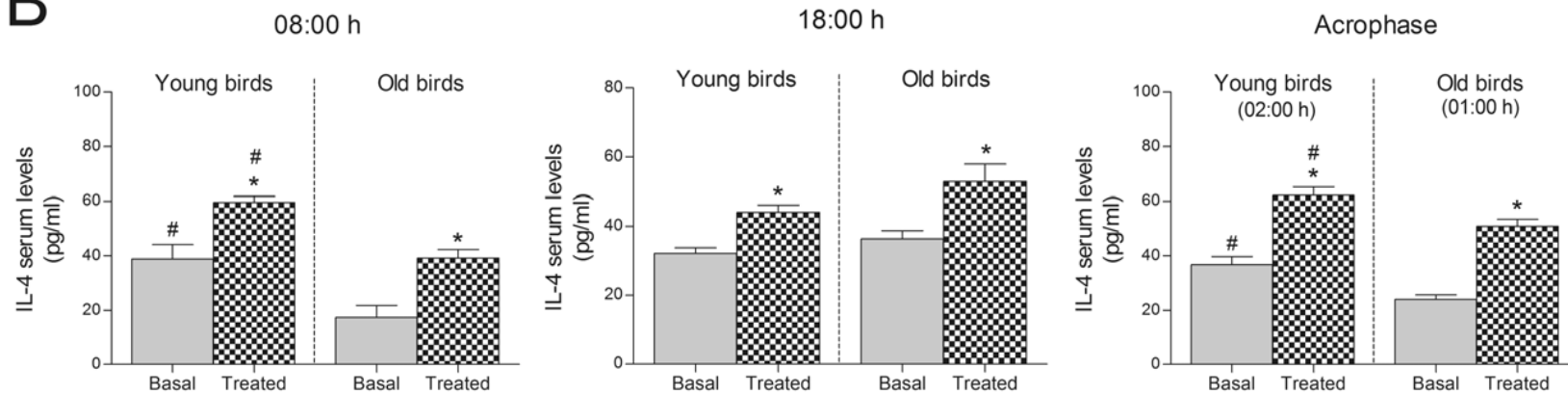


Fig. 3. Serum IL-4 concentrations in rats and ringdoves that consumed a Jerte Valley cherry-based beverage. Serum IL-4 levels in rats (A) and ringdoves (B) before (basal) and after (treated) the consumption of the cherry-based beverage. Samples were analysed at different times, as indicated in Material and methods. Values are expressed as pg/ml, n=8.

* statistically significant regarding basal values, # statistically significant regarding its corresponding value in old animals

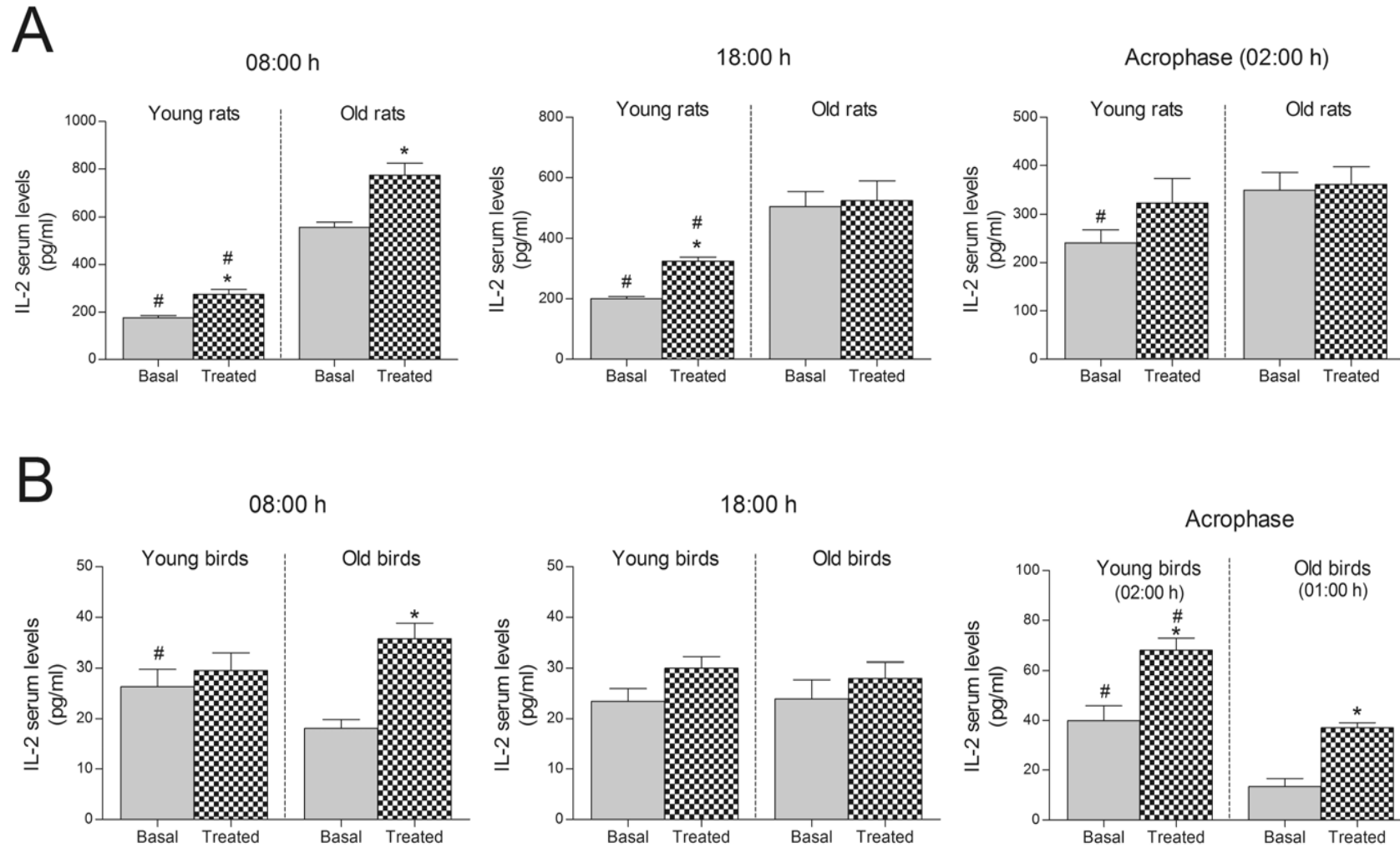


Fig. 4. **Concentrations of IL-2 in rats and ringdoves supplemented with a Jerte Valley cherry-based beverage.** Serum IL-2 levels in rats (A) and ringdoves (B) before (basal) and after (treated) the consumption of the cherry-based beverage. Samples were analyzed at different times, as indicated in Material and methods. Values are expressed as pg/ml, n=8.

* statistically significant regarding basal values, # statistically significant regarding its corresponding value in old animals.

and old ringdoves (Fig. 3B). Like the old rats, old ringdoves showed lower basal levels of IL-4 at dawn and at the time corresponding to the old ringdoves' acrophase of the melatonin rhythm (Fig. 3B).

Finally, serum concentrations of anti-inflammatory IL-2 in treated young rats were clearly raised at dawn (08:00 h) and at 18:00 h compared to young rats in basal conditions, while, in the treated old rats, serum IL-2 levels were only augmented at dawn (Fig. 4A). In this case, old rats exhibited higher IL-2 basal levels at all hours tested than young rats (Fig. 4A). Additionally, the 10-day treatment with the Jerte Valley cherry-based beverage led to increased IL-2 concentrations at dawn (in old ringdoves) and at the time corresponding to each group's acrophase of the melatonin rhythm, i.e. 02:00 h in young ringdoves and 01:00 h in old ringdoves (Fig. 4B). Like basal IL-4, basal levels of IL-2 were much lower in old ringdoves both at dawn and the time corresponding to their acrophase of the melatonin rhythm (Fig. 4B).

DISCUSSION

Consumption of foods rich in antioxidants is associated with a lower incidence of chronic diseases such as cardiovascular disease and cancer (Galassetti and Pontello 2006, González 2006). In this study, we have shown that a 10-day treatment with a Jerte Valley cherry-based beverage modulates the balance of pro- and anti-inflammatory cytokines in both rats and ringdoves by down-regulating the levels of pro-inflammatory (IL-1 β and TNF- α) cytokines and up-regulating the levels of anti-inflammatory (IL-4 and IL-2) cytokines. Our results are consistent with those reported with phenolic compounds in animal models (Tall et al. 2004), preliminary human studies with cherries (Jacob et al. 2003), and *in vitro* studies with phenolic extracts from cherries (Wang et al. 1999). Moreover, the anti-inflammatory effects of cherries have previously been investigated in animal models of arthritis. Thus, He et al. (2006), using male Sprague Dawley mice, showed that animals fed at the highest dose of total cherry anthocyanins (40 mg/kg) exhibited significantly lower TNF- α and prostaglandin E₂ (PGE₂) levels, thereby providing preliminary evidence of the potential role of cherries in reducing the inflammatory response in those with inflammation-related chronic illness.

As cytokine serum levels may vary throughout the day (Paredes et al. 2009b), it is important to note that the time of sample collection may affect the results obtained so that inaccurate conclusions may be drawn. Actually, our findings show that the cherry-based

beverage was able to moderate the inflammatory load of our animal subjects depending on the time at which serum samples were taken. For instance, no changes were observed after the 10-day treatment with the cherry-based beverage in IL-1 β and TNF- α serum levels of old birds at dawn (08:00 h), while a reduction was showed in samples taken at 18:00 h or 01:00 h.

A common finding in the elderly population is a chronic subclinical inflammatory status that coexists with immune dysfunction, and these interconnected processes are of sufficient magnitude to impact health and survival time (Han and Ulevitch 2005). For example, the onset and course of age-associated diseases, such as cardiovascular disease, osteoporosis and arthritis, are influenced by the level of pro-inflammatory cytokines (Gouin et al. 2008). Accordingly, old animals used in this study showed imbalanced levels of inflammatory markers towards a pro-inflammatory status, thereby underlining the fact that ageing is usually accompanied by systemic inflammation and inflammation-related chronic illnesses. Therefore, it is worth noting the possibility that nutritional interventions, like the consumption of the cherry-based beverage, could prevent or delay the functional deterioration of the immune system that accompanies ageing and even, perhaps, return it to that of the "younger" situation, as previously suggested (Dorshkind et al. 2009). In this respect, it has been previously reported that food restriction delays the loss of several cellular immune functions, retards the onset of many diseases during ageing and, consequently, extends significantly both rat and human life-span (Byun et al. 1995, Berger et al. 2005).

Melatonin is known to exhibit immunomodulatory actions that are mainly mediated through the modulation of cytokine production (Carrillo-Vico et al. 2003a) via binding to specific receptors expressed by different immune cells (Carrillo-Vico et al. 2003b). Since Jerte Valley sweet-cherries contain substantial amounts of melatonin (González-Gómez et al. 2010), it is reasonable to assume that the anti-inflammatory properties showed by the Jerte Valley cherry-based beverage in this study may be attributed to melatonin. Nevertheless, as it has been proposed that the beneficial properties of fruits and vegetables come from the additive and synergistic effects of their phytonutrients (Milde et al. 2007), the involvement of other antioxidants, such as phenolic acids, anthocyanins and carotenoids, cannot be ruled out.

Increased dietary intake of vegetable-derived bioactive compounds may retard age-related decrements in immune function and prolong life-span. Therefore, the anti-inflammatory effects of the

cherry-based beverage may be of clinical significance and should be investigated in further studies. In fact, the close relationship between chronic inflammation and poor human health suggests that the Jerte Valley cherry-based beverage is likely to be a beneficial addition to the human healthy diet.

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