

Applied nutritional investigation

# Melatonin in walnuts: Influence on levels of melatonin and total antioxidant capacity of blood

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## Abstract

**Objective:** We investigated whether melatonin is present in walnuts (*Juglans regia* L.) and, if so, tested whether eating walnuts influences melatonin levels and the total antioxidant status of the blood.

**Methods:** Melatonin was extracted from walnuts and quantified by high-performance liquid chromatography. After feeding walnuts to rats, serum melatonin concentrations were measured using a radioimmunoassay and the “total antioxidant power” of the serum was estimated by using the trolox equivalent antioxidant capacity and ferric-reducing ability of serum methods.

**Results:** Mean  $\pm$  standard error melatonin concentrations were  $3.5 \pm 1.0$  ng/g of walnut. After food restriction of rats and then feeding them regular chow or walnuts, blood melatonin concentrations in the animals that ate walnuts were increased over those in the rats fed the control diet. Increases in blood melatonin were also accompanied by increases in trolox equivalent antioxidant capacity and ferric-reducing ability of serum values.

**Conclusions:** Melatonin is present in walnuts and, when eaten, increase blood melatonin concentrations. The increase in blood melatonin levels correlates with an increased antioxidative capacity of this fluid as reflected by augmentation of trolox equivalent antioxidant capacity and ferric-reducing ability of serum values. © 2005 Elsevier Inc. All rights reserved.

## Keywords:

Walnuts; Melatonin; Cardiovascular system; Antioxidant; Trolox equivalent antioxidant capacity; Ferric-reducing ability of serum

## Introduction

Walnuts (*Juglans regia* L.) are receiving increasing interest as a healthy foodstuff because their regular consumption has been reported to decrease the risk of heart disease [1–3]. These findings are sufficiently compelling to the point where the U.S. Food and Drug Administration has affirmed the following health claim for walnuts: “Supportive but not conclusive research shows that eating 1.5 ounces per day of walnuts as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease” (July 14, 2003). The published basic and clinical research on the benefits of walnut consumption on cardiovascular physiology was used to support this claim.

The health benefits of walnuts are usually attributed to their high content of  $\omega$ -3 fatty acids [3–5] and, to a lesser degree, to the amount of vitamin E they contain [6], in particular  $\gamma$ -tocopherol, which promotes the cellular uptake of  $\alpha$ -tocopherol. In addition, walnuts have other components that may be beneficial for health including a low lysine:arginine ratio and high levels of arginine, folate, fiber, tannins, and polyphenols [1,7]. Importantly, walnut consumption does not cause a net gain in body weight when eaten as a replacement food [8].

Several reports that have appeared in the past decade have also identified melatonin, a molecule with beneficial effects on the cardiovascular system [9,10], in edible foods and seeds [11,12]. Melatonin (*N*-acetyl-5-methoxytryptamine) is synthesized and widely distributed in the animal kingdom [13,14], and its presence in plants has prompted investigations into the potential health benefits of consumption of foodstuffs containing this indole. Given the numerous observations regarding the positive effects of walnut consumption on cardiovascular

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pathophysiology [1,2], we anticipated that, in addition to other heart-healthy constituents [4–7], walnuts may contain melatonin. Thus, the goals of the present research were to test whether melatonin is found in walnuts and, if so, to determine whether consumption of walnuts would alter circulating levels of this constituent in rats. Further, because melatonin is an antioxidant, we determined the total antioxidant capacity of blood after rats had eaten walnuts.

## Materials and methods

### Materials

Bulk walnuts (mixed variety) were supplied by the California Walnut Commission. Chromatographically pure melatonin was a gift from the Helsinn Chemical Company (Biasca, Switzerland). Other chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

### Melatonin extraction from walnuts and measurement

Five different 1-g samples of raw walnuts were pulverized with a mortar and pestle by using 15 ml of methanol as the homogenizing solution. The homogenates were centrifuged at 10 000g at 4°C for 30 min. After removing the lipid phase, the supernatant was collected and evaporated under vacuum (20 psi). Residues were redissolved in 1 ml (pH 7.4, 20 mM) of phosphate buffered saline. Two milliliters of chloroform were added to this mixture and horizontally shaken for 2 min. After centrifugation at 1000g for 5 min, the water phase was discarded and the organic phase was evaporated under a vacuum. The residue then was dissolved in 100  $\mu$ L of high-performance liquid chromatography (HPLC) mobile phase for melatonin analysis using HPLC. Before analysis, samples were filtered with a 0.22- $\mu$ m filter under centrifugation at 15 000g for 1 h. Thirty microliters of each sample was injected into the HPLC-ECD system, which consisted of an ESA 580 dual pump, 504 autosampler, and a Coularray 8-channel coulometric array electrochemical detector (ESA, Chelmsford, MA, USA). A C18 reverse-phase column was used to separate melatonin. The mobile phase was constituted with 0.1 mM potassium phosphate buffer (pH 4.5) with acetonitrile (20%) at a flow rate of 1 mL/min. Applied potentials were initiated at 200 mV for channel 1 and was increased by 100 mV for each remaining channel.

### Serum melatonin study and assay

Two-month-old male Sprague-Dawley rats (mean body weight 150–175 g at time of purchase) were purchased from Harlan (Indianapolis, IN, USA). During a 1-wk period of acclimation, animals were kept under a 12-h:12-h light:dark cycle (lights off at 7:00 PM and on at 7:00 AM daily) with an

ambient temperature of  $22 \pm 1^\circ\text{C}$  and 55% humidity. During this interval animals had free access to Purina rodent chow and fresh tap water. Rats were then assigned to one of two groups (control fed and walnut fed) of eight rats each and fasted for 24 h; after the period of fasting (at 9:00 AM), control rats were given access to rodent chow, whereas the remaining eight animals were given walnuts to eat. After 4 h, all animals were killed by decapitation and trunk blood was collected. Serum was separated by centrifugation and stored at  $-80^\circ\text{C}$  until assayed for melatonin.

Serum melatonin was estimated by means of a direct radioimmunoassay [15,16] using the following parameters. A 250- $\mu$ L serum sample was combined with 100  $\mu$ L of antiserum (initial dilution 1:9000; Batch G/S/704-8483; Stockgrand Ltd., Guilford, UK) and 100  $\mu$ L of [ $^3\text{H}$ ]-melatonin (TRK 798 Amersham, Buckinghamshire, UK; approximately 2000 cpm/tube) in an assay buffer (0.1 M tricine buffer, pH 8.0, containing 0.1% gelatin, 0.9% NaCl and 0.1%  $\text{NaN}_3$ ). After an overnight incubation period (18 h) at 4°C, 500  $\mu$ L of chilled dextran-coated charcoal solution was added (0.5 g of charcoal and 0.05 g of dextran in 100 mL of assay buffer). After vortexing and incubation for 15 min at 4°C, tubes were centrifuged at 15 000g for 15 min at 4°C. Thereafter, 750  $\mu$ L of the supernatant was added to 7.5 mL of scintillation fluid (Liquiscini, National Diagnostics, Manville, NJ, USA) and radioactivity in the tubes was counted for 10 min in a beta-counter. Assay sensitivity was better than 2 pg/mL, with intra- and interassay variations of 7.8% and 12.8%, respectively.

### Trolox equivalent antioxidant capacity and ferric-reducing ability of serum studies and assays

Two-month-old male Sprague-Dawley rats were purchased from Harlan. Rats initially had free access to food and water and were maintained under conditions as described above. After a 1-wk acclimation period, animals were assigned to a control group ( $n = 8$ ) and into a walnut-fed group ( $n = 12$ ) and subsequently deprived of food for 24 h. After the period of fasting, animals were provided Purina rodent chow (control fed) or walnuts to eat. After 5 h 30 min, all rats were killed by decapitation and trunk blood was collected. Blood samples were centrifuged and serum was assayed for trolox equivalent antioxidant capacity (TEAC) and ferric-reducing ability of serum (FRAP) values. The TEAC and FRAP assays were performed precisely as described by Re et al. [17] and Benzie and Strain [18], respectively.

### Statistical analyses

Data are expressed as mean  $\pm$  standard error. Unpaired  $t$  test was used to compare differences between control-fed and walnut-fed rats.  $P \leq 0.05$  was considered statistically significant.

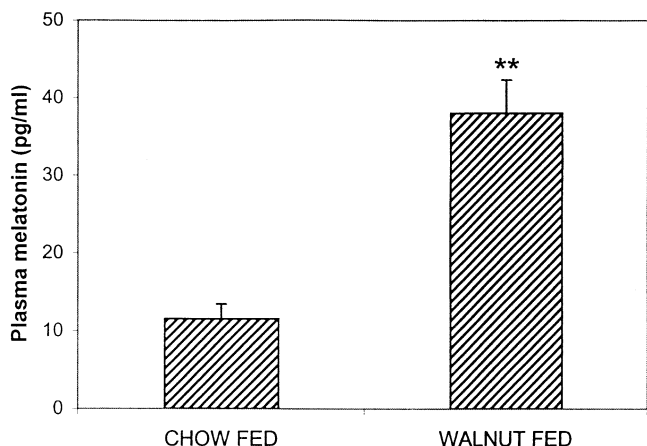


Fig. 1. Serum melatonin levels in blood from rats that were fasted overnight and then given regular rodent chow or walnuts to eat. As shown in this report, walnuts contain melatonin and their consumption is associated with increased circulating levels of melatonin.  $**P < 0.01$ .

## Results

Using the described method of extraction and after correction of the values for the recovery rate (about 40%), the calculated concentrations of melatonin in walnuts was  $3.5 \pm 1.0$  ng/g.

All procedures for the treatment of animals described in this report were approved by the institutional animal care and utilization committee. After fasting for 24 h, feeding of the control diet (rodent chow) or walnuts, and then death, the stomach of each animal was inspected. In both experiments and for all rats, the stomach was filled with food at this time. Judging from the contents of the stomach, it was estimated that each rat had eaten 3.0 to 3.5 g of food during the interval from the initiation of food availability to tissue collection.

After consumption of rodent chow or walnuts, walnut-fed animals had significantly higher blood melatonin levels at the conclusion of the experiment (Fig. 1). The mean value of melatonin in the rodent chow group was  $11.5 \pm 1.9$  pg/mL and that of the walnut group was  $38.0 \pm 4.3$  pg/mL.

As with melatonin levels, consuming walnuts significantly increased the serum TEAC and FRAP values over those of the chow-fed rats (Fig. 2A and 2B, respectively), although the FRAP values appeared to increase more markedly than the TEAC levels after walnut consumption, in both cases  $P < 0.01$ .

## Discussion

Using an HPLC method, melatonin was identified in extracts of walnuts in the present study. The first reports describing melatonin in plant material appeared less than a decade ago [11,19]. Since then, only a few plant products have been analyzed for their melatonin levels, and what has

become apparent is that the concentration of this indoleamine varies widely according to the plant species studied [11,12,20]. The highest concentrations of plant melatonin reported to date exist in Chinese herbal products [20–22]. To our knowledge, the walnut is the first commonly eaten tree nut in which melatonin has been measured.

The function of melatonin in plants is presumably similar to that in animals, where, among a variety of functions, it is a free radical scavenger and antioxidant [23–25]. Its presence in the walnut may be to protect the highly oxidizable lipids from oxidation, thereby preserving the viability of the nut (seed) so it will subsequently successfully germinate. Manchester et al. [26] proposed this function for melatonin in seeds.

Whether melatonin in plant products is synthesized in situ or whether it is taken up from the soil remains unknown. If the latter is the case, it may be possible to increase the melatonin concentration in plants by adding it to the soil in which they grow. However, there is a modicum of evidence that suggests that plants have the enzymatic machin-

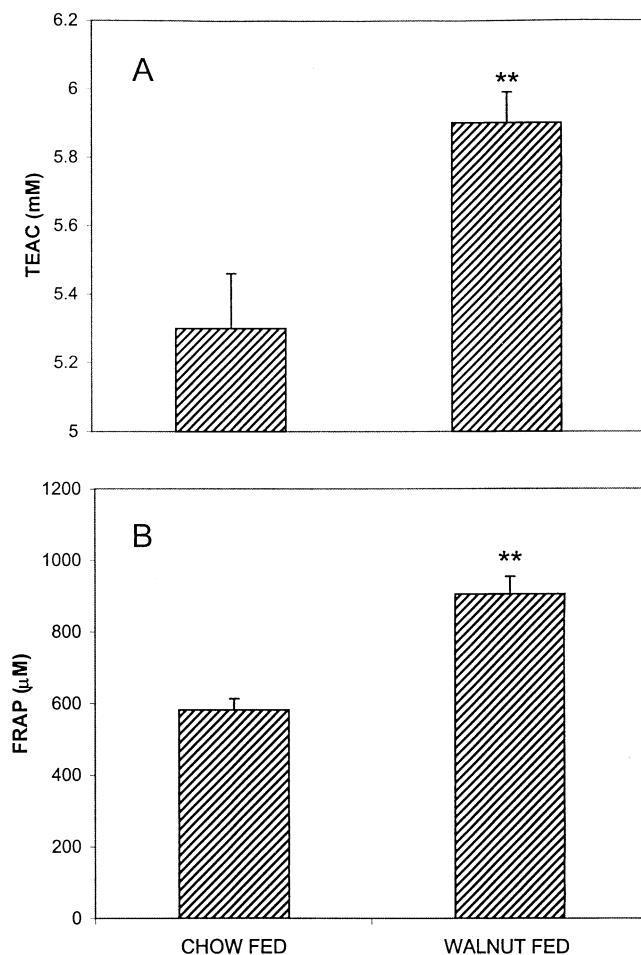


Fig. 2. (A) TEAC and (B) FRAP values of blood from rats that were fasted overnight and then given regular rodent chow or walnuts to eat. TEAC and FRAP values increased in the blood of rats that consumed walnuts.  $**P < 0.01$ . FRAP, ferric-reducing ability of serum; TEAC, trolox equivalent antioxidant capacity.

ery to synthesize melatonin [27]. Further, one study has reported that melatonin levels may be synthesized more abundantly at night [27], in at least one plant species, as in animals [13].

The present study also shows that when walnuts are consumed, the levels of this constituent in the blood increase. Certainly, melatonin taken orally in pure form is readily absorbed by the gastrointestinal tract [28,29]. The present study in a mammal is consistent with a report in which chickens that consumed melatonin-containing food products also had measurably increased melatonin levels in their blood [11].

Rats were food restricted for 24 h and then given exclusively walnuts to eat for 4 h before blood was collected. This experimental design was used because, had normal chow also been available, the rats may not have consumed a sufficient quantity of walnuts to significantly influence blood melatonin concentrations. The purpose of the present study was to test whether melatonin from consumed walnuts is absorbed by the gastrointestinal tract. Blood levels of melatonin increased roughly three-fold after walnut consumption. The final levels achieved (about 40 pg/mL) were significantly lower than those normally present in the blood of most mammals at night [13].

The present study also documented that increased melatonin levels in the blood after walnut consumption positively correlate with an increase in total antioxidant capacity of the serum as reflected by increases in TEAC and FRAP. Heretofore it has been shown that in mammals, including humans, fluctuations in blood melatonin concentrations strongly correlate with the ability of the blood to detoxify toxic free radicals and related reactants [30,31]. It should be noted that in the present study the increases in TEAC and FRAP levels were not necessarily or exclusively related to the increase in melatonin. Walnuts contain other important antioxidants, e.g., vitamin E [6] and polyphenols [7], that are also absorbed from the gut and influence the total antioxidant capacity of serum.

The repeated demonstration that walnuts, if consumed on a regular basis, decrease cardiovascular risk factors has been ascribed to  $\omega$ -3 fatty acids that walnuts contain [1–4]. Although this is very likely the case, melatonin may contribute to the beneficial cardiovascular actions of walnuts because it has been found to have important cardioprotective effects [9]. These beneficial actions of melatonin are apparent at physiologic levels of the indole [10], suggesting that the increase in blood melatonin levels after walnut consumption would increase protection of the heart against oxidative damage, which is the basis of a variety of deteriorative cardiac conditions.

In addition to its direct protective actions against oxidative damage to the cardiovascular system, melatonin has been shown to synergize with other antioxidants, e.g., vitamin E [32–34], which are found in walnuts. Thus, the composite action of a variety of beneficial molecules in

walnuts, e.g.,  $\omega$ -3 fatty acids, vitamin E, and melatonin, may be involved in their antioxidative actions.

In addition to melatonin's beneficial actions on the heart, this indole decreases the initiation of cancer by limiting oxidative damage to DNA [35], and it curtails the growth of tumors once they are established [36]. Melatonin achieves these actions by a number of means including inhibiting the uptake of growth factors such as  $\omega$ -6 fatty acids by cancer cells [36,37]. Further, melatonin's effects on cancer inhibition are achieved at physiologic concentrations [38], and phytemelatonin, such as that in walnuts, has been proposed as a potential means of limiting the growth of established tumors.

Of interest is that melatonin [36] and one of the  $\omega$ -3 fatty acids found in walnuts [4], i.e., eicosapentaenoic acid, act by a similar mechanism to inhibit tumor growth [39]. Thus, these molecules decrease the uptake and metabolism of  $\omega$ -6 linoleic acid by acting on membrane receptors, thereby stimulating an inhibitory G-protein and decreasing intracellular levels of cyclic adenosine monophosphate [39]. The decrease in cyclic adenosine monophosphate decreases linoleic acid transport into cancer cells. Linoleic acid is a growth factor for a variety of tumors. Given their common mechanisms of action on tumor growth, melatonin and eicosapentaenoic acid, both of which are present in walnuts, may act synergistically to inhibit cancer cell proliferation.

In conclusion, melatonin is present in walnuts, and when these nuts are eaten blood levels of the indoleamine increase to values where they could be protective against cardiovascular damage and cancer initiation and growth.

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