Basic nutritional investigation

Effect of whole walnuts and walnut-skin extracts on oxidant status in mice

Mónica Bulló Ph.D. a,b,*, M. Rosa Nogués Ph.D. c, Patricia López-Uriarte B.Sc. a, Jordi Salas-Salvadó Ph.D. a,b,d, Marta Romeu Ph.D. c

a Human Nutrition Unit, Department of Biochemistry and Biotechnology, Facultat de Medicina i Ciències de la Salut, ISPV, Universitat Rovira i Virgili, Reus, Spain
b CIBER Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III, Santiago de Compostela, Spain
c Unit of Pharmacology, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain
d Nutrition and Dietetics Unit, Internal Medicine Department, Hospital Universitari de Sant Joan, Reus, Spain

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A B S T R A C T

Objective: To evaluate the effect of the intake of whole walnuts and walnut fractions on the oxidant status in mice.

Methods: Thirty-six C57BL/6J male mice were randomized to be fed one of three diets: 1) a standard diet (control group), 2) a standard diet with 10% of whole walnuts (walnut-diet group), or 3) a standard diet with 2% of walnut skins (walnut-skin-diet group) for 8 wk. The plasma antioxidant capacity was measured by oxygen radical-absorbance capacity and plasma ferric-reducing antioxidant potential. Conjugated diene formation and reduced glutathione levels were also analyzed.

Results: We observed no changes in plasma oxidation capability between the walnut and walnut-skin groups with the exception of conjugated dienes. Plasma total antioxidant capacity and the ratio between reduced and oxidized forms of glutathione were lower in the walnut and walnut-skin groups than in the control group.

Conclusion: The decrease in the antioxidant burden observed in enzymatic and non-enzymatic antioxidant systems after sustained consumption of a whole-walnut or a walnut-skin diet in mice may be related to the plasma oxidation capability being maintained in the groups consuming the walnut diets.

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Introduction

Cardiovascular disease is the major cause of death in the developed world. Since Goldstein et al. [1] established the oxidative hypothesis for atherosclerosis, oxidative stress has been considered to be one of the major risk factors for atherosclerosis and cardiovascular disease. Therefore, improving the whole-body antioxidant capacity may help to prevent many chronic and age-related diseases, including cardiovascular disease.

Antioxidants are derived from intrinsic and extrinsic antioxidant systems and are constitutively present in human blood and tissue. Diet is a natural source of such extrinsic antioxidants as vitamins, flavonoids, carotenoids, or proteins and, in recent years, greater interest has been paid to the involvement of nutritional compounds in the development or progression of oxidative diseases. Several epidemiologic studies have supported the dietary antioxidant hypothesis and demonstrated that high consumption of plant foods or nutritional factors derived from plants protect against cardiovascular disease because of the numerous phytochemical compounds they contain [2]. Recently, nuts have been included in this group of potentially healthy foods. In this respect, many observational studies in large cohorts have consistently shown a negative and dose-dependent association between nut intake and the risk of cardiovascular disease, which suggests that regular nut consumption reduces the risk of cardiovascular disease by 30–60% in several population groups, independently of other confounding lifestyle factors. The US Food and Drug Administration has supported these findings and claimed that walnuts have a role in reducing the risk of heart disease.

The healthy benefits of nuts, especially walnuts, are mainly attributed to their high content of ω-3 fatty acids and low saturated fatty acids, and their favorable effects on lipid profiles. Moreover, their high content of vitamin E, polyphenols, flavonoids, arginine, and fiber means that they can have

*Corresponding author. Tel.: +34-977-75-93-12; fax: +34-977-75-93-22.
E-mail address: monica.bullo@urv.cat (M. Bulló).
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a considerable modulatory effect on the antioxidant system because they decrease the oxidative damage caused by lipids and lipoproteins and thus help prevent the evolution of atherosclerotic plaque. However, the unsaturated fatty acids in nuts are highly susceptible to oxidation. Thus, the effect of nut consumption on the balance between the pro-oxidant and antioxidant capacities in the body could be crucial to determining the real effect of nuts on cardiovascular health.

Because antioxidant bioavailability depends on the amount of food ingested and the food matrix, it should be taken into account when the antioxidant effects of nuts or nuts extracts are tested. For this reason, the aim of the present study was to evaluate the effect of the consumption of whole walnuts and walnut-skin fractions on oxidant status in mice.

Materials and methods

Animals and diets

Thirty-six C57BL/6J male mice 5 wk of age (Charles River Laboratories, Barcelona, Spain) were housed and maintained in an environmentally controlled room (20–22 °C; 12-h alternating light/dark cycle, and a relative humidity of 60%). Twelve mice were fed a standard diet (control group), another group of 12 was fed a standard diet with 10% of whole walnuts (walnut-diet group), and a third group of 12 was fed a standard diet with 2% of walnut skins (walnut-skin-diet group). A total of 10% of nuts in the diet is the caloric equivalent of the daily nutritional recommendations of nuts in humans (30 g/d). Nuts were mechanically crushed and added to the standard feed. Walnut skins were removed from the walnut by hot water blanching while the walnuts were being prepared and they are generally treated as a waste product. The percentage of 2% was calculated using the normal ratio between whole nuts and the cover fraction so that the amount of antioxidant consumption was the same. The diets were administered ad libitum for 8 wk (Panlab, Barcelona, Spain). Borges S.A. (Reus, Spain) donated the walnuts and walnut-skin extracts used in the study.

Food consumption was measured twice a week and animal weight once a week. Nutritional differences between diets are presented in Table 1.

At the end of this period, and after an overnight fast, the mice were anesthetized with an intraperitoneal injection of ketamine-xylazine (100–10 mg/kg, respectively) and then decapitated with an intracardiac injection of potassium chloride. Blood was obtained by heart puncture and the plasma samples were preserved in acid and frozen at –80 °C. The samples were diluted 1:10 in phosphate-EDTA buffer, pH 8.0. The final assay mixture contained 100 µL of the diluted plasma, 1.8 mL of phosphate-EDTA buffer, and 100 µL of o-phthaldialdehyde solution (Sigma Chemicals Co.). After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a cuvette. The GSSG assay was performed in 50 µL of plasma samples incubated with 20 µL of 0.04 M N-ethylmaleimide (Merck) for 25 min at room temperature. 

To this mixture, 430 µL of 0.1 N NaOH was added to obtain a pH of 12.0. A total of 100 µL of this mixture was taken to measure GSSG, using the procedure outlined above for the GSH assay, except that 0.1 N NaOH was used as diluent instead of phosphoric acid. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the fluorescein decay curve and were expressed as nanomoles per minute per milliliter of plasma. Neither wheat nor soy were added to the diet, considering the amount of antioxidant consumption was the same. The diets were administered ad libitum for 8 wk (Panlab, Barcelona, Spain). Borges S.A. (Reus, Spain) donated the walnuts and walnut-skin extracts used in the study.

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Plasma antioxidant capacity (ORAC assay)

Plasma samples were diluted 500-fold in 75 mM potassium phosphate buffer (pH 7.4) before analysis. The reaction mode used 20 µL of the sample and 370 µL of fluorescein 48 mM in a multwell plate. The reagents were mixed and incubated for 10 s before the initial fluorescence was recorded. Peroxyl radicals were generated by 10 µL of 2.2′-azobis(2-amidinopropane) dihydrochloride reagent. Fluorescence readings were taken every minute for 120 min at 485-nm (λ excitation) and 538-nm (λ emission) wavelengths on a Fluoroskan Ascent fluorescence plate reader ( Labsystems, Helsinki, Finland). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the fluorescein decay curve and were expressed as Trolox equivalents per liter of plasma (millimoles of Trolox equivalents per liter of plasma) [5,6].

Table 1

<table>
<thead>
<tr>
<th>Diet Type</th>
<th>Control</th>
<th>Walnut</th>
<th>Walnut skins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total energy (kcal/kg)</td>
<td>2900</td>
<td>3323</td>
<td>3264</td>
</tr>
<tr>
<td>Carbohydrates (%)</td>
<td>60</td>
<td>40.5</td>
<td>50.6</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>3.1</td>
<td>9.6</td>
<td>6.8</td>
</tr>
<tr>
<td>Saturated (%)</td>
<td>12.9</td>
<td>13.5</td>
<td>15.6</td>
</tr>
<tr>
<td>Monounsaturated (%)</td>
<td>22.58</td>
<td>17.5</td>
<td>18.1</td>
</tr>
<tr>
<td>Polysaturated (%)</td>
<td>40.29</td>
<td>60.4</td>
<td>66.3</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>16.1</td>
<td>15.8</td>
<td>15.7</td>
</tr>
<tr>
<td>Fiber (%)</td>
<td>15.1</td>
<td>15.1</td>
<td>16.3</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>5.1</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Others (%)</td>
<td>2</td>
<td>5.7</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Results

Figure 1A shows the ORAC values of the walnut-skin extracts and the whole walnuts used to enrich the mice’s diets. Walnut-skin extracts showed greater antioxidant capacity than whole-walnut extracts in their hydrophilic fraction, which indicates the presence of numerous potential antioxidant compounds in walnut skin. The ORAC values observed in the walnut and walnut-skin diets used in this study were higher than in the control diet, largely because of the hydrophilic fraction (Fig. 1B). No significant differences were observed in mouse growth and in the amount of food consumption among the three groups during the study (Table 2).

Figure 2 shows the results of the different plasma oxidation markers in the three groups of mice at the end of the interventions. The plasma antioxidant capacity in the groups fed with walnut-skin diets was significantly lower than in the control group. Mice fed with the walnut-skin diet showed significantly lower ORAC and FRAP values than the mice in the control group. In the mice on the walnut diet, only the plasma FRAP values significantly differed from the control group.

Plasma GSH concentrations were lower in the groups that received walnut or walnut-skin diets in comparison with the control group, although these differences were only statistically significant in the case of the mice fed with the walnut-skin diet ($P = 0.020$). No differences were observed in plasma concentrations of GSSG between groups. Therefore, the GSSG/GSH ratio was significantly higher in the walnut-skin group.

The diene oxidation rate and diene lag phase were not significantly different among groups. However, the mice fed with the walnut diet showed a plasma concentration of conjugated dienes that was significantly higher than that of the mice in the control and walnut-skin groups ($P < 0.03$).

Discussion

This is the first chronic study to evaluate the effect of whole-walnut and walnut-skin consumption on oxidative stress markers and the plasma antioxidant capacity in mice. The aim was to investigate whether the oxidation of polyunsaturated fatty acids (PUFAs) from nuts could be compensated for or not by the antioxidant properties attributed to their antioxidant compounds. Our results show that the antioxidant capacity at the plasma level was decreased in the mice on the walnut and walnut-skin diets in comparison with the control group. Despite this, the plasma oxidizing capability seemed to be preserved.

The putative health benefits of walnuts have been mainly attributed to their antioxidant capacity, which is associated with their content of flavonoids—mainly distributed in the skin—and fiber. However, most of the supportive evidence is based on in vitro experiments or in vivo feeding studies that measured the effect of a single potential antioxidant compound. Because the susceptibility of fatty acids to oxidation is thought to be directly dependent on their degree of unsaturation [9] and nuts are rich in monounsaturated fatty acids and PUFAs [10], nuts could per se be prone to easy oxidation independently of their content of antioxidant compounds. Therefore, the debate about whether whole nuts can or cannot improve oxidative status in vivo is still ongoing.

Only two studies have evaluated the acute effect of nut consumption on oxidative status in animals. In relation to control groups, an increase was observed in the postprandial plasma antioxidant capacity in walnut-fed rats [11] and the lag time of

Statistical analysis

The variable normal distribution was tested by the Kolmogorov-Smirnov test. Analysis of variance was used to compare mean groups. Results were expressed as mean ± standard error of the mean. The level of statistical significance for all tests was $P < 0.05$. Statistical analyses were performed with SPSS (SPSS, Inc., Chicago, IL, USA).

Table 2

| Characteristic of body weight and food consumption during the study† |
|------------------|------------------|------------------|------------------|------------------|
|                  | Control           | Walnut            | Walnut skins     | $P^1$            |
| Body weight (g)  |                  |                  |                  |                  |
| Basal            | $22.18 \pm 0.51$  | $21.68 \pm 0.26$  | $21.39 \pm 0.41$ | 0.419            |
| After 8 wk       | $29.24 \pm 0.82$  | $28.04 \pm 0.48$  | $28.72 \pm 0.59$ | 0.432            |
| Differences      | $7.05 \pm 0.52$   | $6.36 \pm 0.50$   | $7.33 \pm 0.86$  | 0.561            |
| Food intake/mouse (g) |              |                  |                  |                  |
| Basal            | 2.84              | 4.02              | 4.04             |                  |
| After 8 wk       | 4.08              | 6.21              | 3.55             |                  |

† Mean ± SE.

$^1$ Analysis of variance.
Fig. 2. Values of plasma oxidation markers in the three groups of mice at the end of the interventions. (A) Plasma antioxidant capacity measured by ORAC and FRAP. (B) Enzymatic antioxidant capacity related to glutathione. (C) Plasma conjugated diene formation. Results are expressed as mean ± SE. * P < 0.05 versus control group, # P < 0.03 versus walnut diet group. The white bar represents the control group (n = 12), the gray bar the walnut-diet group (n = 12), and the black bar the walnut-skin diet group (n = 12). C, control diet; FRAP, ferric-reducing antioxidant potential; GSH, reduced glutathione; GSSG, oxidized glutathione; ORAC, plasma antioxidant capacity; TE, Trolox equivalent; W, walnut diet; WS, walnut-skin diet.
low-density lipoprotein (LDL) oxidation 180 min after administering 40 μM of gallic acid equivalents from almonds by stomach gavage in hamsters [12].

In contrast, long-term clinical trials in humans evaluating the effect of nut consumption on oxidative status have produced controversial results. Although some studies have reported a significant decrease in such oxidative markers as plasma malondialdehyde and isoprostanes after nut consumption [13,14], others have failed to find any positive effect on conjugated diene synthesis in LDL or even in the antioxidant enzymatic activity [15–17].

Although the walnut and walnut-skin diets have shown a higher antioxidant capacity in vitro, in the present study we found that the total plasma antioxidant capacity, measured by ORAC and FRAP, was significantly lower in the animals that were chronically fed with a walnut diet or a walnut-skin diet in comparison with the control group. Although walnuts are very rich in antioxidant compounds, the total amount of fat to be oxidized is greater. Walnuts are also richer in ω-6 PUFAs than in monounsaturated fatty acids, which are present in larger amounts in other nuts. The ω-6 PUFAs are more easily oxidized than monounsaturated fatty acids [18–21] and ω-3 PUFAs [10,22]. These differences in oxidizing capacity between types of fats could partly explain our results in those mice fed with a walnut diet.

We also observed that, in comparison with the control group, the formation of total plasma-conjugated dienes was significantly higher, whereas no changes in the lag phase were observed between groups. Moreover, although the mice on the walnut-skin diet had higher levels of conjugated dienes than the control group, they had significantly lower levels than the mice on the walnut diet. Once again, those animals consuming larger amounts of ω-6 PUFAs derived from walnuts showed a higher synthesis of oxidized products. Similar negative results on conjugated diene formation in LDL were observed in four clinical trials performed in humans to evaluate the chronic effects of nut consumption on oxidative status have produced controversial results. Although some studies have reported a significant increase in the maximal rate of oxidation of conjugated dienes in LDL, it could be related to the plasma oxidizing capability being maintained in the groups consuming the walnut diets.

Conclusion

The decrease in the antioxidant burden observed in enzymatic and non-enzymatic antioxidant systems after the sustained consumption of a whole-walnut or a walnut-skin diet in mice could be related to the plasma oxidizing capability being maintained in those mice fed with a walnut diet.

Acknowledgments

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susceptibility of plasma low density lipoproteins to oxidative modification. 


