Lower Dose of Almonds Exhibits Vasculo-protective Effect when Given in Empty Stomach

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ABSTRACT

The cardiovascular disease (CVD) protection by almonds is well-established. Where most clinical studies used high dose almonds with food, some recent indications suggest almonds’ use as snacks (without accompanying foods). Pharmacological effects of multiple almond-doses, given with or without food had not been explored. In animal models, we simultaneously compared the effect of food on medicinal properties of multiple almond-doses. Study-1 (tylospol model) determined the minimum effective dose in rats. Group 1-2 received Normal Diet (ND). Groups 3-8 received 1, 3 and 10 g kg⁻¹ American and Pakistani almond varieties. After four weeks, tylospol was injected to all groups except group 1 which received saline. Study-2 and 3, on American and Pakistani almonds, respectively explored the effect of food on almonds’ efficacy, in High-Fat Diet (HFD) rat model. Group-1 received ND and all other groups received HFD. For four weeks almonds (doses 0.5, 1 and 2 g kg⁻¹) were given with and without food, to groups 3-8, after which blood was drawn. Almonds inhibited hyperlipidemia, hyperuricemia, hyperphosphatemia, increase in serum alkaline phosphatase, gamma-glutamyl transferase, aspartate and alanine aminotransferase. With food, anti-hyperlipidemia was at 3 and 10 g kg⁻¹, in tylospol model and 2 g kg⁻¹ in HFD model. Low dose (1 g kg⁻¹), ineffective when given with food, significantly prevented hyperlipidemia, hepatic and vascular dysfunction when coupled with 2 h fasting. In conclusion, medicinal value of almond can be maximized by consuming low doses in empty stomach. This study impacts the cost associated with life-long use of nuts for preventing chronic disorders like CVDs.

Key words: Animal models, hyperlipidemia, functional food, nuts, fasting, high-fat diet

INTRODUCTION

Regular nut consumption reduces cardiovascular disease (CVD) risk (Ternus et al., 2006). Almonds prevent dyslipidemia (Jalali-Khanabadi et al., 2010; Phung et al., 2009), diabetes (Li et al., 2011; Lovejoy et al., 2002; Sweaen et al., 2014), obesity (Foster et al., 2012; Wien et al., 2003), oxidative stress (Jenkins et al., 2008; Li et al., 2007) and inflammation (Liu et al., 2013; Rajaram et al., 2010). In most clinical trials, almonds were generally incorporated to foods-like cereals (Mori et al., 2011), breads (Jenkins et al., 2006; Josse et al., 2007), meals (Li et al., 2011; Liu et al., 2013), pizza (Rajaram et al., 2010; Sabate et al., 2003), desserts (Li et al., 2011), muffins (Jenkins et al., 2002, 2008) or cookies (Lovejoy et al., 2002). More recent studies suggest the use of almonds as snack (Tan and Mattes, 2013). Food matrix is shown to hinder almond digestion and reduced content bioavailability (Wickham et al., 2010).

Doses used in different trials, also varied from 25-168 g almonds per day (Phung et al., 2009) and only some of the recent clinical trial have used the US FDA recommended dose of 42 g (1.5 ounce) (Sweaen et al., 2014). Though,
dose-dependent effect has been reported, we observed that the mode of almond consumption for different doses, were dissimilar.

In countries where almonds have been traditionally used for centuries, (as a health tonic), the general practice is eating seven almonds (around 10 g) early in the morning, before breakfast, presumably in empty stomach. This is consistent with the recent reports suggesting the use almonds as snacks (Tan and Mattes, 2013). Yet, the effect of food on efficacy of multiple almond-doses had not been elaborated.

We aim to use animal models for simultaneously comparing different doses of almonds, given with and without food. Preference of animal models over clinical trial was based on the feasibility of managing several groups alongside; precision of dietary monitoring; relative low-cost and ease of validation.

MATERIALS AND METHODS

Animals and almonds: Adult Sprague-Dawley (SD) rats of either gender weighing 150-200 g were housed at Aga Khan University Animal House. Experiments were performed, in accordance with the Guideline for Care and Use of Laboratory Animals provided by The National Research Council (Institute for Laboratory Animal Research, 1996). Approval was obtained from The Ethical Committee for Animal Use and Care, Aga Khan University, Karachi, Pakistan. Diets and water were provided ad libitum, until otherwise mentioned.

Imported American almonds called California Sheld almonds (Karmal) were purchased from local market whereas Pakistani almond variety (Talwar) which is grown around the region of Quetta, were purchased with the help of Professor Muddasir Israr, Dean Faculty of Life Sciences, University of Balochistan.

The United States Food and Drug Administration (US FDA) recommended formula was used for dose translation among species (Reagan-Shaw et al., 2008). Human doses of 5, 10, 20, 30 and 100 g/person were converted to equivalent rat doses of 0.5, 1, 2, 3 and 10 g kg⁻¹. Weighed pieces of almonds, corresponding to individual body weights, were given to each rat. A set of log doses (1, 3 and 10 g kg⁻¹) of both American and Pakistani almonds were used in tyloxapol model. Based on the results of this study, another set of log dose (narrow range: 0.5, 1 and 2 g kg⁻¹) were used in subsequent experiments.

Study designs: The first study was designed to evaluate the minimum effective dose in rats. Three log doses (1, 3 and 10 g kg⁻¹) of almonds were used in tyloxapol-induced hyperlipidemia model. After four weeks of almond supplementation (see Table 1 for grouping), saline or tyloxapol was injected. Blood was drawn after 18 h (Zanwar et al., 2012).

<table>
<thead>
<tr>
<th>Group (n = 7)</th>
<th>Almonds (for 4 weeks)</th>
<th>Intra-peritoneal injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>Tyloxapol (500 mg kg⁻¹)</td>
</tr>
<tr>
<td>3</td>
<td>American (1 g kg⁻¹)</td>
<td>Tyloxapol (500 mg kg⁻¹)</td>
</tr>
<tr>
<td>4</td>
<td>American (3 g kg⁻¹)</td>
<td>Tyloxapol (500 mg kg⁻¹)</td>
</tr>
<tr>
<td>5</td>
<td>American (10 g kg⁻¹)</td>
<td>Tyloxapol (500 mg kg⁻¹)</td>
</tr>
<tr>
<td>6</td>
<td>Pakistani (1 g kg⁻¹)</td>
<td>Tyloxapol (500 mg kg⁻¹)</td>
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<tr>
<td>7</td>
<td>Pakistani (3 g kg⁻¹)</td>
<td>Tyloxapol (500 mg kg⁻¹)</td>
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<tr>
<td>8</td>
<td>Pakistani (10 g kg⁻¹)</td>
<td>Tyloxapol (500 mg kg⁻¹)</td>
</tr>
</tbody>
</table>

Table 2: Design of Study-2 and 3 using American and Pakistani almonds, respectively in high-fat diet model

<table>
<thead>
<tr>
<th>Groups (n = 7)</th>
<th>Diet (for 4 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal Diet (ND)</td>
</tr>
<tr>
<td>2</td>
<td>High-Fat Diet (HFD)</td>
</tr>
<tr>
<td>3</td>
<td>HFD+almond 0.5 g kg⁻¹</td>
</tr>
<tr>
<td>4</td>
<td>HFD (withdrawn 2 h)+almond 0.5 g kg⁻¹</td>
</tr>
<tr>
<td>5</td>
<td>HFD+almond 1 g kg⁻¹</td>
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<tr>
<td>6</td>
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</tr>
<tr>
<td>8</td>
<td>HFD (withdrawn 2 h)+almond 2 g kg⁻¹</td>
</tr>
</tbody>
</table>

In the second and third study, a different set of log doses (narrow range: 0.5, 1 and 2 g kg⁻¹) were given in high-fat diet model and the influence of food was explored. The normal chow diet (Gilani et al., 2006) was given to the normal controls i.e. group 1. This diet was modified by addition of 2% cholesterol, 0.5% cholic acid and 5% butterfat (Siddiqi et al., 2012) and given to remaining groups. Second and third study inspected American and Pakistani almonds, respectively (Table 2).

Rats’ diet was withdrawn for 90 min; almond pieces were given and food was given back 30 min after the almond was eaten by rats. This was to ensure maximum absorption of almonds’ nutrients, presumably in empty stomach. In the “with-food” groups (study 2 and 3) fasting (food withdrawal) was not performed.

Biochemical estimations: At the end of four weeks, blood was collected through tail vein puncture (Thomson et al., 2013), which was centrifuged at 4000 rpm for 15 min. Serum was separated and analysed for concentrations of Total Cholesterol (TC), Low Density Lipoprotein (LDL) and triglyceride (TG). Serum from the study on Pakistan almond was also analysed for concentrations of biomarkers associated with vascular dysfunction: Uric Acid (UA), phosphorus (phosp) and alkaline phosphatase (ALP) and hepatic dysfunction: Gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Automated analyser Roche cobs-c111 was used for all the biochemical tests, with the commercially available enzymatic kits.

Statistical analysis: The software Graph PrismPad 4 was used for statistical analysis. One way ANOVA followed by Tukey’s multiple comparison tests was applied. p-values <0.05 were considered significant and values were represented as Mean+SEM.
RESULTS

Dose-independent effect of American and Pakistani almond varieties on tyloxapol-induced hyperlipidemia: Tyloxapol led to a drastic increase in TC (13.4 fold), LDL (10.7 fold) and TG (10.5 fold) as shown in Fig. 1. Both American and Pakistani almond varieties at 3 and 10 g kg\(^{-1}\) doses, partially inhibited hyperlipidemia (p<0.01), with TC concentrations 29-39% less, LDL concentrations 38-51% less and TG concentrations 39-49% less, compared to the tyloxapol group (Fig. 1). The low dose (1 g kg\(^{-1}\)) of either almond variety was ineffective in reducing these serum lipids, indicating that the minimum effective dose of almond given with food, is somewhere between 1 and 3 g kg\(^{-1}\).

Apparently, the lipid lowering effect obtained by the two higher doses was similar (p>0.05) and not dose-dependent. Comparison of the two varieties also showed that both were equally efficacious (p>0.05) in inhibiting hyperlipidemia.

Dose-independent effect of American almonds on HFD-induced hyperlipidemia: As shown in Fig. 2, HFD increased TC, LDL and TG to 4.8, 6.6 and 2.3 folds higher than controls. Consistent with the results of the tyloxapol model, when given with food, almond-dose of 1 g kg\(^{-1}\) and lower (0.5 g kg\(^{-1}\)), were ineffective in inhibiting hyperlipidemia (Fig. 2). However, at 2 g kg\(^{-1}\) dose, almonds significantly inhibited the HFD-induced increase in TC, LDL and TG. An all-or-none response was observed, where 3 and 10 g kg\(^{-1}\) were equally effective (in tyloxapol model) and 0.5 and 1 g kg\(^{-1}\) were equally ineffective (in HFD model). Interestingly, when given after 2 h fasting, 1 g kg\(^{-1}\) dose (ineffective with food) significantly inhibited the rise in TC, LDL and TG with concentrations close to normal (p>0.05).

Low dose Pakistani almonds prevent HFD-induced hyperlipidemia only when given after fasting: In study-3, high-fat diet also led to increase in TC, LDL and HDL to 6.6, 6.2 and 2.6 folds, respectively (p<0.001). In line with the results obtained with American almonds, 0.5 and 1 g kg\(^{-1}\) doses of Pakistani almond variety given with food, had no influence on hyperlipidemia; the resultant concentrations were similar to HFD group. Only 2 g kg\(^{-1}\) dose, given with food, prevented HFD-induced hyperlipidemia (Fig. 3).

Also authenticating prior observations with American almonds, when coupled with fasting, 1 g kg\(^{-1}\) Pakistani almonds inhibited hyperlipidemia resulting in TC, LDL and TG concentrations to be, respectively 34, 31 and 35% less

![Graphs showing lipid levels](image-url)
Fig. 2(a-c): Anti-hyperlipidemic effect of different doses of American almonds given with food or with fasting, in high-fat diet model. Serum concentration of (a) Total cholesterol (TC), (b) Low-density lipoprotein (LDL), (c) Triglyceride (TG), ND: Normal diet, HFD: High-fat diet. All values are expressed as Mean±SEM (n = 7 per group). One way ANOVA followed by Tuckey’s post-test is applied (*p<0.05, **p<0.01 and ***p<0.001)

Fig. 3(a-c): Anti-hyperlipidemic effect of different doses of Pakistani almonds given with food or with fasting, in high-fat diet model. Serum concentration of (a) Total cholesterol (TC), (b) Low-density lipoprotein (LDL), (c) Triglyceride (TG), ND: Normal diet, HFD: High-fat diet. All values are expressed as Mean±SEM (n = 7 per group). One way ANOVA followed by Tuckey’s post-test is applied (*p<0.05, **p<0.01 and ***p<0.001)

compared to HFD. The almonds doses -1 g kg⁻¹ with fasting and 2 g kg⁻¹ with or without fasting had serum lipids concentrations similar to normal (p>0.05) as evident from Fig. 3.

Effects of Pakistani almond variety on HFD-induced vascular dysfunction: Possible protective effects of Pakistan almond variety on cardiovascular system had not been reported. We analysed certain serum biomarkers associated with vascular dysfunction like Uric Acid (UA), phosphorus and alkaline phosphatase (ALP). The high-fat diet significantly elevated these biomarkers to 0.8, 1 and 3.8 folds, respectively as shown in Fig. 4. Excitingly, supplementation of Pakistani almond inhibited these elevations (p<0.05). The effective doses were 1 g kg⁻¹ (with fasting) and 2 g kg⁻¹ (with food and with fasting). As shown in Fig. 4, serum concentrations of UA, phosphorus and ALP in these groups were similar to normal (p>0.05).

Effects of Pakistani almond variety on HFD-induced hepatic dysfunction: High-fat diet led to hepatocellular damage indicated by increase in serum GGT, AST and ALT
Fig. 4(a-c): Vascular protection by different doses of Pakistani almond variety given with food or fasting, in high-fat diet model. Serum concentration of (a) Uric acid (UA), (b) Phosphorus (Phosp.), (c) Alkaline phosphatase (ALP). ND: Normal diet, HFD: High-fat diet. All values are expressed as Mean±SEM (n = 7 per group). One way ANOVA followed by Tuckey’s post-test is applied (*p<0.05, **p<0.01 and ***p<0.001)

Fig. 5(a-c): Hepatic protection by different doses of Pakistani almond variety given with food or fasting, in high-fat diet model. Serum concentration of (a) Gamma-glutamyltransferase (GGT), (b) Aspartate aminotransferase (AST), (c) Alanine aminotransferase (ALT). ND: Normal diet, HFD: High-fat diet. All values are expressed as Mean±SEM (n = 7 per group). One way ANOVA followed by Tuckey’s post-test is applied (*p<0.05, **p<0.01 and ***p<0.001)

DISCUSSION

This is the first study in rat models showing that the medicinal effects of almonds, on multiple organ systems, can be enhanced by consuming low doses in empty stomach. We found absence of a dose-dependent effect in tyloxapol model. We also report that almonds (Pakistani variety in this study) potentially prevents vascular and hepatic dysfunction by inhibiting hyperuricemia, hyperphosphatemia, elevation of serum ALP, GGT, AST and ALT. Previously,
while demonstrating the mechanistic basis for the medicinal use of almonds in multiple rat models of cardiovascular disorders, we have shown similar protection of liver and vasculature by American almonds (Jamshed and Gilani, 2014).

Selection of these biomarkers was based on the evidence indicating that hyperuricemia potentially induces endothelial dysfunction (Kanlis and Kang, 2005) via vascular smooth muscle cell proliferation (Corry et al., 2008) whereas hyperphosphatemia and increased serum alkaline phosphatase (ALP) are associated with vascular calcification (Giachelli et al., 2005; Jono et al., 2000; Limas and Cohn, 1973; Narisawa et al., 2007). Conversely, serum GGT is not only an indicator of hepatic damage, like the two aminotransferases AST and ALT but is also found in atherosclerotic plaques (Franzini et al., 2009), inferred to be involved in lipid oxidation (Dominici et al., 2003).

Results from tyloxapol model indicated that Pakistani and American almonds are equally efficacious in preventing hyperlipidemia. In addition to the presentation of a dose-independent effect, this model also indicated the possible minimum effective dose in rats. Based on use of wide-range doses in different clinical trials, we selected human log doses of 10, 30 and 100 g/person and translated to rat doses of 1, 3 and 10 g kg⁻¹. Given with food 1 g kg⁻¹ was ineffective; hence, in the subsequent experiments, we selected a narrow range of log doses around 1 g kg⁻¹.

The absence of dose-dependent effect observed in tyloxapol model was in line with the previous reports (Rajaram et al., 2010). Rajaram et al. (2010) also reported that both low and high doses of almonds, produced similar responses against inflammatory markers of CVDs.

In contrast, however, two clinical trials (Jenkins et al., 2002; Sabate et al., 2003) described dose-dependent reduction in serum lipids by almonds—though the mode of administration of the two almond doses seem inconsistent. In the former study (Jenkins et al., 2002), high dose was given as snacks but low dose was accompanied with muffin—which could potentially interfere with almond’s digestion (Ellis et al., 2004). In the later study (Sabate et al., 2003), almonds were added to foods like cold salads and hot pizza. High temperatures could increase acrylamide content which is a potential carcinogenic neurotoxin (Lukac et al., 2007). Heat stress also causes oxidative deterioration of almond lipids, specifically the unsaturated fatty acids (Buransompob et al., 2003; Saleedo et al., 2010) which are generally held responsible for the lipid-lowering actions of almonds (Berryman et al., 2011). We counterbalanced such variability of consumption pattern, by using animal models whereby dietary modifications can be unified.

Our results in rat models have shown that doses as low as 1 g kg⁻¹ which corresponds to around 10 g/person could be effective in preventing cardiovascular disease. Tamizzifar et al. (2005) showed lipid lowering effect of a relatively low almond dose (25 g/person). However, almonds were used in the form of finely ground powder. Evidence suggests that nutrient release from grinded powder, is higher (Mandalari et al., 2008a) and whole almonds are hard to digest (Ellis et al., 2004). But there are merits of digestion-opposition by almonds, like satiety-induction (Holli and Mattes, 2007) which assures limited food intake thereby preventing weight-gain (Fraser et al., 2002; Wien et al., 2003). Delayed residence of almonds in gut also provides general health benefits via prebiotic action (Mandalari et al., 2008b). We advocate the traditional mode of almond consumption where seven almonds (around 10 g) are chewed before breakfast (in empty stomach). The phenomenon of mastication further signifies the preferential use of whole almonds. The physical act of munching is associated with lesser food intake and augmented salivary flow which induces satiety (Cassady et al., 2009), further preventing weight gain and obesity.

CONCLUSION

Our study provides the scientific basis for the folkloric recommendation of almond consumption. Results have shown that the lipid lowering, vascular and hepatic protection provided by almonds can be maximized by consuming even low doses in empty stomach. This could have significant implications in terms of the cost effectiveness of using almonds for cardiovascular disease prevention.

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