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Design: Thirty healthy volunteer men (age 45.57±7.14 years and body mass index 24.29±2.15 kg/m2) with mild hyperlipidemia received 60 g almond daily for 4 weeks.

Outcome measures: Overnight fasting blood samples were obtained before and after supplementation. Serum levels of lipids, lipids lipoproteins, and apolipoproteins and the copper-induced serum lipid oxidation were determined. Lipid oxidation was followed by monitoring of the change of conjugated dienes in diluted serum after addition of Cu2+. A number of quantitative parameters including lag-time, maximal rate of oxidation (V-max), and maximal amount of lipid peroxide products (OD-max) were evaluated.

Results: After 4 weeks almond supplementation significantly decreased low density lipoprotein cholesterol (LDLc), total cholesterol (TC) and apolipoprotein B100 (apo-B100). At baseline, there were little correlation between lipid risk factors with lipid oxidation parameters, but there were observed positive correlation between TC and lag-time (r=0.6, P=0.001), negative correlation between TC with V-max and OD-max (r=-0.65, P<0.001 and r=-0.61, P=0.001), and also positive correlation between apo-B100 and...
with V-max and OD-max (r=0.48, P=0.01 and r=0.54, P=0.003) after almond supplementation. Conclusion: These results demonstrated that almond supplementation in addition to lowering effects on serum levels of CHD lipid risk factors, may contribute to a dramatic change on the relation of lipid risk factors and susceptibility of serum lipids to oxidative modification. This may be due to the distribution of different almond phenolic antioxidants in different components of serum including non-lipoprotein molecules such as serum albumin.
Effects of Almond Dietary Supplementation on Coronary Heart Disease Lipid Risk Factors and Serum Lipid Oxidation Parameters in Mild Hyperlipidemic Men

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Running title: Almond, CHD lipid risk factors and lipid oxidation
Abstract

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Introduction

Coronary heart disease (CHD) is the leading cause of death in many populations. One of the considerable strategies to control CHD is to identify and modify the most serious and modifiable local risk factors. There are several groups of risk factors for atherosclerosis and cardiovascular diseases. High plasma level of lipids and lipid peroxidation are closely implicated in the development and progression of atherosclerosis.

During the last decades, considerable interest has been directed toward investigation of plasma lipids susceptibility to oxidation in healthy and different diseased conditions. Another area of interest is evaluation of the protective effects of different medication or specific diet supplementation on the susceptibility of isolated and whole serum lipids to oxidation.

Epidemiologic and clinical studies have shown that consumption of nuts is associated with favorable plasma lipid profiles and reduced cardiovascular risk. Almond nuts have been used in a wide variety of food formulations and some research indicated its plasma lipids lowering effect on isolated low density lipoprotein (LDL) oxidation.

Whether the monounsaturated fatty acid or nonfat (protein, fiber, flavonoids) components of almonds are responsible for the beneficial effect of the nut on cardiovascular risk is not fully elucidated. On the other hand, there is little available data on the effect of dietary almond supplementation on the serum lipid oxidation parameters and their relation to lipid risk factors for CHD in un-fractionated serum.

This study was undertaken to assess the effect of almond supplementation on in-vitro copper induced lipid oxidation parameters in diluted whole serum, and its relation with lipids, lipoproteins and apolipoproteins levels in mild hyperlipidemic men.
Materials and methods

Subjects and study design

Study population consisted of 30 healthy volunteer men (age 45.57±7.14 years and body mass index 24.29±2.15 kg/m²) with mild hyperlipidemia (serum cholesterol and triglycerides 200-300 mg/dl) that received daily 60 g almond for 4 weeks. The participants were weekly given a packet including 7 sachets containing 60 g of raw almond nuts and they were instructed to use two times daily as snack for next week.

During the study, subjects followed their own normal diets to which included the supplement. None of subjects had clinical or biochemical evidence of diabetes, cardiovascular, liver or renal disease, and no one was taking the medications, antioxidant supplements or smoking cigarettes. During the study, subjects were asked not to consume any additional nuts or nut products or alter consumption of dietary fiber, and also to maintain their level of physical activity.

Measures and biochemical analyses

An overnight fasting blood samples and body weight were obtained before and after the supplementation. Serum levels of lipids, lipoproteins, and apolipoproteins were determined, and the in-vitro copper-induced profiles of serum lipids oxidation were evaluated. Serum total cholesterol (TC) and triglycerides (TG) concentration were determined by enzymatic methods, cholesterol oxidase and glycerol oxidase respectively. High density lipoprotein cholesterol (HDLc) concentration was determined with dextran-sulfate-magnesium chloride precipitation of betalipoproteins, followed by the same enzymatic method for TC. Low density lipoprotein cholesterol (LDLc) was calculated using Friedewald formula. Apolipoprotein-A1 (apo-A1) was determined by immunoturbidimetry method. Apolipoprotein-B100 (apo-B100) and lipoprotein(a) [Lp(a)] were determined by the method of electroimmunoassay.

Specific anti-Lp(a), anti-apo-B100, anti-apo-A1 antibodies, primary standards and controls were from DAKO (DK-2600 Glostrup, Denmark). Copper-induced lipid peroxidation was estimated in a 60-fold diluted serum in 20 mM phosphate buffer containing 150 mM NaCl and 720 µM citrate, PH=7.4. The lipid oxidation procedure was
conducted at 37°C and was initiated by the addition of CuCl$_2$ to give a final concentration of 60 µmol/L.  

The kinetics of conjugated dienes formation were monitored spectrophotometrically (Perkin-Elmer UV.VIS Double beam spectrophotometer 505S) by measuring absorbance in a 1-cm quartz cuvette at 245 nm every 10 min for 300 min. Microsoft excel software was used for plotting of the kinetic curves of the accumulation of lipid peroxide products (change of absorbance at 245 nm over time in min), and a number of quantitative oxidation parameters including lag-time (the time interval between the addition of CuCl$_2$ to the serum and the beginning of extensive oxidation), maximal rate of oxidation (V-max), and maximal amount of lipid peroxide products accumulation (OD-max) were evaluated. Before the processing of samples, method of serum lipid oxidation was optimized and an inter-individual coefficient of variations (CV) of 6% (for lag-time, n = 10), 7.4% (for OD-max, n = 10) and 7.5% (for V-max, n = 10) was obtained.  

**Data analysis**  
All values are reported as mean ± SD. Differences between before and after supplementation were assessed by paired t-test. The Pearson correlation test was used for evaluation of relationship between oxidation parameters and other variables in the study population. P-value < 0.05 was considered as significant. All P-value were two-tailed. All the tests were performed by The SPSS package Version 11 (SPSS Inc., Chicago, IL, USA).  

**Ethical Considerations**  
An informed consent was obtained from each participant. They could quit the study freely, whenever they liked. All the patients were continuing their usual diet. The Research Ethics Committee of the Shahid Sadoughi University of Medical Sciences approved the research proposal of the study.  

**Results**  
Almond nut supplementation significantly decreased serum levels of LDLc, TC and apo-B100. Comparison of serum lipids, lipoproteins and apolipoproteins before and after supplementation are summarized in table 1.
The Kinetics analysis of conjugated diene production revealed that almond supplementation prolonged the lag time of conjugated diene formation (from 60.5±34 to 79±50 min), but this and other changes in oxidation parameters were not statistically significant.

Serum lipid oxidation parameters before and after intervention are compared in table 2. At baseline, a significant association was found between TC and lag time, but after almond supplementation, there was observed a significant positive correlation between these two variables.

Table 3 shows the relationship between some cardiovascular lipid risk factors and lipid oxidation parameters of serum in the study population before and after the intervention. No significant changes were found for Lp(a) and apo-A1 following the almond supplementation.

**Discussion**

Consumption of 60 g almond nuts daily as a supplement in the diet of mild hyperlipidemic men reduced LDLc and apo-B100. These data suggest a favorable effect of almonds in modifying lipid risk factors for CHD. These benefit effects have been demonstrated by other authors over the last decade and may be due to unsaturated fat contents of almonds.17-19

A noticeable finding of our study was a significant change in the association of lipid CHD risk factors with lipid oxidation parameters of serum in the study population following the almond supplementation. The most effective changes have been observed on the TC and related parameters such as TC/HDLc ratio following the supplementation. TC indicated a negative correlation with lag-time before intervention, while there was seen a strong positive correlation between TC and lag-time after intervention. Lag-time is an index for resistance of serum lipid to oxidation, and longer lag-time indicated more resistance of lipid to initiation of oxidation. Therefore, in subjects with normal diet, increased serum levels of TC have been associated with increased susceptibility of serum lipids to oxidation, while following almond supplementation serum lipids are more resistant to initiation of copper-induced oxidation. Minimal changes also were observed on LDLc and LDLc/HDLc ratio following the supplementation. This is predominantly related to lag-time and there is a shift from negative correlation of these lipid risk factors
with lag-time toward a more positive correlation after supplementation. Association of serum TC and LDLc with susceptibility of serum to lipid oxidation also have been reported by others.\textsuperscript{20, 21} Our results show that almond supplementation reversed this effect, and in subjects with this intervention increased serum levels of TC and LDLc are associated with decreased susceptibility of whole serum lipids to oxidation.

On the other hand, TC had little or no correlation with other oxidation parameters (OD-max and V-max) before the intervention, while there were seen an extensive negative correlation between TC with OD-max and V-max after the supplementation. V-max and OD-max are indexes for the rate and final extent of lipid oxidation products accumulation in the course of in-vitro copper-induced profile of serum lipid oxidation respectively. Therefore, in subjects with supplementation, increased serum levels of TC are associated with decreased rate and extent of lipid oxidation products accumulation in lipid oxidation profile at experimental condition.

As compared to TC and LDLc, reverse changes have been observed on apo-B100 and related parameters following the supplementation. However apo-B100 had no statistical significant correlation with lipid oxidation parameters before intervention, but there were seen a weak negative correlation of apo-B100 with lag-time and a strong positive correlation between apo-B100 with OD-max and V-max after intervention. This results indicated that in subjects after supplementation, serum higher levels of apo-B100 is associated with more susceptible to initiation and higher rate and extent of lipid oxidation in the course of copper-induced serum lipid oxidation. Apparently, supplementation causes more effectiveness of apo-B100 in the process of serum lipid oxidation in this experimental condition. Thus following the almond supplementation, serum lipids susceptibility to oxidation is more sensitive to the variation of serum apo-B100 concentration. Chen et al. in an in-vitro study on isolated LDL and apo-B100 demonstrated that phenolic compounds from almond reduced the oxidative modification of apo-B100 and stabilized LDL conformation.\textsuperscript{22} In this study, we assessed un-fractionated serum for lipid susceptibility to oxidation after supplementation and observed a shift to more negative association of apo-B100 and lag-time. Therefore, in our experimental condition some of almond phenolic compounds with antioxidant properties may be bind on molecules other than lipoproteins including serum albumin. Therefore,
this reversed effect on apo-B100 in comparison to TC that we observed after supplementation may be due to this profile of antioxidant serum distribution.

In this study, we assessed the effects of almond supplementation on serum levels of CHD lipid risk factors and also their relationship with lipid oxidation parameters. However, almond supplementation had beneficial effects on CHD lipid risk factor levels, but its effect on the relation of lipid risk factors to lipid oxidation parameters was controversial. After supplementation the most effective change in the relation of lipid risk factor and parameters of serum lipid oxidation were observed in TC, moderate change for LDLc and apo-B100. This controversy may be due to the effects of different components of almonds and its distribution in serum. Beneficial effect of almonds on the serum levels of CHD risk factors is mainly related to its unsaturated fatty acids and fiber contents, while its different effects on the relationship of lipid risk factors and lipid oxidation parameters in whole serum may be due to its phenolic contents and distribution of these antioxidant molecules in various components of serum.

These results demonstrated that almond supplementation in addition to beneficial effects on serum levels of CHD lipid risk factors, may contribute to a dramatic change on the relation of lipid risk factors and susceptibility of serum lipids to oxidative modification. We suggest that different effects of almond supplementation on the relation of lipid risk factor levels and serum lipid susceptibility to oxidation may be due to the distribution of different almond phenolic antioxidants in different components of serum including albumin. Further studies are needed to clarify the exact distribution of almond antioxidant in serum and their antioxidant potential against serum lipid oxidation.

**Acknowledgement**

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**Conflicts of interests, source of funding and authorship**

The authors declare that they have no conflicts of interest. This study was fully funded by the Department of Research Administration, SSUMS in Yazd, Iran.
Dr B.A. Jalali is a Senior lecturer in Department of Biochemistry at SSUMS, participated in designing and supervising this study and writing the manuscript. Dr H. Mozaffari-Khosravi is the head of Human Nutrition Department at SSUMS, participated in design, data analysis and writing the manuscript. N. Parsaeyan participated in case selection, patient visits and supervising laboratory analyses. All authors critically reviewed the manuscript and approved the final version submitted for publication.

References


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Table 1: comparison of serum lipids, lipoproteins and apolipoproteins levels in study population before and after almond supplementation

<table>
<thead>
<tr>
<th>Serum parameters</th>
<th>Before Mean ± SD</th>
<th>After Mean ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/L)</td>
<td>6.61 ± 0.68</td>
<td>5.99 ± 1.03</td>
<td>0.01</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>3.15 ± 0.99</td>
<td>2.96 ± 1.15</td>
<td>0.38</td>
</tr>
<tr>
<td>HDLc (mmol/L)</td>
<td>1.01 ± 0.21</td>
<td>1.01 ± 0.27</td>
<td>0.96</td>
</tr>
<tr>
<td>LDLc (mmol/L)</td>
<td>4.38 ± 0.70</td>
<td>3.76 ± 0.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lipoprotein(a) (g/L)</td>
<td>0.255 ± 0.225</td>
<td>0.239 ± 0.215</td>
<td>0.36</td>
</tr>
<tr>
<td>apo-A1 (g/L)</td>
<td>1.33 ± 0.190</td>
<td>1.35 ± 0.167</td>
<td>0.56</td>
</tr>
<tr>
<td>apo-B100 (g/L)</td>
<td>1.26 ± 0.305</td>
<td>1.11 ± 0.246</td>
<td>0.009</td>
</tr>
</tbody>
</table>

TC: total cholesterol, TG: triglycerides, HDLc: high density lipoprotein cholesterol, LDLc: low density lipoprotein cholesterol, apo-B100: apolipoprotein B100, apo-A1: apolipoprotein A1
Table 2: Comparison of parameters of serum lipid oxidation in study population before and after almond supplementation.

<table>
<thead>
<tr>
<th>Oxidation parameters</th>
<th>Before</th>
<th>After</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>60.5 ± 34</td>
<td>79 ± 50</td>
<td>0.21</td>
</tr>
<tr>
<td>OD- max</td>
<td>153.5 ± 70.7</td>
<td>160.8 ± 73.6</td>
<td>0.57</td>
</tr>
<tr>
<td>V-max</td>
<td>0.92 ± 0.41</td>
<td>0.90 ± 0.37</td>
<td>0.93</td>
</tr>
<tr>
<td>T-max (min)</td>
<td>149.5 ± 73</td>
<td>160 ± 71</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Lag-time: the time needed (in min) to initiation of lipid oxidation products accumulation during the lipid oxidation course after addition of CuCl₂. OD-max: maximal amount of lipids peroxide products accumulation in µmol/L of conjugated dienes per mmol of LDLc during the lipid oxidation course, V-max: maximal rate of oxidation in µmol/L of conjugated dienes production per mmol of LDLc per minute in during the lipid oxidation course, T-max: time needed (in min) to gained the maximal rate of lipid peroxide products accumulation during the lipid oxidation course.
Table 3: Correlation coefficient (r) between parameters of serum lipid oxidation with some cardiovascular lipid risk factors before and after almond supplementation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lag-time Before</th>
<th>OD-max Before</th>
<th>V-max Before</th>
<th>Lag-time After</th>
<th>OD-max After</th>
<th>V-max After</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>TC</td>
<td>-0.41</td>
<td>0.04</td>
<td>-0.43</td>
<td>0.03</td>
<td>-0.33</td>
<td>0.11</td>
</tr>
<tr>
<td>TG</td>
<td>-0.03</td>
<td>0.90</td>
<td>-0.13</td>
<td>0.55</td>
<td>-0.15</td>
<td>0.48</td>
</tr>
<tr>
<td>LDLc</td>
<td>-0.37</td>
<td>0.07</td>
<td>-0.59</td>
<td>0.002</td>
<td>-0.49</td>
<td>0.01</td>
</tr>
<tr>
<td>apo-B100</td>
<td>0.37</td>
<td>0.06</td>
<td>0.06</td>
<td>0.75</td>
<td>0.25</td>
<td>0.22</td>
</tr>
<tr>
<td>apo-B100/apo-A1</td>
<td>0.30</td>
<td>0.14</td>
<td>-0.08</td>
<td>0.72</td>
<td>0.29</td>
<td>0.15</td>
</tr>
<tr>
<td>LDLc/HDLc</td>
<td>-0.33</td>
<td>0.11</td>
<td>-0.53</td>
<td>0.007</td>
<td>-0.46</td>
<td>0.02</td>
</tr>
<tr>
<td>TC/HDLc</td>
<td>-0.29</td>
<td>0.16</td>
<td>-0.44</td>
<td>0.03</td>
<td>-0.37</td>
<td>0.07</td>
</tr>
<tr>
<td>HDLc</td>
<td>0.04</td>
<td>0.83</td>
<td>0.30</td>
<td>0.14</td>
<td>0.32</td>
<td>0.12</td>
</tr>
</tbody>
</table>

TC: total cholesterol, TG: triglycerides, LDLc: low density lipoprotein cholesterol, HDLc: high density lipoprotein cholesterol, apo-B100: apolipoprotein B100, apo-A1: apolipoprotein A1, LDLc/HDLc: low density lipoprotein cholesterol to high density lipoprotein cholesterol ratio, TC/HDLc: total cholesterol to high density lipoprotein cholesterol ratio.