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Curcuminoids Modulate Pro-Oxidant–Antioxidant Balance but not the Immune Response to Heat Shock Protein 27 and Oxidized LDL in Obese Individuals

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Curcuminoids have potentially important functional qualities including anti-inflammatory and antioxidant properties. In this randomized double-blind placebo-controlled cross-over trial, the effects of a curcuminoid supplement on serum pro-oxidant–antioxidant balance (PAB) and antibody titres to Hsp27 (anti-Hsp27) and oxLDL (anti-oxLDL) were investigated. Thirty obese individuals were randomized to receive either curcuminoids (1 g/day) or placebo for a period of 30 days. After a wash-out period of 2 weeks, subjects were crossed over to the alternate regimen for another 30 days. Serum PAB along with anti-Hsp27 and anti-oxLDL titres was measured at the beginning and at the end of each study period. There was no significant carry-over effect for any of the assessed parameters. Curcuminoid supplementation was associated with a significant decrease in PAB ($p = 0.044$). However, no significant change was observed in serum concentrations of anti-Hsp27 or anti-oxLDL ($p > 0.05$). These findings suggest that oral curcuminoids supplementation (1g/day) is effective in reducing oxidative stress burden, though this needs to be validated in larger study populations. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: curcumin; heat shock protein 27; oxidized low-density lipoprotein; oxidative stress.

INTRODUCTION

Curcumin (diferuloyl methane; $C_{21}H_{20}O_{6)}$ is a hydrophobic polyphenol that may be extracted from dried rhizomes of the herb *Curcuma longa* L. (turmeric). Turmeric is widely used as a spice in Indian, Middle Eastern and South Eastern Asian cooking, and its diverse medicinal properties have been applied in several traditional systems of medicine (Lao *et al*., 2006; Anand *et al*., 2008). Recent studies have shown that curcumin has potentially important biological activities including anti-inflammatory, antioxidant, immunomodulatory, neuro- and cardioprotective properties. It has therefore been proposed for the treatment of various types of cancer, arthritis, cardiometabolic, cystic fibrosis and pulmonary disorders (Liu *et al*., 1997; Bharti *et al*., 2003; Duvoix *et al*., 2003; Uddin *et al*., 2005; Ak and Gülçin, 2008; Goel *et al*., 2008; Aftab and Vieira, 2010; Ma *et al*., 2010; Malhotra *et al*., 2010; Chandran and Goel, 2012; Panahi *et al*., 2012a, 2012b; Tu *et al*., 2012; Sahebkar, 2012).

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Oxidative stress is a condition arising from a physiological imbalance between pro-oxidant and biological antioxidant species (Choi and Rothman, 1990). Oxidative stress plays a significant role in the pathogenesis of various disorders including atherosclerosis and subsequent cardiovascular disease (Ashok and Ali, 1999). One of the principal mechanisms for the involvement of oxidative stress in the pathogenesis of atherosclerosis is oxidation of LDL and formation of oxidized LDL (oxLDL) particles. Upon oxidation, the affinity of LDL for its receptor decreases whilst it becomes more prone to interact with scavenger receptors of macrophages and promote foam cell formation (Steinberg, 1997). OxLDL has well-known immunogenic properties and induces an immune response that is related to the severity of atherosclerosis (Shoenfeld *et al*., 2004). OxLDL is toxic for many of cells such as endothelial cells and impairs the integrity of such cells (Hessler *et al*., 1983). OxLDL also triggers chemotaxis of circulating monocytes which is regarded as an important step in the progression and exacerbation of atherosclerosis (Quinn *et al*., 1987).

Another important consequence of oxidative stress is inducing the expression and release of heat shock proteins (Hsps). Hsps are abundant intracellular proteins that protect cells under stress. The upregulation of these proteins during stressful conditions activates the cell protective mechanisms (Pourghadamyari *et al*., 2011).

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Hsps comprise several families of molecules expressed in cells under physiological and environmental stress conditions. Free radicals, toxins, infections, ischemia, mechanical and oxidative stress are among the known inducers of Hsps (Ghayour-Mobarhan *et al.*, 2005; Riganò *et al.*, 2007). Hsp27 is a member of the small Hsp family that is constitutively expressed in many cell types including cardiomyocytes and endothelial cells (Tucker *et al.*, 2009; Gernold *et al.*, 1993; Pauli *et al.*, 1990). Several investigations have reported an elevation of circulating levels of Hsp27 and its corresponding antibodies (anti-Hsp27) with coronary artery disease (CAD) (Pourghadamyari *et al.*, 2011), acute coronary syndrome (ACS) (Heidari-Bakavoli *et al.*, 2012; Ghayour-Mobarhan *et al.*, 2008) and metabolic syndrome (Sahebkar *et al.*, 2011).

Obesity is a major health problem in the world (Mohammadi *et al.*, 2012) and is closely associated with type 2 diabetes, cardiovascular disorders, hypertension, hyperglycemia, atherosclerosis and cancer (Esmaillzadeh *et al.*, 2006; Bibbins-Domingo *et al.*, 2007; Furukawa *et al.*, 2004). Obese individuals are known to be at a higher risk of dyslipidemia and inflammation compared to subjects with normal body mass index (BMI) (Bibbins-Domingo *et al.*, 2007; Wilson *et al.*, 2005). Although the global awareness on the deleterious effects of overweight and obesity has increased in recent decades, the prevalence of morbidity and mortality due to these conditions has yet an increasing trend (Levi *et al.*, 2007; Kuczmarski *et al.*, 1994). In Iran, approximately 60% of the total population is either overweight or obese (Esmaillzadeh *et al.*, 2005). With respect to Mashhad as the second largest city in Iran, the statistics have shown a prevalence of about 28.9% and 11.7% for overweight and obesity, respectively (Nematy *et al.*, 2009).

The potential role of immune responses to oxLDL and Hsp27 in the pathogenesis of atherosclerosis, and the well-documented anti-inflammatory, antioxidant and immunomodulatory effects of curcumin led to the hypothesis being tested in, the present study, whether curcumin supplementation affects the circulating levels of anti-oxLDL and anti-Hsp27 in obese individuals. The impact of curcumin therapy on the overall burden of oxidative stress was determined by means of a simple and rapid assay of pro-oxidant–antioxidant balance (PAB).

MATERIALS AND METHODS

Subjects. Individuals who were aged 18–65 years were recruited from the Ghaem Hospital, Mashhad, Iran. Inclusion criteria were any of the following conditions [based on the NCEP-ATP III guidelines (Manjunatha and Srinivasan, 2007)]: (i) patients with $<$ 2 risk factors (except diabetes mellitus) for coronary heart disease (CHD) and 160 mg/dL $<$ LDL-C $<$ 190 mg/dL, (ii) patients with BMI $\geq$ 30 and (iii) patients with $\geq$ 2 risk factors (except diabetes mellitus) for CHD and 130 mg/dL $<$ LDL-C $<$ 160 mg/dL. Cardiovascular risk factors were obesity, defined as BMI [BMI $\geq$ 30 kg/m^2], age $>$ 65 years, male sex, hypertension (defined as taking any anti-hypertensive medication; or systolic blood pressure $\geq$140 mmHg or diastolic blood pressure $\geq$ 90 mmHg), smoking, positive family history of CHD and diabetes mellitus (defined as fasting blood sugar $\geq$ 126 mg/dL).

Exclusion criteria were: a history of systemic disease, history of CHD, or of taking any lipid lowering drugs or supplements within the previous 6 months. Each subject gave informed written consent to participate in the study, which had previously been approved by the Mashhad University of Medical Science Ethics Committee. Participants were advised to continue their normal medication schedule.

Study design. The study comprised a 10-week randomized and double-blind trial with a two-arm two-period (2×2) cross-over design (Fig. 1). Eligible participants were assigned to receive curcuminoids (1 g/day) or matched placebo for 30 days and then crossed over to the alternate treatment following a 2-week washout period. The number of randomized subjects in each treatment arm was 15. Four overnight fasting blood samples were collected from each subject before and after each treatment period. Administered curcuminoid capsules contained 500 mg C3 Complex® curcuminoids formula + 5 mg bioperine® (Sami Labs LTD, Bangalore, India). Bioperine® is an extract obtained from black pepper (*Piper nigrum* L.) or long pepper (*Piper longum* L.), and contains 95% piperine, a well-known bioavailability booster. Placebo capsules contained piperine (5 mg) and were matched with curcuminoids capsules regarding shape and size. Four overnight fasting blood samples and anthropometric data were collected from each participant before and after each dietary treatment phase.

Anthropometric measurements. Anthropometric parameters including weight, height, BMI and weight were measured with the subjects dressed in light clothing after an overnight fasting using a standard scale. BMI was calculated as weight (kg) divided by height squared (m^2).

Blood sampling. Blood samples for each subject were collected at baseline and at the end of each treatment period after a 12-h fasting. Blood was collected in Vacutainer® tubes and centrifuged at 10,000g for 15 min

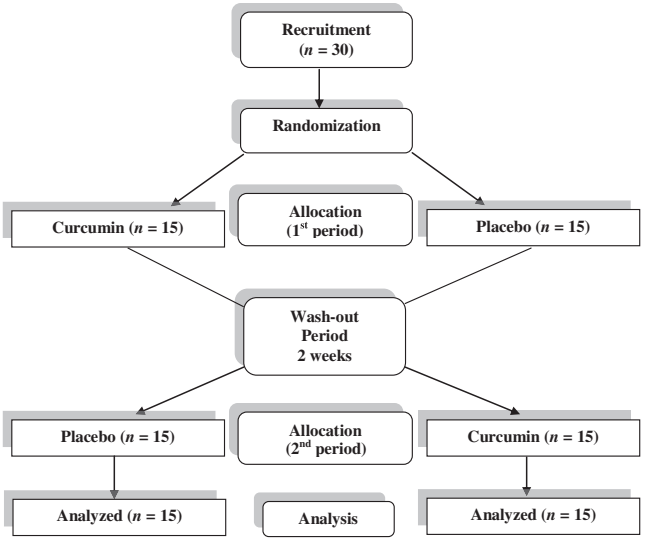

Figure 1. Flow chart of trial.

for laboratory assays to obtain serum. Aliquots of serum were frozen at −80 °C until analysis.

Serum anti-Hsp27 assay. An in-house ELISA assay was applied to determine serum anti-Hsp27 levels as described previously (Sahebkar *et al*., 2011). In brief, microtitre plates were coated with recombinant human Hsp27. After repeated washing with phosphate buffered saline (PBS), serum was diluted 1:100 with 2% goat serum in PBS (for blocking and reduction of non-specific binding), added to each well in duplicate and incubated for 30 min at room temperature (RT). Afterwards, wells were washed and treated with peroxides conjugated-goat anti-human IgG (Sigma-Aldrich, Poole, UK) diluted with 2% goat serum in PBS. Following an incubation for 30 min at RT and subsequent washing, tetramethylbenzidine (TMB) substrate solution was added per well and plates incubated for 15 minutes in the dark at RT. The reaction was terminated by adding HCl, and optical density was read at 450 nm with a reference wavelength of 620 nm.

Serum anti-oxLDL assay. Serum antibody titers to oxLDL were measured by a sandwich ELISA technique using a commercially available kit (Immunodiagnostics Co.). All samples and reagents were brought to RT and mixed prior to the assay. Then, pre-diluted samples and standards were added into wells of microtitre plate coated with oxLDL. Following 2 h of incubation at RT on a shaker, wells were aspirated and repeatedly washed. Then, peroxidase-labeled conjugate was added to each well, and covered plates were incubated for 1 h on a shaker. Finally, the wells were aspirated, washed and treated with TMB as a peroxidase substrate. After 10–20 min of incubation in the dark, the colorimetric reaction was terminated by adding sulfuric acid stop solution followed by vigorous mixing. Immediately, the intensity of appeared yellow color, which is directly proportional to the anti-oxLDL concentration, was read at 450 nm using a microplate ELISA reader. Anti-oxLDL concentration in each sample was calculated from a calibration curve of standards. All assay steps were conducted at RT.

PAB assay. A modified PAB assay was applied based on a previously described method (Alamdari *et al*., 2008).

Statistical analysis. Values were expressed as means ± SD or, in the case of non-normally distributed data, as median and interquartile range. The Wilcoxon signed rank test or paired *t* test were done for comparison between pre- and post-treatments. Data obtained from independent variables analyzed using Mann–Whitney U test (for those without a normal distribution) or using Student's *t* test (for those with a normal distribution). Categorical data were compared using chi-squared test. Mixed model analysis of variance for 2 × 2 cross-over studies were fitted when assumption for normality were met. We performed all analysis with the Statistical Analysis Software (SAS version 9.1). A two-sided *p* value of <0.05 was considered statistically significant.

RESULTS

The mean age, BMI, height, weight, PAB, anti-Hsp27 and anti-oxLDL of study participants were 38.43 ± 10.84 years, 32.60 ± 3.58 kg/m^2, 158.62 ± 7.82 cm, 82.10 ± 11.58 kg, 93.74 ± 3.04, 0.26 ± 0.22 and 1.00 ± 0.60, respectively. Five of the subjects were male (16.7%). For none of the assessed parameters was there a significant carry-over effect between the trial periods in either of the groups. The results failed to reveal any significant effect of curcumin supplement on weight, BMI, anti-Hsp27 and anti-oxLDL values ($p > 0.05$). However, curcumin supplementation was associated with a significant reduction in serum PAB ($p = 0.044$). Baseline and post-trial values for anthropometric parameters, PAB, anti-Hsp27 and anti-oxLDL in each study period are summarized in Table 1.

DISCUSSION

There has been a large body of evidence supporting the antioxidant functions of curcumin. This phytochemical has been shown to inhibit the generation of ROS in several cell types including macrophages and erythrocytes. In addition, curcumin effectively scavenges different classes of free radicals, most importantly, superoxide and hydroxyl anions. The metal-chelating activity of curcumin is an important mechanism for its radical scavenging effects and blocks the promotion of metal ion-induced radical generating chain reactions (Baum and Ng, 2004; Daniel *et al*., 2004). Interestingly, metal complexes of curcumin are reported themselves to be effective radical scavengers (Baum and Ng, 2004). Curcumin can also inhibit different types of nitric oxide synthase enzymes, thereby decreasing NO bioavailability and conferring protection against NO-driven reactive intermediates (Amin and Bano, 2012). It has been demonstrated that curcumin treatment can efficiently mitigate lipid peroxidation and upregulate the expression and activity of different antioxidant enzymes such as activated protein-1, heme oxygenase-1, superoxide dismutase, glutathione peroxidase and catalase Manikandan *et al*., 2004; Rukkumani *et al*., 2004; Panahi *et al*., 2012). These antioxidant effects of curcumin might protect against formation of oxLDL and its associated antibodies. To the current knowledge, oxLDL is the leading triggering factor for atherogenesis. Deleterious effects of oxLDL are exerted through induction of leukocyte adhesion and infiltration, smooth muscle cell proliferation and migration, vasoconstriction, endothelial cell dysfunction and apoptosis, and platelet adhesion and aggregation (Mertens and Holvoet, 2001).

Although the effects of curcumin on the pro-oxidant–antioxidant status have been reported by a few *in-vitro* studies (Ahsan *et al*., 1999; Banerjee *et al*., 2008), there has been no previous cross-over trial designed to verify the antioxidant properties of curcumin. In previous studies, PAB values have been shown to be elevated in patients with established CAD and ACS (Alamdari *et al*., 2008). Furthermore, it has been suggested that PAB may be a potential cardiovascular risk predictor (Alamdari *et al*., 2008). Several trials have applied the PAB assay described here as an index of oxidative stress

Table 1. Effect of curcumin supplementation on evaluated anthropometric and biochemical measures.

	Study group	N	First period		Second period		*p*-value	
			Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Period effect	Treatment effect
Weight (kg)	Curcumin-Placebo	15	84.1 ± 13.5	84.3 ± 14.6	83.6 ± 15.1	82.9 ± 14.9	0.71	0.23
	Placebo-Curcumin	15	80.1 ± 9.3	78.7 ± 8.7	78.6 ± 9.2	78.3 ± 10.2		
BMI (kg/m^2)	Curcumin-Placebo	15	33.4 ± 3.7	33.4 ± 4.4	33.2 ± 4.8	32.9 ± 7.8	0.61	0.21
	Placebo-Curcumin	15	31.8 ± 3.4	31.2 ± 3.1	31.2 ± 3.3	31.0 ± 3.4		
PAB (AU)	Curcumin-Placebo	15	95.26(83.33–130.66)	94.1(77.18–122.01)	87.99(71.87–110.72)	78.3 (54.54–102.98)	0.15	0.04
	Placebo-Curcumin	15	73.45(64.12–87.18)	82.69(55.53–111.98)	60.63(55.56–84.74)	57.4 (46.28–74.24)		
Anti-Hsp27 (OD)	Curcumin-Placebo	15	0.23(0.09–0.45)	0.23(0.17–0.51)	0.27(0.13–0.42)	0.3 (0.14–0.43)	0.35	0.64
	Placebo-Curcumin	15	0.21(0.1–0.4)	0.25(0.12–0.4)	0.17(0.07–0.38)	0.3 (0.07–0.43)		
Anti-oxLDL (OD)	Curcumin-Placebo	15	0.8 (0.6–1.3)	0.9 (0.73–1.25)	0.8 (0.59–1.39)	0.5 (0.49–1.33)	0.21	0.3
	Placebo-Curcumin	15	0.8 (0.6–1.1)	0.7 (0.44–1.09)	0.6 (0.46–1.00)	0.6 (0.49–1.01)		

All values are expressed as mean ± SD or median (interquartile range). Hsp27: Heat shock protein 27; ox-LDL: oxidized low-density lipoprotein.

(Ghayour-Mobarhan *et al*., 2009; Yazdanpanah *et al*., 2011; Parizadeh *et al*., 2011a, 2011b). While it still remains to be determined by future studies if PAB could be used as a sensitive and specific marker of oxidative stress-related disorders, current available evidence clearly indicate that PAB values are reduced following antioxidant therapy (Tara *et al*., 2010; Parizadeh *et al*., 2011a, 2011b; Boskabadi *et al*., 2010). PAB values have been reported to show a linear response in response to increasing concentrations of various pro-oxidants (PAB elevation) and antioxidants (PAB decline). The results of PAB assay have also been shown to correlate with those of other routinely applied oxidative stress tests such as carbonyl assay, advanced glycation end products assay and advanced oxidative protein products assay (Alamdari *et al*., 2007). Hence, PAB reduction by curcumin may imply that this phytochemical has cardioprotective effects. Contrary to our expectations, mitigation of oxidative stress by curcumin was not accompanied by concomitant reduction in serum anti-oxLDL titres. This might have been due to insufficient dose or duration of treatment of the supplementation with curcumin.

Another parameter that was evaluated in the present study was serum antibody titres to Hsp27. Hsps have been reported to be overexpressed in response to stressful conditions such as presence of some risk factors of CHD, e.g. toxins, infections, ox-LDL, hypertension and oxidative stress (Pauli *et al*., 1990). Hsps have immunogenic properties and are present in serum of patients with CHD. Furthermore, Hsps can form immune complexes with their corresponding antibodies. These complexes play a role in the progression of atherosclerosis because of their pro-inflammatory properties. Hsp27 is present in atherosclerotic plaques, and its antibodies have been reported in plasma of patients with atherosclerosis (Riganò *et al*., 2007). There is also evidence indicating elevated serum levels of both Hsp27 and anti-Hsp27 in patients with myocardial infarction and unstable angina (Pauli *et al*., 1990). It has been suggested that high levels of anti-Hsp27 could be considered as a risk factor for CHD (Mertens and Holvoet, 2001). Hence, factors capable of reducing anti-Hsp27 to the normal level could potentially prevent the progression of atherogenesis. So far, no clinical study has explored the effect of curcumin on circulating antibody titers to Hsp27 or any other type of Hsp. The findings of the present study failed to detect any significant impact of curcumin supplementation on serum anti-Hsp27 titres. Therefore, it appears that curcumin modulates cardiovascular risk through mechanisms other than blocking the immune response toward small Hsps.

The present trial had several limitations that are worth noting. First, the trial recruited a relatively small number of patients. This might have negatively impacted the statistical power of study to detect significant differences in the assessed biochemical parameters. Second, duration of curcumin therapy in the current trial was 30 days that may not be sufficiently long to observe significant changes, if any, in the immunologic parameters such as anti-HSP27 and anti-oxLDL titres. Third, dietary records were not obtained from the study participants. As intake of micronutrients, in particular antioxidants, could alter serum antibody titres to Hsps and PAB, future studies in this field are greatly

recommended to monitor and normalize dietary intakes between curcumin and placebo supplementation groups and/or periods. Finally, it would be interesting to test if higher doses of curcumin would be associated with significant changes in serum anti-oxLDL and anti-Hsp27 titres, and also whether the observed curcumin-induced change in PAB is dose dependent.

CONCLUSION

The findings of the present study indicated that curcuminoid supplementation (1 g/day for 30 days) leads to a significant reduction in PAB but not serum anti-Hsp27 and anti-oxLDL concentrations. While these findings need to be confirmed in future by larger scale trials and studies over longer durations of follow-up, they suggest that modulation of systemic oxidative stress is an important mechanism that might account, at least in part, for the well-known cardioprotective effects of curcumin.

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Conflict of Interest

The authors have no competing interest to declare.

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