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Saffron in metabolic syndrome: its effects on antibody titers to heat-shock proteins 27, 60, 65 and 70

Abstract

Background: The metabolic syndrome is the most important risk factor for cardiovascular disease. The heat shock proteins (HSPs) are highly conserved families of proteins expressed by a number of cell types following exposure to stressful environmental conditions include several known risk factors for cardiovascular disease. Recent studies have shown the potential of constituents of saffron in the treatment of atherosclerosis. We aimed on investigating the effect of saffron on antibody titers to HSP in patients with metabolic syndrome.

Methods: This was a randomized, placebo-controlled clinical trial. One-hundred and five subjects with metabolic syndrome were randomly allocated to one of the three groups: the case group received 100 mg/day saffron, the placebo control group received a capsule of placebo and a non-placebo control group received no capsule, for 12 weeks.

Results: Antibodies against heat shock proteins 27, 60, 65 and 70 were determined in all patients before (week 0) and after (week 6 and 12) intervention. At 12 weeks, saffron produced a significantly decrease in AntiHSP27, 70 levels. Saffron can decrease AntiHSP27, 70 levels in patients with metabolic syndrome.

Conclusions: The results of this study indicate the efficacy of saffron in the improvement of some markers of autoimmunity HSPs in patients with metabolic syndrome.

Keywords: metabolic syndrome, anti heat shock proteins, saffron, cardiovascular disease (CVD), heat shock proteins (HSPs), American Heart Association (AHA), waist circumference (Wc), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), fasting blood glucose (FBS)

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Introduction

The heat shock proteins (HSPs) are families of highly conserved protein chaperons that are expressed by cells in response to a variety of stressful stimuli that include free radicals, heat, sheer stress, toxins, ischemia (hypoxic stress, reperfusion and oxidative stress), oxidized low-density lipoprotein (ox-LDL), mechanical stress, oxidants and cytokine stimulation, via the activation of heat shock transcription factor 1 [1]. HSPs have been classified into seven major families on the basis of their molecular mass and include: HSP10 (HSPE), small HSPs [15–30 kDa, of which HSP27 (HSPB1) is a member], HSP40 (DNAJ), HSP60 (HSPD), HSP70 (HSPA), HSP90 (HSPC) [2], and HSP100. Several HSPs function as molecular chaperones [3]. As new proteins are being produced by ribosomes, HSPs assist in correct folding of polypeptide chains into functional proteins, and after a stress event, HSPs assist in refolding of partially denatured proteins, preventing protein

aggregation and targeting improperly folded proteins to specific degradative pathways [4]. Among the factors that have been shown to induce HSPs are several known potential risk factors for CVD that stimulate HSP expression by macrophages and smooth muscle cells [5]. Therefore, there is a potential relationship between serum HSPs, their antibody concentrations, and CVD that has been the subject of several investigations in the past two decades. Clinical studies have reported positive associations between plasma antibody titers to HSPs and cardiovascular disease [6]. HSPs have been shown to be expressed in atherosclerotic plaques of both experimental animals and humans [7]. It has been shown that antibody titers to HSP60/65 are associated with the severity and progression of cardiovascular disease [8, 9]. Interest has been particularly focused on HSP60 and HSP70 [10, 11].

The metabolic syndrome refers to the clustering of cardiovascular risk factors that include diabetes, obesity, dyslipidemia and hypertension [12, 13]. The metabolic syndrome has been used as a clinical tool for the detection of individuals at increased risk of atherosclerotic cardiovascular disease. It has been estimated that people with the metabolic syndrome are at twice the risk of developing cardiovascular disease compared to those without the syndrome [14]. Oxidative stress, specifically the oxidation of LDL, has long been suspected of having a critical role in the development of atherosclerosis [15]. The relationship between Ox-LDL and the progression of atherosclerosis has recently become more fully understood. So the antioxidants, which decrease the level of Ox-LDL temporarily, have been expected to have potentials similar to antiatherogenic agents [16].

Saffron (*Crocus sativus*) is a bulbous perennial of the iris family (Iridaceae) valued for its golden-colored, pungent stigmas, which are dried and used to flavor and color foods as well as a dye [20]. The value of saffron is determined by the existence of three main secondary metabolites: crocin and its derivatives which are responsible for color; picrocrocin, responsible for taste; and safranal responsible for odor [17]. Indeed, saffron contains over 150 volatile and aroma-yielding compounds [18]. It also contains non-volatile active components, many of which are carotenoids, including zeaxanthin, lycopene, bcarotenes and polysaccharides. It has been reported that *C. sativus* has anti-inflammatory, anticancer and antiplatelet effects [19]. Saffron has also reported to have antioxidative and hypolipidemic effects [15]. Recent studies have shown the potential of saffron derived compounds in the treatment of cardiovascular risk factors [16, 20].

Because of the relationship between cardiovascular risk factors and elevated expression of HSPs, and the potential of saffron constituents in the prevention of atherosclerosis and related diseases, saffron may be expected to have an effect on serum HSP concentrations. In the present study, we have investigated the changes in antibody titers to HSP60, 65, 70 and 27 after saffron consumption in subjects with metabolic syndrome.

Materials and methods

This was a 12-week randomized and double-blind clinical trial. The investigation was conducted in the Nutrition clinic of Qaem Teaching Hospital, Mashhad, Iran, between September 2010 and March 2011.

Participants

A total of 105 subjects with metabolic syndrome (defined by using the International Diabetic Federation criteria, 2005), aged 18–75, were recruited. Participants were provided with information on the study protocol by verbal explanation and written information sheets. Patients with systemic diseases (such as diabetes mellitus, AIDS, rheumatoid arthritis) and pregnant women were excluded from the study. All patients provided written informed consent and the study protocol satisfied Mashhad University of Medical Sciences Ethics Committee requirements.

Study design

Patients were randomly divided into three groups with 35 individuals in each using a computer-generated code: a case group, received capsules of saffron 50 mg twice daily; a placebo control group, receiving a capsule of placebo (BD) and further control group, received no capsule at all; for 12 weeks for each study arm. All patients were also given dietary advice according to the AHA guidelines during the study. Compliance was monitored by three-weekly follow-up sessions, as assessed by counting capsules; subjects who did not take their capsules regularly or who were intolerant were excluded from the study. Antibody titers to HSP60, 65, 70 and 27 were determined in all patients before (week 0) and after (week 6 and 12) the intervention.

Saffron capsule preparation

Crocus sativus L. stigma was supplied by the Novin Saffron Co. (Mashhad, Iran). It was formulated as a capsule containing 50 mg of dried saffron stigma. Placebo capsules were matched for size, shape and volume of content and manufactured by the same company.

Data collection

The following data were collected from each participant.

Questionnaires: Questionnaires were administered to collect information on socio-demographic status, occupation, smoking behavior, medical history and medication. Physical activity level was self-reported as mild, moderate and heavy.

Anthropometry: For all patients, anthropometric parameters including weight, height, WC and hip circumference were determined using a standard protocol after an overnight fasting. Height was measured without shoes to the nearest 0.1 cm. Weight was measured in light clothing without shoes to the nearest 0.1 kg. Hip circumference was measured at the level of maximum extension of the buttocks and waist circumference was measured mid-way between the lateral lower rib margin and the iliac crest with the scale to the nearest ± 0.1 cm.

Blood pressure: Blood pressure was measured while the patients were seated and rested for 15 min, using a standard protocol.

Blood sampling: Blood samples were collected from each subject in the morning after a 12 hour fast. Hemolysed samples were excluded from analysis. After separation, aliquots of serum were frozen at −80°C until analysis.

Laboratory assays: A full fasted lipid profile comprising of total cholesterol, triglycerides, HDL-C and LDL-C was determined for each subject. Serum lipid and FBS concentrations were measured enzymatically using commercial kits.

Serum HSP27 antibody titers were measured using an in-house ELISA assay. Microtiter plates (Nunc Maxisorp, Nottingham, UK) were coated with 100 ng per well of recombinant human HSP27 dissolved in 50 μL of carbonate buffer, pH = 9.6, incubated for 18 h at 4°C under humidified conditions. The wells were washed three times in a wash buffer (PBS containing 0.05% Tween-20). Non-specific binding was reduced by blocking each well with 2% of goat serum in PBS and 250 μL added to each well and incubated for 30 min in 37°C and 30 min at room temperature. The wells were washed three times with PBS. Serum was diluted 1:100 with 2% goat serum in PBS and 100 μL added to the each well in duplicated and incubated for 30 min at room temperature. After washing (four times in wash buffer and two times in PBS), 100 μL peroxidase conjugated-goat antihuman IgG (Sigma-Aldrich, Poole, UK) diluted 1:500 with 2% goat serum in PBS was added to each well and incubated for 30 min at room temperature. After washing (four times in wash buffer and two times in PBS), 100 μL of TMB substrate (100 μL of 6 mg/mL TMB in DMSO was added to 10 mL of 50 mM acetate buffer, pH = 4.5, containing 3 L H_2O_2) was added per well and plate incubated for 15 min in the dark at room temperature. The reaction was terminated by adding 50 μL 2 M HCl per well. Optical density at 450 nm was measured using a Labsystems iEMS Reader MF Microtiter plate reader with a reference wavelength of 620 or 570 nm. The within-assay and between-assay precision was 3.5% and 5.2% respectively. After correction for the non-specific background absorbance (subtracting the absorbance of uncoated wells from the antigen-coated wells for each sample), the results were expressed in optical density units.

Serum HSP60, 65 and 70 antibody titers were measured by using in-house enzyme-linked immunosorbent assays. In brief, a 96-well microtiter plate (Nunc Immunoplate Maxisorp; Scientific Laboratory Supplies Ltd, Nottingham, United Kingdom) was coated with human recombinant HSP60, 65, or 70 by adding 10 ng recombinant HSP in phosphate-buffered saline (PBS) to the wells of a microtiter plate and incubating overnight at 4°C. Plates were washed with PBS, blocked with Superblock (Pierce & Wariner, Chester, United Kingdom), and washed three times with PBS containing 0.05% (by vol) Tween-20. Plasma samples were diluted 1:15 with PBS containing 0.1% Tween-20 and 1% bovine serum albumin (PBT; Sigma-Aldrich Inc, Poole, United Kingdom), and 100 L/well in quadruplicate was incubated for 30 min at 37°C. After washing, bound HSP antibodies were detected by the addition of peroxidase-conjugated goat anti-human immunoglobulin G, which was diluted 1:100 with PBT. After washing with PBS/Tween-20, *o*-phenylenediamine (0.04%) [dissolved in 0.05 mol citrate/L with 0.1 mol phosphate buffer/L (pH 5) and containing 10 L of a 30% solution of H_2O_2/25 mL] was added and incubated for 5 min at room temperature. The reaction was terminated by the addition of 3 mol hydrochloric acid/L. The absorbance was read at 492 nm by using a plate reader with GENESIS 2 software (version 2; Life Sciences, Basingstoke, United Kingdom).

Statistical analysis

All statistical analyses were performed using the SPSS for Windows™, version 11.5 software package (SPSS Inc., Chicago, IL, USA). Data were assessed for normality by using the Kolmogorov–Smirnov test. Data were expressed as mean ± SD or median and interquartile range. Group comparisons were performed using ANOVA or Kruskal–Wallis test. Data that were normally distributed were analyzed using one-way analysis of variance (ANOVA). Data found not to be normally distributed were analyzed using the non-parametric Kruskal–Wallis test. Categorical data were compared using Chi-square test. A two-sided p-value < 0.05 was considered as statistically significant.

Results

Comparison of the baseline characteristics of the study and control groups

Ninety one subjects (26 individuals from the study group, 30 from the placebo control group and 35 from the non-placebo control group) completed the trial; 14 participants were excluded; 1 due to symptoms of saffron-induced allergy, 2 because of pregnancy, 2 due to the need for an operative procedure for an unrelated reason and 9 others because they were not taking their capsules regularly.

No significant differences were identified between patients randomly assigned to the study or the two control groups regarding baseline characteristics ($p>0.05$) (Table 1).

Effect of saffron on anthropometric parameters and lipid profile

Weight, waist and hip circumference decreased significantly after 12 weeks in all three groups. Serum total cholesterol, low-density lipoprotein cholesterol (LDL-C) and triglyceride decreased while high-density lipoprotein cholesterol (HDL) increased in all three groups. Reduction in serum TC and LDL-C were significant in the case group alone.

Effect of saffron on serum HSPs antibody titers

After 12 weeks of treatment initiation serum HSP27, HSP60, HSP65 and serum HSP70 antibody titers fell in all three groups (Table 2), though a significant reduction

Table 1 Baseline characteristics.

	Case group	Placebo control group	Non-placebo control group
Women, n	21	19	23
Men, n	5	11	12
Age, year	42.19 ± 11.52	43.60 ± 9.05	47.03 ± 13.82
Weight, kg	80.63 ± 11.65	79.30 ± 16.43	78.78 ± 16.62
Height, cm	161.65 ± 9.53	161.90 ± 7.30	160.68 ± 7.52
WC, cm	105.76 ± 9.01	103.36 ± 12.09	104.45 ± 11.80
HC, cm	115.61 ± 9.49	112.60 ± 10.89	113.75 ± 10.39
Smokers, %	11.5	10	7
Diabetics, %	23.1	20	20
Hypertensive, %	23.1	23.3	22.9
Hyperlipidemic, %	23.1	23.3	22.9
Triglycerides, mg/dL	139.76 ± 70.14	139.00 ± 73.52	154.05 ± 73.12
Total cholesterol, mg/dL	214.15 ± 27.30	227.16 ± 33.34	235.65 ± 43.28
HDL-C, mg/dL	39.03 ± 5.34	39.13 ± 8.00	39.54 ± 8.54
LDL-C, mg/dL	120.03 ± 30.01	125.16 ± 22.33	126.03 ± 32.73
FBS, mg/dL	109.69 ± 24.02	108.33 ± 22.26	117.80 ± 37.96
SBP, mmHg	11.73 ± 1.04	11.61 ± 0.94	11.42 ± 1.19
DBP, mmHg	7.69 ± 1.04	7.78 ± 1.09	7.95 ± 0.84

WC, waist circumferences; HC, hip circumferences; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FBS, fasting blood sugar; DBP; diastolic blood pressure, SBP; systolic blood pressure. Values are expressed as mean ± SD. Chi-square, one-way analysis of variance (ANOVA), and Kruskal–Wallis tests were used to compare qualitative and quantitative (normal and non-normal) variables, respectively.

Table 2 Changes in serum HSP27, 60, 65 and 70 antibody titers in different groups (median ± SD).

	Week 0–6	Week 6–12	Week 0–12
Case:			
Serum HSP27 antibody titer	0.012 ± 0.092	0.011 ± 0.057	0.041 ± 0.108[a]
Serum HSP60 antibody titer	0.034 ± 0.188	−0.003 ± 0.176	0.028 ± 0.262
Serum HSP65 antibody titer	0.007 ± 0.1398	0.013 ± 0.1922	0.090 ± 0.1776
Serum HSP70 antibody titer	0.120 ± 0.267	−0.065 ± 0.217	0.020 ± 0.165[a]
Positive control group:			
Serum HSP27 antibody titer	−0.001 ± 0.190	0.013 ± 0.207	0.009 ± 0.163
Serum HSP60 antibody titer	0.042 ± 0.123	0.0460 ± 0.201	0.050 ± 0.153
Serum HSP65 antibody titer	0.007 ± 0.1153	0.010 ± 0.1268	0.007 ± 0.1644
Serum HSP70 antibody titer	0.045 ± 0.286	−0.050 ± 0.232	0.005 ± 0.306
Negative control group:			
Serum HSP27 antibody titer	0.033 ± 0.199	0.026 ± 0.228	0.022 ± 0.238
Serum HSP60 antibody titer	−0.006 ± 0.073	0.000 ± 0.091	0.010 ± 0.091
Serum HSP65 antibody titer	0.001 ± 0.186	−0.008 ± 0.1374	0.012 ± 0.2010
Serum HSP70 antibody titer	0.000 ± 0.104	−0.016 ± 0.092	−0.009 ± 0.096

[a] $p < 0.05$.

in serum HSP27 and HSP70 antibody titers were seen in the saffron-treated group ($p < 0.05$) alone.

Serum HSP27 antibody titer decreased by 0.041 ± 0.108 (mg/dL) (median ± SD), 0.009 ± 0.163 and 0.022 ± 0.238, in the saffron, placebo control and non-capsule control groups, respectively. Serum HSP60 antibody titer decreased by 0.028 ± 0.262 (mg/dL) (median ± SD), 0.050 ± 0.153 and 0.010 ± 0.091; serum HSP65 antibody titer decreased by 0.090 ± 0.1776 (mg/dL) (median ± SD), 0.007 ± 0.1644 and 0.012 ± 0.2010 and serum HSP70 antibody titer decreased by 0.020 ± 0.165 (mg/dL) (median ± SD), 0.005 ± 0.306 and −0.009 ± 0.096 in the same groups, respectively (Table 2).

Discussion

In this study, the serum antibody titers to HSP27, 60, 65 and 70 fell in all three groups and probably relates to the dietary changes recommended to all subjects. However, this reduction was only significant for the anti-HSP27 and 70 antibody. Considering the power of this study to show difference between the three groups of 30 subjects each and with a 95% confidence interval, the effect of saffron on anti-HSP70 titers was 0.111. In order to reach a power of 80%, approximately 200 subjects would be required. To the authors' knowledge, no study has yet been performed on the relationship between saffron and anti-HSPs but the effect of other food-derived nutrients has been studied on HSPs in animal models. Kelly et al. [21] have shown that vitamin E deficiency for 16 weeks results in the expression of HSP27 in mice. Andres et al. [22] and Andres and Cascales [23] revealed that cyclosporine A which stimulates the expression of HSP70 in mice hepatocytes by the release of active oxygen species, reduces significantly in the presence of vitamin E (in vitro). It has been reported that vitamin C results in the increased expression of HSP60 and HSP70 (in vitro) but its mechanism of action is still unknown [24]. Romano et al. [25] have reported that a diet rich in saturated fatty acids leads to the expression of HSP25, HSP60 and HSP70 in the mouse spleen lymphocytes. It has also been shown that the anti-HSP65 titer in rabbits being fed by a cholesterol-rich diet is increased.

In 2005 the first human study in this field and with the aim of investigating whether receiving dietary food fats and antioxidants is associated with changes in the HSP antibody in cases with dyslipidemia or not was performed on 238 patients affected with dyslipidemia and 188 controls. The results were as follows: in the dyslipidemic patients, the antibody titer of HSP60, 65 and 70, serum level of hsCRP and vitamin E, dietary protein, total fat and single saturated fat intake were significantly higher than for non-dyslipidemic controls. It was also reported that an inverse correlation was found between dietary vitamin E intake and the anti-HSP60 titer in patients and anti-HSP65 titer in controls. In addition, there was a positive relationship between dietary vitamin C intake and anti-HSP60 and 65 titers in patients. This was the first study on the anti-HSP plasma titer and dietary components in humans. It revealed that there is a correlation between dietary food components and an

immune response to HSP in patients with cardiac risk factors [26]. In our study saffron supplementation was associated with significant decrease in the anti-HSP27 and 70 titers, and this may indicate a protective effect of saffron on the immune response to HSPs in patients with cardiovascular risk factors.

Based on the suggested probable mechanism about the association between HSPs and atherosclerosis, the positive effect of saffron on HSPs could be induced through interfering with the body's immune response and the inflammatory reactions or by modulating the stress-inducing factors (mainly elevated oxidized-LDL, LDL-C or TC or TG levels) which result in the increased expression and release of soluble HSPs.

Conclusions

We have shown that saffron can decrease serum Hsp27 and 70 antibody titers in patients with metabolic syndrome. As this study was conducted on a small number of patients with metabolic syndrome, it is highly recommended that these findings are confirmed in a larger sample size.

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Conflict of interest statement

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