



Simvastatin Treatment is not Associated with Changes in Serum Concentrations of Heat Shock Proteins -60 and -70 in Patients with Dyslipidemia

Roya Aryanpour^{1,2}, Seyed Mohammad Reza Parizadeh², Mohsen Moohebbati^{1,2}, Shima Tavallaie², Amirhossein Sahebkar^{3,4}, Shabnam Mohammadi⁵, Majid Ghayour-Mobarhan^{2*}, Gordon AA. Ferns⁶

¹Cardiovascular Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.

²Biochemistry of Nutrition Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.

³Biotechnology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.

⁴Metabolic Research Centre, Royal Perth Hospital, School of Medicine and Pharmacology, University of Western Australia, Perth, Australia.

⁵Department of Basic Sciences, Faculty of Medicine, Gonabad University of Medical Sciences, Gonabad, Iran; Neurogenic Inflammation Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

⁶Division of Medical Education, Mayfield House, University of Brighton, BN1 9PH, United Kingdom.

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ABSTRACT

Background: Increased heat shock protein (Hsp) expression is associated with atherogenesis. The statin group of cholesterol lowering drugs reduces cardiovascular events and this may be related to their pleiotropic effects that may include their anti-inflammatory properties. The aim of this study was to evaluate the effect of 40 mg simvastatin on serum levels of Hsp-60 and -70 in dyslipidemic individuals. **Methods:** Patients ($n=102$) were treated with simvastatin (40mg/day), or placebo in a randomized, double-blind, placebo-controlled, cross-over trial. Lipid profile and serum Hsp-60 and -70 antigen levels were measured before and after each treatment period. Seventy-seven subjects completed the study. Data were analyzed using Independent-Samples t-test for parametric data and Mann-Whitney test for non-parametric data and chi-square test for categorical data. **Results:** There was a reduction in the level of LDL-C ($p<0.001$), total cholesterol ($p<0.001$), and triglycerides ($p<0.05$). However, simvastatin therapy did not significantly alter the serum level of HDL-C, Hsp-60 and -70 ($p>0.05$). **Conclusions:** We found that statin therapy did not significantly affect the serum level of HSP-60 and -70.

Introduction

Heat shock proteins (Hsps) are a group of highly conserved proteins expressed by all organisms in response to stressful stimulants, including oxidative stress, inflammatory cytokines and high temperature.^{1,3} These proteins have intracellular functions including roles as molecular chaperones and inhibitors of apoptosis.^{2,3} In addition, some studies have shown that Hsp-60 and -70 expression increases in human atherosclerotic plaque.⁴ Atherosclerosis is a chronic disease that underlies cardiovascular disease (CVD). Several lines of evidence have consistently shown that inflammation plays an important role in the progression of atherosclerosis.^{3,5} Aside from inflammation, there is ample evidence for the activation of both humoral and cellular immune systems in atherosclerosis.⁶ The immune-inflammatory nature of atherosclerosis has prompted the use of anti-inflammatory and immunomodulatory agents as potential therapies.⁶ 3-Hydroxyl-3-methylglutaryl coenzyme A reductase inhibitors (statins) are currently the most potent class of

cholesterol-lowering agents² and are extensively used in the management of coronary risk.⁷ In addition to the lipid-lowering effects, statins possess anti-inflammatory^{8,9} and antioxidant¹⁰ properties, decrease CRP levels¹¹ and enhance the stability of atherosclerotic plaque.^{12,13} We have previously shown that statin therapy is associated with reduction in anti-Hsp-60 and 70 titres in dyslipidemic patients.³ However, it is not clear if the reduction of anti-Hsp titres is due to the attenuation of Hsp antigen release into the circulation, or a direct immunosuppressive effect. To our knowledge, there is little data investigating the effect of statin on Hsps. Therefore, in the present study we examined the effect of simvastatin on serum Hsp-60 and 70 levels in a randomized, double-blind, placebo-controlled, cross-over trial.

Materials and methods

Subjects

Eligible subjects were those not originally taking lipid lowering agents who had at least one of the following

conditions: 1) one cardiovascular risk factor and 160 mg/dL < LDL-C < 190 mg/dL, 2) ≥ 2 cardiovascular risk factors and 130 mg/dL < LDL-C < 160 mg/dL, and 3). Cardiovascular risk factors were defined as age more than 65 years old, hypertension, hyperlipidemia, diabetes mellitus, and positive family history of CVD, a current smoking habit, male gender, physical inactivity and obesity. Exclusion criteria were a history of malignancy, infections, connective tissue disorders or treatment with immune-modulatory drugs (for instance: corticosteroids), liver and renal disease, leukocytosis (white blood cell count > 10,000 $10^9/L$), thrombocytosis (platelet count > 450,000 $10^9/L$) and anemia (hematocrit < 40%). This study was approved by Ethics committee of MUMS and each subject completed consent form before the study.

Study design

The study was designed as a randomized double-blind placebo-controlled cross-over trial. In crossover study, the influence of confounding covariates decrease because each crossover subjects serves as own control. Besides, this study needs fewer patients than do non-crossover studies. Subjects who met the eligibility criteria ($n = 102$) were randomly assigned to either simvastatin-placebo or placebo-simvastatin sequence. Each sequence of treatment with simvastatin and placebo was 4 weeks, intermitted by a 2-week wash-out. The dose of simvastatin was 40 mg/day and remained unchanged throughout the trial.

Anthropometric and measurements

Anthropometric parameters (height, weight and body mass index) were measured at the baseline and the each period. Height was measured by the nearest 0.1 cm. After an overnight fasting, Weight was measured by the nearest 0.1 kg. BMI was calculated by division weight (kg) by height (m^2). Blood pressure was measured twice, using a standard sphygmomanometer. The appearance of the first sound (Korotkoff phase 1) was considered as the systolic blood pressure and disappearance of the sound (Korotkoff phase 5) the was considered as diastolic blood pressure.

Blood sampling

Blood samples were collected from participants after a 12-h fast at 4 time points i.e. at the baseline and endpoint of simvastatin and placebo periods. After clotting, samples were centrifuged at 2500 rpm for 15 minutes at room temperature. Hemolyzed samples were excluded and serum was stored at $-20^\circ C$ until analysis.

Biochemical analysis

For each subject, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) was determined. The serum LDL-C concentration was determined using a commercial kit.

Determination of serum Hsp-60 and 70 concentrations

A commercial kit (stressGen Biotechnologies) was used for evaluation of Hsp-60 concentration. An in-house enzyme-linked immunosorbant assay (ELISA) was used for determination of serum Hsp-70 concentration. Plates were incubated at $4^\circ C$ after coating with primary antibody (rabbit monoclonal anti Hsp70, Stressgen, Canada) (100 μ l; 5 μ g/ml) diluted in 0.1 M the carbonate buffer (pH9.6). Then, plates were washed with phosphate-buffered saline and incubated with 300 μ l of PBS/T containing 0.1%BSA (PBS/T/BSA) for 2 hours at $37^\circ C$. After washing, 100 μ l of second antibody (rabbit polyclonal anti Hsp70, Stressgen, Canada) (1/400) diluted in PBS/T containing 4% mouse serum was added. After 1hour at $37^\circ C$, plates were washed and incubated with 100 μ l of an anti-rabbit immunoglobulin peroxidase conjugate (Sigma – Aldrich, USA) in PBS/T/BSA (1/10,000) for 1hour at $37^\circ C$. Plates were then washed and 100 μ l of TMB substrate were added. After 7 minutes and at $37^\circ C$, the reaction was stopped with 50 μ l of 2N HCL and the absorbance determined at 450 nm in an ELISA reader. Serum Hsp70 levels were determined by comparing sample absorbance with the absorbance of a standard reference.

For Hsp70 (in- house ELISA) : The average intra-assay variation was 6% and the average inter-assay variation was 9%.

For Hsp 60 (Hsp 60 ELISA Kit, assay Designs): The Intra-assay Coefficient of variation of of Assay Designs Hsp ELISA has been determined to be <10 %. The Inter-assay coefficient of variation of the ssay for Hsp ELISA was been determined to be <10 %.

Statistical analysis

Normally distributed values are expressed as median and non-normally distributed data was expressed as median and inter-quartile range. After assuming normality, a mixed model analysis of variance for 2x2 cross-over studies was used and data were analyzed using the Statistical Analysis Software (SAS) version 8. A two-sided p -value of < 0.05 was considered as significant. Data were analyzed using Independent-Samples t-test for parametric data and Mann–Whitney test for non-parametric data and chi-square test for categorical data.

Results

Twenty five of one hundred and two subjects (24.5%) did not complete the study. The reasons for drop-out included non-compliance with the study protocol ($n=21$), drug intolerance ($n=2$) and moving to another city ($n=2$). Therefore, the number of final sample is 77 (78.18%).

Effects of simvastatin vs. placebo on serum lipid profile

As you can see in Figure 1 and 2, although treatment

with simvastatin and placebo was not associated with a notable reduction in median serum HDL-c levels ($p > 0.05$), there was a significant reduction in median serum TC, LDL-C and TG levels after 4 weeks of treatment with simvastatin ($p < 0.01$) (2). Triglycerides (~14%), total cholesterol (~25%) and LDL-C (~34%) were reduced significantly after 4

weeks of treatment with simvastatin. In placebo-statin group, changes were ~19% decrease in total cholesterol, ~23% decrease in LDL-C, and ~15% increase in triglycerides. HDL-C did not change significantly in both groups.

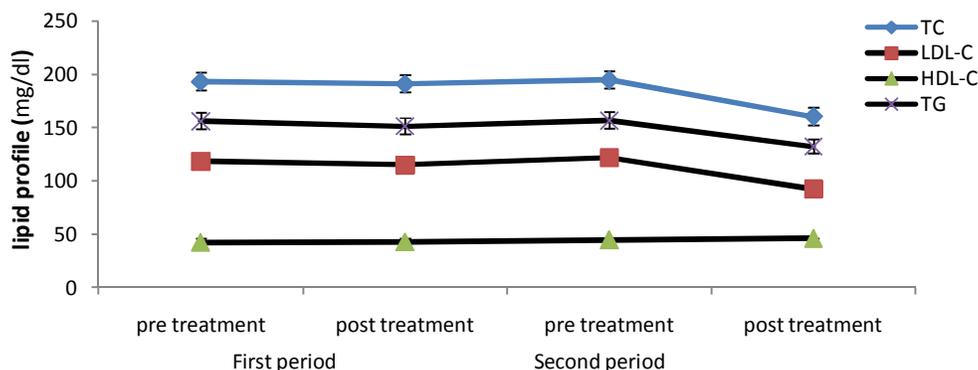


Figure 1. Effect of simvastatin therapy in placebo-statin group
 Values expressed as mean± SD for normally distributed data; TC: total cholesterol; LDL: low-density lipoprotein; TG: triglyceride; HDL: high -density lipoprotein.
 a $P < 0.001$ compared to mean value of **TC** after placebo-statin treatment
 b $P < 0.001$ compared to mean value of **LDL-C** after placebo-statin treatment
 c $P = 0.002$ compared to mean value of **TG** after placebo-statin treatment

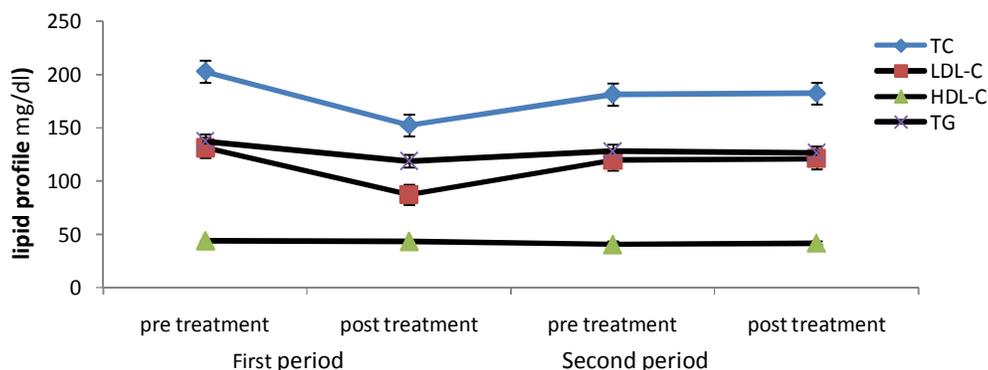


Figure 2. Effect of simvastatin therapy in statin-placebo group
 Values expressed as mean± SD for normally distributed data; TC: total cholesterol; LDL: low-density lipoprotein; TG: triglyceride; HDL: high -density lipoprotein.
 a $P < 0.001$ compared to mean value of **TC** after statin-placebo treatment
 b $P < 0.001$ compared to mean value of **LDL-C** after statin-placebo treatment
 c $P = 0.002$ compared to mean value of **TG** after statin-placebo treatment
 Treatment with simvastatin and placebo was not associated with a notable reduction in serum HDL-c levels but there was a significant reduction in serum TC, LDL-C and TG levels after treatment with simvastatin

Effects of simvastatin vs. placebo on serum Hsp-60 and 70 levels

Statistical analysis (determining of non-normally distributed data, as median and inter-quartile range) showed that treatment with simvastatin (40mg/day) for 4 weeks was not associated with a significant reduction in serum HSP-60 and -70 levels and in two groups (placebo-statin and statin-placebo); p-value is more than 0.05 (Table 1).

Discussion

The aim of this study was the investigation of simvastatin effect on serum Hsp-60 and -70 levels in cardiovascular patients. We found that simvastatin therapy (40 mg/ day) for 4 weeks did not cause a statistically significant reduction in serum Hsp-60 and -70 levels. Although, few studies have reported effects of simvastatin on anti-Hsp levels^{2,3,5}, to date, there is not any data to evaluate the effect of simvastatin on serum Hsps levels.

The relationship between serum Hsps, Hsp antibody concentrations and CVD was initially explored in the early 1990s. The expression of Hsp-60 and -70 in atherosclerotic lesions was first reported by Kleindienst *et al.* and Berberian *et al.*¹⁴ Kleindienst *et al.* reported that the HSP 60 expression increases with the atherosclerotic severity and that most lymphocytes participating in atherogenesis lesion the alpha/beta T cell receptor. Berberian and colleagues have been found a relationship between elevated Hsp-70 and necrosis and lipid accumulation in human arteries.

According to Pockley *et al.*, anti-Hsp60 titers are present in healthy subjects and are positively associated with atherosclerosis burden, particularly in the early stages of disease.^{15,17} Some soluble Hsps may have pro-inflammatory effects and can induce the expression of tumor necrosis factor (TNF- α), IL-6, IL-12, IL-15; and

exogenous HSP-70 has been reported to upregulate IL-1, IL-6, TNF- α expression in human monocytes^{18,21}. Binding of soluble Hsps to the TLR 4/CD14, stimulate an innate immune response that includes the production of pro-inflammatory cytokines by macrophages and adhesion molecules in endothelial cells via nuclear factor Kappa B (NF- κ B) activation. These data suggest that soluble Hsps might be a danger signal for the innate immune system.²² According to previous studies, statins decrease the activation of NF- κ B, down-regulate the expression of multiple proinflammatory molecules²³ and decrease CD40 expression in vascular cells.^{24,25} By reducing CD40 expression, statins reduce inflammation, resulting in atherosclerotic plaque stabilization.²⁶

Table 1. Effect of simvastatin and placebo therapy on serum HSP-60 and -70 levels

Study groups	First period				Second period				Period effect ^a	Treatment effect	
	Pre treatment		Post treatment		Pre treatment		Post treatment				
	Median	IQR	Median	IQR	Median	IQR	Median	IQR			
Serum Hsp-60 (mg/dl)	Statin-placebo	0.10	0.14	0.11	0.11	0.11	0.13	0.11	0.12	$p=0.199$	$p=0.56$
	Placebo –statin	0.12	0.05	0.12	0.05	0.11	0.05	0.12	0.08		
Serum Hsp-70 (mmg/dl)	Statin-placebo	0.60	0.32	0.54	0.52	0.40	0.26	0.46	0.24	$p=0.16$	$p=0.31$
	Placebo –statin	0.62	0.46	0.59	0.47	0.42	0.44	0.43	0.32		

Values expressed, as median and inter-quartile range as non-normally distributed data; .Hsp-60:Heat shock protein 60; Hsp-70:Heat shock protein 70; IQR: inter-quartile range.

^a Defined as comparison of mean values between the first and second periods.

Effect of simvastatin therapy on anti serum Hsp-60 and -70 levels

In our previous studies^{2,3} we found that simvastatin administration (40 mg/day) for 4 weeks was associated with significant reductions in anti-Hsp60 and 70 serum levels but not Hsp and it was suggested that this may be explained by its immune-modulatory properties of statins. These results may have been because of the small sample size. But in the present study we have positive results in large sample. Shin *et al.* have reported that fluvastatin treatment (80 mg/day) was associated with a remarkable decrease in serum anti-Hsp 60 and anti-HSP70 titers in patients with coronary artery disease.²⁷ Besides, it has been found that statin therapy (10 mg/day) is associated with reduction in plasma anti-Hsp70 titers but not anti-Hsp 60.⁵ Shin explained these results by the underlying differences in the nature of the patients. Some of the patients were treated with lipid-lowering drugs and were undergone revascularization. Pretreatment with lipid-lowering

agents decreased the anti-inflammatory effect of statin and stabilized the level of blood lipids.

Effect of simvastatin therapy on serum Hsp-60 and -70 levels

There was no significant difference between simvastatin therapy versus placebo therapy on serum Hsp-60 and -70 levels ($p > 0.05$). Although, statins cause a significant reduction on anti serum Hsps levels be effecting on humeral immune system, it cannot effect on release of Hsp-60, 70 from antigenic sources. Hence cells involved in atherogenesis express large quantities of Hsps in response to exposure to several stressors that may also promote atherosclerosis. Measurement of HSP expression, including serum antigen, or antibody concentrations may be useful as markers of disease susceptibility. This may be due to complex interactions between Hsp production, release, clearance and autoimmune response.

In line with the present results, expression of Hsp 70 and Hsp 90 remained unchanged after statin therapy in

the cultured cells.²⁸ Besides, Kretz et al. reported that simvastatin treatment did not affect on levels of mRNA for Hsp 70 and Hsp 90 expression after ocular injury.²⁹ On the other hand, in a study by Huang and colleagues on subjects with acute coronary syndrome, it has been reported that simvastatin (80 mg/dL) for 8 weeks normalized Hsp-70 expression.³⁰ Besides, statins have been shown to increase Hsp-70 expression, but it did not stimulate the induction of Hsp-60 in vascular endothelial cells.³¹ Also, atorvastatin may decrease apoptosis by up-regulation Hsp 70 and HSF-1 in coronary endothelial cells.³² A possible reason for the inconsistency between in-vivo and in-vitro results is that in-vitro studies did not evaluate cardio-myocytes as a rich source of Hsps. Besides, in-vitro studies assessed Hsp expression but not release of Hsps into the circulation.

Study limitations

This study had limitations: 1) Twenty-five subjects (of 102 subjects) did not complete the study and have been reduced the numbers of subjects. 2) The dose of simvastatin was 40 mg per day for 4 weeks that increasing of the dose and treatment period may be more effective and longer period exert more dramatic changes.

Conclusion

we demonstrated that simvastatin failed to affect on Hsp-60 and -70 levels in serum while it did reduce the anti-Hsp-60 and Hsp-70 serum levels and this result may indicate that simvastatin plays a role in modulation of cellular function of heart cells, independent to Hsp-60 and -70. We suggest longer term prospective studies in different population with a more careful assessment of the time course of appearance of the Hsps and antibodies relative to the development of clinical events.

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