ORIGINAL RESEARCH

Effects of statin use on total oxidant and antioxidant capacity and ceruloplasmin activity

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Abstract

Purpose: Oxidative damage plays an important role in atherosclerosis development. Statin drugs have anti-oxidant properties, but the clinical value of their antioxidant properties remains unclear. In this study, our aims were: (1) to assess the anti-oxidant effects of statins in patients with coronary artery disease (CAD) using a newly developed valid measure of total oxidant and anti-oxidant capacity; and (2) to identify whether statins influence ceruloplasmin levels.

Methods: Within a cross-sectional study, 67 dyslipidemic CAD patients on atorvastatin for at least three months were compared with 69 age- and gender-matched CAD patients not using atorvastatin. All patients were either newly-diagnosed with or already had established CAD. Patients and controls were selected from among patients who had undergone coronary angiography for a variety of reasons. Immediately prior to angiography, plasma total oxidant and antioxidant capacity and ceruloplasmin (Cp) levels were measured by means of a relatively new and highly-reliable method.

Results: Total oxidant capacity levels were significantly lower and total antioxidant capacity significantly higher in those on atorvastatin; serum ceruloplasmin levels also were significantly increased in the atorvastatin groups (all $p < 0.05$). On multivariate analysis, atorvastatin use was a sig-

nificant determinant of Cp increase, independent of any antioxidant effect.

Conclusions: This study clearly demonstrates increased anti-oxidant capacity and decreased oxidative stress with statin use. Atorvastatin use may also increase Cp levels although this effect appears to be independent of its anti-oxidant effects.

Free radicals and oxidants are produced in metabolic and physiological processes, and their effects are controlled by exogenous and endogenous antioxidants. If the quantity of free radicals exceeds the capacity of anti-oxidant defense mechanisms, oxidative stress occurs.^{1,2} Oxidative stress alters normal endothelial functions, inducing proinflammatory, prothrombotic, proliferative and vasoconstrictor mechanisms that support atherogenic processes.^{2,3}

A number of reports in the literature implicate oxidative stress and/or inadequate antioxidant defences in the pathogenesis of, or as a risk factor for, cardiovascular disease.^{4,5} Ischemic heart disease is an ideal example of a clinical situation in which there is an increased production of oxygen free radicals.⁶ Oxi-

oxidative stress has been regarded as one contributor to the progression of atherosclerosis, since oxidized low density lipoprotein plays a key role in atherosclerosis development.^{2,7}

3-Hydroxy-methyl-glutaril coenzyme A reductase inhibitors (HMG-Co A, statins) reduce cardiovascular risk, mortality and morbidity rates, especially in terms of secondary prevention; that is, in patients with known coronary heart disease or coronary equivalents.⁷ Effects on the cardiovascular system primarily have been attributed to LDL-lowering effects.⁷ Statins have several beneficial effects, aside from their lipid lowering properties, that have been called '*pleiotropic effects*'. These pleiotropic effects include antioxidant properties.^{8,9} In recent studies, lipid-lowering therapy has been shown to reduce oxidized LDL levels by means of increasing anti-oxidant effects.^{10,11}

This study had two aims. The first was to identify the effects of statins on oxidant and anti-oxidant status in patients with coronary artery disease by means of a different and newly developed measurement method that entails measuring both *total oxidant* and *total anti-oxidant capacity* (TOC and TAC). Second, since it is known that ceruloplasmin (Cp) has anti-oxidant properties on LDL by means of blocking Cu^{2+} -mediated lipid oxidation,¹² we measured plasma ceruloplasmin levels in all three subject groups and determined if these correlated with statin use.

Materials and Methods

Subjects and study design

The study was conducted at the Sanliurfa Harran University School of Medicine. Prior to initiating subject recruitment, the study protocol was reviewed and approved by the local ethics committee of the university, in accordance with the ethical principles for human investigations, as outlined in the second Declaration of Helsinki. All subjects provided informed, written consent prior to participating. For this

cross-sectional study, 67 consecutive dyslipidemic coronary artery disease (CAD) patients (38 males, 29 females; mean age: 61 years) who had been using atorvastatin for at least three months were compared with 69 age and gender-matched CAD patients not taking atorvastatin. All patients were either newly-diagnosed with, or had already established, CAD and all had been referred to our center for coronary angiography for such reasons as stable angina pectoris, angina pectoris equivalent symptoms, wide-field hypokinesia of the left ventricle wall and positive stress tests. Those patients on atorvastatin were subdivided further into two groups according to their daily atorvastatin dose, thereby creating three subject groups: Group 1: controls; Group 2: patients taking 20 mg atorvastatin daily; and Group 3: patients taking 40 mg atorvastatin daily. The following otherwise-eligible patients were excluded: patients with any acute or chronic infection or inflammation; patients with extremely high triglyceride levels (> 400 mg/dl); patients who, over the previous 48 hours, had used any drug with anti-oxidant properties (like nebivolol, carvedilol, vitamins E or C, and acetylcysteine); and patients with regular alcohol use or alcohol use within 48 hours.

Coronary angiography and coronary artery disease determination

In all subjects, coronary angiography was performed using the standard Judkin's technique and a Toshiba Infinix Csi (Toshiba Corporation, Japan). The percentage of coronary artery stenosis was determined by handheld caliper measurements. Significant angiographic coronary stenosis was defined as the presence of stenosis $> 50\%$ of the luminal diameter and affecting at least one main coronary artery.

Blood samples

All blood samples were obtained following an overnight fast, just before angiography and after insertion of a femoral catheter sheath connected to a plastic sy-

ringe and without interruption of arterial flow. Twenty ml of blood were drawn and the first few ml were discarded. Ten ml were used for baseline routine laboratories. The residual content of the syringe was transferred immediately to polypropylene tubes. These tubes then were centrifuged at 3000 rpm for 10 minutes at 10-18°C. Supernatant plasma samples were stored in plastic tubes at -80°C pending the assays for serum TOC, TAC and Cp.

Measurement of TOC

The TOS of serum was determined using a novel automated measurement method developed by Erel.¹³ Oxidants that are present in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundant in the reaction medium. The ferric ion generates a coloured complex with Xylenol Orange in an acidic medium. Colour intensity, which can be measured spectrophotometrically, correlates with the quantity of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results expressed in terms of $\mu\text{mol H}_2\text{O}_2$ equiv/L.

Measurement of TAC

Serum TAC was determined using a novel automated measurement method, also developed by Erel.¹⁴ In the assay, ferrous ion solution, present in Reagent 1, is mixed with hydrogen peroxide, which is present in Reagent 2, producing hydroxyl radicals. Sequentially-produced radicals, like the brown-colored dianisidiny radical cation that is produced by the hydroxyl radical, also are potent radicals. Using this method, the anti-oxidative effect of the sample against potent free-radical reactions, which are initiated by the produced hydroxyl radical, can be measured. The assay has excellent precision values of greater than 97%. The results are expressed as mmol Trolox equiv./L.

Measurement of Ceruloplasmin

The enzymatic activity of Cp was measured according to Erel's method.¹⁵ Using this assay, ferrous ion is oxidized to ferric ion via ceruloplasmin ferroxidase activity. The results are expressed as units per gram protein (U/L).

Measurement of other laboratory markers

Fasting serum glucose, urea, creatinine, sodium, and potassium levels were measured, as well as a lipid profile that included total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol, using commercially-available assay kits (Abbott, Illinois, USA) with an Abbott Aeroset auto-analyzer (Abbott). LDL cholesterol was calculated using the Friedwald equation,¹⁶

Statistical analysis

All data analysis was conducted using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA), with group parameters expressed as means $\pm$ standard deviations. Between-group comparisons were conducted by one-way analysis of variance (ANOVA) for continuous variables, with Tukey's HSD test conducted *post hoc*. Pearson's correlation analysis was performed to identify the degree of correlation between continuous variables. Multivariate linear regression analysis was performed to identify significant independent predictors of Cp level. Differences at $p \leq 0.05$ were interpreted as statistically significant. All inferential tests were two-tailed.

Results

The clinical characteristics and hemodynamic, anthropometric and biochemical parameters of the three study groups are presented in Table 1. Age, sex, body mass index, presence of hypertension, systolic and diastolic blood pressure, sodium, potassium, urea, creatinine, fasting glucose, total cholesterol, triglyceride and high density lipoprotein cholesterol (HDL-C) all

TABLE 1. Clinical and demographic properties of the three study groups

Variables	Group 1 (no statin use)	Group 2 (20 mg atorvastatin)	Group 3 (40 mg atorvastatin)	P value
Age	59±11	62±10	59±12	0.359
Sex (F/M)	18/51	16/24	13/14	0.086
DM (+/-)	19/50	14/26	12/15	0.273
HT (+/-)	25/44	22/18	10/17	0.136
Smoking (+/-)	23/46	17/23	13/14	0.352
SBP (mmHg)	130±24	126±27	130±26	0.746
DBP (mmHg)	79±14	78±14	83±12	0.414
BMI (kg/m ²)	27±5	27±8	28±5	0.751
ASA (+/-)	63/6*	40/0*	27/0*	0.047
BB (+/-)	33/36 ^α	29/11 ^α	16/11 ^α	0.042
ACEi (+/-)	29/40	20/20	11/16	0.668
ARB (+/-)	4/65 [¶]	6/34 [¶]	7/20 [¶]	0.023
CCB (+/-)	9/60	1/39	3/24	0.187
Nitrate (+/-)	8/61	11/29	6/21	0.100
Clopidogrel (+/-)	17/52	6/34	2/25	0.118
Diuretic (+/-)	6/63	8/32	2/25	0.155
Insulin (+/-)	5/64	2/38	2/25	0.887
SU (+/-)	15/64	13/27	10/17	0.242
Laboratory parameters				
Na (mg/dl)	134±26	140±3	140±4	0.447
K (mg/dl)	4±0.6	4±0.5	4±0.5	0.262
Urea (mg/dl)	36±15	46±22	42±28	0.185
Creatinine (mg/dl)	0.9±0.4	0.9±0.3	1.0±0.4	0.704
FBG (mg/dl)	151±80	149±75	156±60	0.946
TC (mg/dl)	195±34	196±34	183±26	0.203
LDL-C (mg/dl)	120±27 [§]	119±23 [§]	106±17 [§]	0.042
HDL-C	42±11	42±15	42±9	0.986
Triglyceride (mg/dl)	164±70	178±89	174±85	0.648

From One-Way ANOVA and Chi-square analysis

Abbreviations: DM, diabetes mellitus; HT, hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; ASA, acetylsalicylic acid; BB, beta-blocker; ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin-II receptor blocker; CCB, calcium channel blocker; SU, sulfonylurea; FBG: fasting blood glucose; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein-cholesterol.

* p < 0.05 (ASA use; Group 1 vs. 2, and 1 vs. 3) and p > 0.05 (Group 2 vs. 3).

^α p < 0.05 (Beta blocker use; Group 1 vs. 3, and 2 vs. 3), p > 0.05 (Group 1 vs. 2).

[¶] p < 0.05 (ARB use; Group 1 vs. 2, and Group 1 vs. 3), p > 0.05 (Group 2 vs. 3).

[§] p < 0.05 (Group 1 vs. 3, and Group 2 vs. 3), p > 0.05 (Group 1 vs. 2).

were similar across the three groups (all p > 0.05). Low density lipoprotein cholesterol (LDL-C) levels and acetylsalicylic acid, angiotensin receptor blocker and beta blocker use varied. LDL-cholesterol was lowest in those taking 40 mg atorvastatin daily. No difference in LDL-C was observed between controls

(Group 1) and those on 20 mg statin daily (Group 2). Controls used less ASA, beta-blockers, and angiotensin II receptor blockers than either of the two atorvastatin-using groups (p < 0.05).

The oxidative parameters and Cp levels of all groups are summarized in Table 2. TOC levels were

TABLE 2. Plasma TAC and TOC levels and ceruloplasmin activity in the three study groups

	No statin	20 mg statin	40 mg statin	P value
TOC ($\mu\text{mol H}_2\text{O}_2$ equiv./L)	12.44 $\pm$ 1.91*	11.15 $\pm$ 1.16*	10.48 $\pm$ 1.38*	P < 0.001
TAC (mmol Trolox equiv./L)	0.81 $\pm$ 0.14 [¶]	0.96 $\pm$ 0.16 [¶]	1.06 $\pm$ 0.25 [¶]	P < 0.001
Ceruloplasmin (U/L)	510.34 $\pm$ 56.04 [§]	668.38 $\pm$ 44.74 [§]	718.88 $\pm$ 77.17 [§]	p < 0.001

From One-Way ANOVA. Data presented as means $\pm$ SD.

Abbreviations: OSI: oxidative stress index; TAC: total antioxidants capacity; TOS: total oxidant status.

* p = 0.001 (Group 1 vs. 2), p < 0.001 (Group 1 vs.3), p = 0.15 (Group 2 vs. 3)

[¶] p < 0.001 (Group 1 vs. 2, and Group 1 and 3), p = 0.088 (Group 2 vs. 3)

[§] p < 0.001 (Group1 vs. 2, and Group 1 vs. 3), p = 0.219 (Group 2 vs. 3)

lower in both atorvastatin-using groups (p < 0.001) relative to controls (Table 2, Figures 1a and 1b). The TOC level was lowest in those taking 40 mg atorvastatin; however, when compared to those on 20 mg, the difference failed to achieve statistical significance (p = 0.15) (Table 2, Figure 1a). In contrast, serum TAC levels were significantly higher in those on atorvastatin (p < 0.001), with the TAC level highest in those on 40 mg; however, again, there was no statistically significant difference between the two atorvastatin groups (p = 0.088) (Table 2, Figure 1b). Similarly, plasma Cp was significantly increased in both atorvastatin-using groups (p < 0.001), highest in the

40 mg group, but with no significant difference between the two atorvastatin groups (Table 2, Figure 1c) (p = 0.22).

A weakly negative significant correlation was detected between Cp and LDL-C levels, and weakly positive significant correlations were observed between Cp and atorvastatin, sulfonylurea, and ARB use (Table 3). Upon linear regression analysis, atorvastatin was found to be the only variable significantly predicting serum Cp level (p < 0.0001, R² = 0.40). Neither total oxidative nor anti-oxidative status were found to affect Cp level (p > 0.05).

FIGURE 1. Comparison of TOC and TAC levels and ceruloplasmin activity in all groups

TABLE 3 Results of the linear regression model where the ceruloplasmin level is the dependent variable and the results of bivariate correlations with ceruloplasmin

	Pearson correlation coefficient	P value	β regression coefficient	P value
Statin use	0.583	<0.0001	0.709	<0.0001
LDL-C	-0.206	0.016	-1.123	0.097
ARB	0.213	0.013	1.689	0.195
SU use	0.187	0.029	0.070	0.283

Abbreviations: ARB, angiotensin-II receptor blocker; SU, sulfonylurea

Discussion

This study assessed two main issues, the first being the effect of a given statin drug, atorvastatin, on parameters of oxidative stress. As a determinant of oxidative stress, oxidized plasma concentrations of LDL-C are known to be associated with CAD.^{2,7,17} Therefore, preventing oxidative stress would seem to be important and the anti-oxidant effects of statins might be critical. In previous studies, statins have been shown to reduce oxidative modification of LDL.^{10,11,18} This study differs from previous studies primarily in the way by which oxidant parameters were measured. We studied oxidative stress parameters by means of a more reliable approach, which measures both *total oxidant* and *total antioxidant capacity* rather than measuring separate oxidative stress variables.^{13,14} The separate measurement of different oxidant and anti-oxidant molecules is impractical since there are a great number of oxidants and antioxidants in the body and their effects are additive. Measuring total oxidant-antioxidant status is more valid and reliable.^{13,14} When only a few parameters are measured, the levels of those particular antioxidants may be unchanged or even decreased, yet the actual overall oxidant status may be increased, or *vice versa*. This study clearly demonstrated that statin treatment decreases *total oxidant capacity* and increases *total antioxidant capacity* in CAD. Another point worth mentioning is that levels of Cp were elevated in atorvastatin-using patients. Upon regression analysis, the main determinant of Cp elevation seemed to be statin use, which perhaps is the most important finding of our study.

Cp is an abundant plasma protein that contains seven copper atoms per molecule and accounts for 95% of the total circulating copper in adults.¹⁹ Serum Cp levels have been shown to be increased in numerous situations, diseases and conditions, including physical exercise, third trimester pregnancy, ovarian hyperfunction, epilepsy, chronic inflammatory processes, some malignant tumors and, especially, cardiovascular diseases like arteriosclerosis, coronary artery disease and abdominal aortic aneurysms.²⁰⁻²²

Based upon the data mentioned above, in this study there are two main factors that could have caused the elevation of Cp levels. The first is the presence of cardiovascular disease and, in particular, atherosclerosis. In several studies, elevated Cp levels have been identified in cardiovascular diseases.^{23,24} The second probable causative factor is the anti-oxidant effects of statins. The anti-oxidant effects of statins are well-known.⁸⁻¹¹ Therefore, atorvastatin might be the cause of the elevation of Cp levels, since Cp itself has antioxidant properties.^{12, 25-28} These results are not consistent with those of Ghayour-Mobarhan *et al.*, who reported a decrease in Cp levels after atorvastatin treatment. That study sample size, however, was rather low (nine patients) and the same effect was not observed in simvastatin-using patients.²⁹

In summary, it seems unlikely that coronary artery disease, in itself, was causative in this study, because all patients in all three study groups had CAD and there little difference in group characteristics (except for the use of ASA, beta blockers and ARB). According to our regression analysis, statin use seems to be an independent determinant of Cp elevation. On that point, the question should arise: was this effect due

the statin's anti-oxidant properties? Multivariate analysis found Cp levels to not be correlated with either total oxidant or total anti-oxidant capacity. Moreover, neither ARB and ACEi use (because they were found to be correlated with Cp, these two variables were included in multivariate analyses) nor total oxidant and antioxidant capacity were predictive. Therefore, we believe that atorvastatin caused a beneficial increase in Cp levels in a manner that is different to what has been ascribed to statins previously.

We would be remiss not to point out the limitations of our study, the first being that the study sample was relatively small. On the other hand, given that a statistically significant difference is harder to detect in a small study, the differences we observed obviously were quite sizeable. As such, we feel confident in our inferences. A second limitation relates to the cross-sectional nature of this study, such that we did not know baseline Cp levels; however, we had a clinically similar, age-matched control group, and the differences relating to Cp level were quite prominent.

In conclusion, in this study, which differs from other prior studies with respect to utilizing newly developed measurement of overall oxidant status, we feel that we have demonstrated the anti-oxidant effects of atorvastatin. These antioxidant effects seem to be more prominent with increased atorvastatin dose; however, it is not possible to say the antioxidant effects are dose-dependent. Secondly, atorvastatin seems to affect Cp levels independent of its antioxidant properties, which might be a pleiotropic effect: this possibly clearly warrants validation in future studies.

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