

# Antioxidant Effect of Atorvastatin in Type 2 Diabetic Patients

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## ABSTRACT

Evidence has long been existed regarding the relationship between oxidative stress and diabetes. The present study was conducted to assess the effect of atorvastatin on selected oxidative stress parameters and its effect on lipid profile parameters in dyslipidaemic type 2 diabetic patients. Fifty nine dyslipidaemic type 2 diabetic patients were included in this study. A full history was taken and general examination was performed. The patients were taking an oral hypoglycaemic drug (glibenclamide) during the study. The patients were followed up for 60 days and divided randomly into 2 groups. Group I (n = 31) received no drug and served as dyslipidaemic diabetic control. Group II (n = 28) received atorvastatin tablets 20 mg once daily at night. Blood samples were drawn from the patients at the beginning and after 60 days of follow up between 8:30 and 10:30 am after at least 12-14 hours fasting. Fasting blood glucose, lipid profile, selective oxidative stress parameters, glutathione S reductase (GSH), malondialdehyde (MDA) levels, glutathione S transferase (GST) and catalase (CAT) activities were measured. Renal and hepatic functions were also assessed. The results showed that atorvastatin treatment produced significant increase in serum levels of GSH and High Density Lipoprotein (HDL), while serum levels of MDA, Total Cholesterol (TC), Triglyceride (TG), Low Density Lipoprotein Cholesterol (LDL-C) and Very Low Density Lipoprotein (VLDL) were significantly decreased. However, no significant effect was observed regarding CAT and GST activity. There were insignificant correlations between atorvastatin induced changes in the oxidation markers and the observed changes of the lipid profile. In conclusion, the antioxidant effect of atorvastatin could be unrelated to its hypolipidemic action as there was insignificant correlation between changes in lipid profile and oxidative stress in this study.

**Keywords:** Atorvastatin, Type 2 Diabetes, Oxidative Stress, Dyslipidaemia

## 1. Introduction

Oxidative stress is defined as tissue injury resulting from a disturbance in the equilibrium between the production of reactive oxygen species (ROS) (also known as free radicals) and antioxidant defense mechanisms [1]. Under physiologic conditions, the antioxidant defenses are able to protect against the deleterious effects of ROS, but under conditions where either an increase in oxidant generation, a decrease in antioxidant protection or a failure to repair oxidative damage, accumulation of free radicals ensues, leading to cellular and tissue damage [2]. Excess generation of ROS in oxidative stress have pathological consequences including damage to polyunsaturated fatty acids in membrane lipids, proteins, DNA and ultimately

cell death [3]. ROS have been implicated in many disease state including neurodegenerative disease like

Alzheimer's and Parkinson's disease, atherosclerosis, inflammatory conditions, certain cancers, diabetes mellitus (DM), cataract in the eye, pulmonary, renal, heart diseases and the process of aging [4,5]. Diabetes mellitus is a group of metabolic disorders with one common manifestation: hyperglycaemia associated with defects in insulin secretion, action or both. Traditionally it has been classified into two forms Type 1 DM and Type 2 DM [6]. Type 2 DM which is known to be multifactorial, resulting from combination of various factors such as impaired fatty acid metabolism, central fat deposition leading to insulin resistance in various tissues (liver, muscles, adipose) [7], beta-cell secretory defect and obesity [4]. Evi-

dence has long existed regarding the relationship between oxidative stress and DM [8]. Eisei N. *et al.* postulated that oxidative stress is involved in the onset and progression of diabetes, initiation and exacerbation of micro- and macrovascular complications in diabetes and recently oxidative stress status markers have been associated directly with the severity and prognosis of diabetes [9]. There are multiple sources of oxidative stress in DM, including non enzymatic (glucose autoxidation, non enzymatic glycation of proteins), enzymatic (NADPH oxidase, nitric oxide synthase) and mitochondrial pathway [10].

Dyslipidaemia is used to describe a group of conditions in which there are abnormal levels of lipid and lipoprotein in the blood [11]. In type 2 diabetes, dyslipidaemia is characterized by elevated circulating levels of TG, decreased circulating levels of HDL and usually accompanied by an elevation of small dense LDL-cholesterol particles [12]. There is an evidence indicating that hyperlipidaemia is associated with enhanced oxidative stress [13]. Atorvastatin belongs to 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, (or statins) which are potent inhibitors of cholesterol biosynthesis that are used extensively to treat patients with hypercholesterolaemia [14,15]. Atorvastatin is a synthetic lipid lowering agent [16]. It is a competitive inhibitor of HMG-CoA reductase which catalyzes the conversion of HMG-CoA to mevalonate, an early rate limiting step in cholesterol biosynthesis resulting in depletion the intracellular supply of cholesterol [17]. Inhibition of cholesterol biosynthesis is accompanied by an increase in hepatic LDL receptor on the cell surface which promotes uptake and clearance of circulating LDL. Thus the end result is a reduction in plasma cholesterol by both lowered cholesterol synthesis and by increased catabolism of LDL [15]. Atorvastatin also reduces VLDL-C, TG and produces variable increase in HDL-C [18]. Atorvastatin is safe and generally well tolerated [19]. Mild gastrointestinal side effects like dyspepsia, flatulence, abdominal pain, diarrhea and constipation may occur. Other side effects include headache, rash, pruritus and malaise. The most detrimental adverse effect of atorvastatin is hepatotoxicity and myopathy [20].

Munford [21] and Shishehbor *et al.* [22] stated that the overall clinical benefits observed with atorvastatin therapy appear to be greater than what might be expected from changes in lipid levels alone, suggesting effects beyond cholesterol lowering called pleiotropic effects. Vishal *et al.* indicated that some of the cholesterol-independent effects of atorvastatin involve improving endothelial function, enhancing the stability of atherosclerotic plaques, decreasing oxidative stress, decreasing inflammation, improve insulin resistance, inhibiting the throm-

bogenic response in the vascular wall and impede tumor cells. Further more statin have other extrahepatic beneficial effects on the immune system, central nervous system and bone [23]. Atorvastatin possesses antioxidant properties by reducing lipid peroxidation and ROS production [23]. It reduces the susceptibility of lipoproteins to oxidation both in vitro and in vivo, *i.e.*, they decrease the LDL oxidation [23].

The Aim of This Study was to clarify the effect of atorvastatin on selected oxidative stress parameters namely (reduced glutathione (GSH), lipid peroxidation product malondialdehyde (MDA) levels, glutathione  $-S$ -transferase (GST) and catalase (CAT) activities) and lipid profile in dyslipidaemic type 2 diabetic patients.

## 2. Materials and Methods

### 2.1. Materials

Atorvastatin (Atorfit 20, Ajanta Pharma Limited, India.), EDTA & GSH (Biochemicals Co. Ltd.), DTNB (Sigma Co. Ltd.), Trichloroacetic acid (TCA) & Thiobuteric acid (TBA) (Merk Co. Ltd.) were obtained as a gift samples. CDNB,  $K_2HPO_4$ ,  $KH_2PO_4$ ,  $Na_2HPO_4$ ,  $H_2O_2$  (Analar company) were purchased commercially and used as received.

### 2.2. Patients

Fifty nine patients (age:  $57.16 \pm 1.34$  years; 32 men & 27 women) with type 2 DM and dyslipidaemia were included in this study after obtaining their written consent and formal approval of the human experimentation committee at the Faculty of Medicine, Kufa University. The mean fasting blood glucose was ( $7.91 \pm 0.7$  mmol/l), with a mean duration of diabetes of ( $8.4 \pm 1.08$  years). The mean LDL-C level was ( $5.48 \pm 0.72$  mmol/l). The patients were chosen randomly from Al- Hakeem center for researches and treatment of DM at Al-Sadr Teaching Hospital in Najaf City in the period between 5<sup>th</sup> Nov. 2006 to 24<sup>th</sup> June 2007. These patients underwent full history and complete physical examination. Patients with the following criteria were excluded from the study: 1) Patients who use any vitamin preparations, or statins in the last three months [24]; 2) Patients with renal insufficiency, defined as a serum creatinine level equal to or more than 1.8 mg/dl [24]; 3) Patients with liver disease [22]; 4) Hypertensive patients, as this condition affects oxidative stress [13] and antihypertensive drugs may affect lipid profile and oxidative stress in hypertensive patients [25,26]; 5) Patients with chronic inflammatory diseases [24]; 6) Alcoholics & smokers were also excluded [27]. The patients were using glibenclamide (Glibesyn, Medochemie LTD-Cyprus; Glibils, Hikma-Jordon) (an oral hypoglycaemic agent) during the study

as a treatment for their diabetes. According to the design of the study, type 2 diabetic patients were followed up for 60 days and divided randomly into two groups: Group I (n = 31) received no drug and served as dyslipidaemic diabetic control. Group II (n = 28): received atorvastatin tablets 20 mg once daily at night (Atorfit 20). Only forty six patients continued to the end of the study while thirteen patients were withdrawn (eight patients from Group I and five patients from Group II) because of non compliance. The patients were put on diet control and followed up every two weeks during the time of the study in order to make sure that they were using the medication properly and to regularly checking fasting blood glucose. Values of fasting blood glucose before, during and after the study were controlled within the previously mentioned range. Blood samples were drawn from the patients at the beginning and after 60 days of follow up between 8:30 & 10:30 am after at least 12-14 hours fasting. Fasting blood glucose, lipid profile, selected oxidative stress parameters (GSH, MDA levels, GST, CAT activities) were measured. Renal and hepatic functions were also assessed.

### 2.3. Sample Preparation

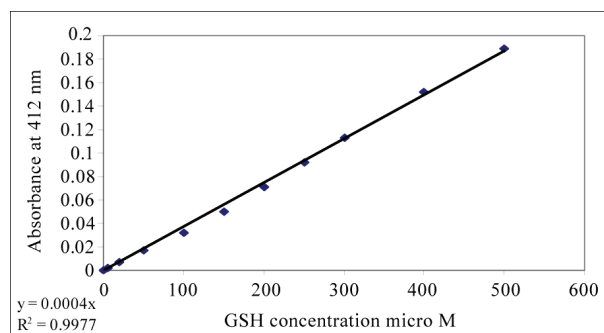
From each patient, 3 ml of blood was obtained without using heparin after an overnight fasting. The blood was placed in serum tube and left to stand for 30 minutes. The serum was prepared by centrifugation at 3000 rpm for 10 minute, 1.5 ml of serum was obtained for determination of experimental parameters which included GSH, MDA (the byproduct of lipid peroxidation), GST enzyme, CAT enzyme and lipid profile.

### 2.4. Serum Reduced Glutathione (GSH) Assay

Serum GSH was estimated according to a modified method of Ellman [28]. Briefly, Stock standard solution was prepared fresh daily by dissolving 0.0307 gm of GSH in a final volume of 100 ml of (0.2M) EDTA solution. Dilutions were made in EDTA solution to 50, 100, 150, 200, 250, 300, 400, 500  $\mu$ M. The absorbance of this series of known concentrations was determined spectrophotometrically using Shimadzu UV-1650P (UV-visible) at 412 nm to construct the calibration curve, "Figure 1".

### 2.5. Serum Lipid Peroxidation Product (MDA) Assay

The level of serum MDA was determined by a modified procedure described by Guidet and Shah [29]. Add 1 ml of TCA 17.5% and 1 ml of 0.6% thiobarbituric acid (TBA) to 0.15 ml of serum sample. The solution was mixed well by vortex, incubated in boiling water bath for 15 minutes, then allowed to cool. 1 ml of 70% TCA was



**Figure 1. Standard curve for determination of reduced glutathione level.**

added, and the mixture was allowed to stand at room temperature for 20 minutes, centrifuged at 2000 rpm for 15 minutes, and the supernatant was taken out for scanning spectrophotometrically at 532 nm using Shimadzu UV-1650P (UV-visible) Spectrophotometer.

$$\text{The concentration of MDA} = \frac{\text{Absorbance at 532 nm}}{L \times \epsilon} \quad (1)$$

L: Light path (1 cm)

$\epsilon$ : Extinction coefficient  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$

$$D: \text{dilution factor} = \frac{\text{Total volume}}{\text{Volume of the sample}}$$

### 2.6. Serum GST Enzyme Activity Assay

The absorption technique [30] includes mixing of 2.7 ml of Phosphate buffer (pH = 6.25) with 100  $\mu$ L of serum and 100  $\mu$ L of CDNB (1-chloro, 2, 4-dinitro benzene) and then after 3 minutes with 100  $\mu$ L of GSH solution. After mixing the solutions, the absorbance was read every minute for 10 minutes at wave length 340 nm.

Calculation:

$$\text{Activity of GST} \left( \frac{U}{L} \right) = \frac{\frac{\Delta A}{10} \times V_t \times 1000}{\epsilon \times V_s \times b} \quad (2)$$

$\Delta A$  = absorbance difference between the first and tenth minute

$V_t$  = The total volume

$V_s$  = Sample volume

$\epsilon$  = 9.6 mM<sup>-1</sup>cm<sup>-1</sup>

$b$  = 1 cm.

$$\text{Activity of GST} = \frac{\frac{\Delta A}{10} \times 3 \times 1000}{9.6 \times 0.1 \times 1} \quad (3)$$

### 2.7. Serum Catalase Activity Assay

The absorption technique [31] include mixing of 2 ml of

diluted serum with 1ml of H<sub>2</sub>O<sub>2</sub>. The solution was mixed well and the first absorbance (A1) was read after 15 seconds (t1) then the second absorbance (A2) after 30 seconds (t2) at a wave length 240 nm. 1 ml of Phosphate buffer solution was used instead of H<sub>2</sub>O<sub>2</sub> for the blank solution.

Calculation:

$$K = \frac{V_t}{V_s} \times 2.3 \times \log \frac{A_1}{A_2} \times 60 \quad (4)$$

K = rate constant of the reaction

Δt = (t2 – t1) 15 seconds

A1 = absorbance after 15 seconds

A2 = absorbance after 30 seconds

Vt = total volume (3ml)

Vs = volume of the sample

## 2.8. Serum Lipid Profile Assay

Total cholesterol, Triglyceride and HDL were measured according to procedures supplied by Spinreact company kits, using Shimadzu UV-1650P (UV-visible) Spectrophotometer. Serum LDL measure according to the following equation [32]

$$\text{LDL} = \text{total cholesterol} - \text{HDL} - \text{VLDL} \quad (5)$$

$$\text{VLDL} = \text{TG}/2.2. \quad (6)$$

## 2.9. Statistical Methods

The data expressed as mean ± SEM unless otherwise stated. Statistical analyses were done by using paired t-test. Pearson's correlations were also performed with significant difference set at P < 0.05.

## 3. Results

### 3.1. Effect of Atorvastatin on Oxidative Parameters

Atorvastatin treatment increased serum GSH and reduced MDA level significantly (P 0.05) while no significant change in serum GST and CAT activity was observed (P

0.05). There was no significant change (P 0.05) in oxidative stress parameters in diabetic control group apart from significant increase in MDA level "Table 1".

### 3.2. Effect of Atorvastatin on Lipid Profile

A significant decrease in serum TC, TG, LDL and VLDL and significant increase in HDL levels after atorvastatin treatment (P 0.05) while no significant change in lipid profile in diabetic control group was observed "Table 2".

### 3.3. Correlations between Observed Changes in Oxidation Markers and Observed Changes in Lipid Parameters in Atorvastatin Group

There were no significant correlations between atorvastatin induced changes in the oxidation markers and the observed changes in the lipid profile (P 0.05) "Table 3".

## 4. Discussion

### 4.1. Effect of Oxidative Stress Parameters

The significant increase in GSH and significant decrease in MDA levels (P 0.05) following atorvastatin treatment is supported by the findings of Save *et al.* [33] and Koter *et al.* [34], respectively. The most likely explanation for the increment of GSH and reduction of MDA by atorvastatin was attributed to the antioxidant mediated effect of atorvastatin which results from inhibition of mevalonate pathway. This effect results in a reduction in the synthesis of important intermediates including isoprenoids ((farnesyl pyrophosphate & geranylgeranyl pyrophosphate). The latter serve as lipid attachments for intracellular signaling molecules in particular inhibition of small GTPase binding proteins (Rho, Rac, Ras and G proteins) whose proper membrane localization and function are dependent on isoprenylation. These proteins modulate a variety of cellular processes including signaling, differentiation and proliferation [35,36]. Atorvastatin attenuates endothelial reactive oxygen species (ROS)

**Table 1. Effect of atorvastatin (20 mg/day) on oxidative stress parameters after 60 days of treatment and changes in dyslipidaemic diabetic control (n = 23 in each group).**

Parameters	Diabetic control		Atorvastatin	
	Before treatment	After treatment	Before treatment	After treatment
GSH(mmol/l)	0.24 ± 0.0079	0.22 ± 0.0036*	0.23±0.0034	0.40±0.0009**
MDA(mol/l)	1.25 × 10 <sup>-4</sup> ± 0.0146	1.59×10 <sup>-4</sup> ±0.0210**	1.24×10 <sup>-4</sup> ±0.0178	0.24×10 <sup>-4</sup> ± 0.004**
GST(U/l)	13.68 ± 0.18	13.87 ± 0.2194*	13.95 ± 0.234	14.07 ± 0.212*
CAT(K/ml)	0.49 ± 0.0123	0.5001 ± 0.016*	0.48 ± 0.0133	0.483 ± 0.012*

\*p > 0.05; \*\*p < 0.01; Values expressed as mean ± SEM.

**Table 2. Effect of atorvastatin (20 mg/day) on lipid profile after 60 days of treatment and changes in dyslipidaemic diabetic control (n = 23 in each group).**

Parameters	Diabetic control		Atorvastatin	
	Before treatment	After treatment	Before treatment	After treatment
TC(mmol/l)	6.99 ± 0.1173	6.74 ± 0.0332*	7.49 ± 0.0230	4.38 ± 0.0189**
TG(mmol/l)	2.78 ± 0.0645	2.68 ± 0.0159*	2.84 ± 0.0145	1.78 ± 0.0069**
HDL(mmol/l)	0.81 ± 0.0300	0.76 ± 0.0114*	0.76 ± 0.0159	1.06 ± 0.0109**
LDL(mmol/l)	4.90 ± 0.1300	4.75 ± 0.0339*	5.43±0.0243	2.51 ± 0.0168**
VLDL(mmol/l)	1.26 ± 0.0129	1.21 ± 0.0031*	1.29±0.0029	0.81 ± 0.0013**

\*p > 0.05; \*\*p < 0.01; Values expressed as mean ± SEM.

**Table 3. Pearson's correlation for changes in the oxidative markers and lipid parameters in the atorvastatin group.**

parameter	GSH	MDA	GST	CAT
TC	-0.232	0.042	0.427	-0.119
TG	-0.172	-0.105	0.039	-0.135
HDL-C	-0.223	-0.068	0.220	-0.139
LDL-C	-0.083	0.088	0.329	-0.032
VLDL-C	-0.157	0.001	-0.031	-0.055

P > 0.05 non significant.

formation, through attenuating endothelial superoxide anion production, by inhibition of NAD(P)H oxidase activity via Rho dependent mechanism. Some of antioxidant effects of atorvastatin may be due to its metabolites such as hydroxyl metabolites which have direct antioxidant effect. Atorvastatin improves and preserves the level of vitamin C, E and endogenous antioxidant such as reduced glutathione [16]. The protective effects of atorvastatin on ROS including cholesterol dependent and non cholesterol dependent antioxidative properties [16].

Serum GST enzyme activity did not significantly change in atorvastatin treatment and this finding was consistent with Passi *et al.* [37] who concluded that atorvastatin had no effect on GST activity.

It was also noted that atorvastatin showed insignificant change in the CAT activity which is in agreement with Passi *et al.* [37]. However, our results conflict with the findings of Wassmann *et al.* who concluded that atorvastatin caused a significant increase in the CAT activity [38]. This confliction may be due to the fact that the sample size may be relatively small permitting chance observations to exert substantial effects.

#### 4.2. Effect on Lipid Profile

A significant decrease in serum level of TC, TG, LDL

and VLDL and significant increase in serum level of HDL by atorvastatin treatment are in agreement with the findings obtained by Diabetes Atorvastatin Lipid Intervention study group [39] and Save *et al.* [33]. The mechanism involved is most likely attributed to the ability of atorvastatin to impair cholesterol synthesis via inhibiting the enzyme HMG-CoA reductase, which is the rate limiting step in cholesterol biosynthesis. This leads to both, decrease circulating lipoproteins and increase their uptake by up regulating hepatic LDL-C receptors. The overall lipid lowering effect include increase uptake and degradation of LDL-C, inhibition of LDL-C oxidation, reduction in cholesterol accumulation and esterification and decreases lipoprotein secretion and cholesterol synthesis [22,40].

#### 4.3. Correlations between the Observed Changes in the Oxidation Markers and the Improvement of Lipid Profile in Atorvastatin

According to this study there were insignificant correlations between the observed changes in the pleiotropic effect of atorvastatin, regarding antioxidant properties and the improvement in the lipid profile. The same findings was reported by Sakabe *et al.* [41]. This pleiotropic effect of atorvastatin is due, predominantly, to inhibition of isoprenoids but not cholesterol synthesis [42].

From the results of this study, we can conclude that, atorvastatin increased GSH, reduced MDA levels and had no effect on CAT and GST activities. Atorvastatin reduced TC, TG, LDL-C, VLDL-C and increased HDL-C levels. Also there were no correlations between the observed changes in the oxidation markers and the improvement of the lipid profile in the atorvastatin.

#### 5. Conclusions

The antioxidant effect of atorvastatin could be unrelated to its hypolipidemic action as there was insignificant

correlation between changes in lipid profile and oxidative stress in this study.

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