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# Improve Markers of Oxidative Stress and Coagulation Parameters in Response to Atorvastatin Therapy

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author BIM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors NRH and HMJ managed the analyses of the study. Authors DAJ and HAAA managed the literature searches and wrote the final draft. All authors read and approved the final manuscript.*

***Original Research Article***

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## ABSTRACT

**Aims:** This study was undertaken to investigate the antioxidant effects of atorvastatin in treating cases of atherosclerosis associated with hyperlipidemia.
**Methodology:** Forty local domestic rabbits were assigned to five groups (eight rabbits in each group): After two weeks acclimatization period, a group of 8 rabbits (Group I) were used as the baseline values of the study parameters. Another 8 rabbits were selected and maintained on standard chow diet (4% fat, 18% protein, 60% carbohydrate, and 4% fibers) throughout the experiment (12 weeks) and served as a normal diet control (Group II). The rest 24 rabbits were fed on an atherogenic diet for 8 weeks to induce atherogenesis. At the end of 8th week, a group of 8 atherogenic rabbits (Group III) were separated and sacrificed and served as an atherogenic-baseline group. The remaining 16 atherogenic rabbits were randomly allocated into two groups; first group received

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atherogenic diet only for the next four weeks and served as an atherogenic control (Group IV). The other group received atorvastatin (Group V).
**Results:** Blood samples were collected for serum lipids, coagulation parameters and oxidation parameters. Results showed a significant improvement in the coagulation parameters and oxidation parameters in the atorvastatin treated group compared to the atherogenic control group (*P*= .01).
**Conclusion:** This study illustrated the beneficial anti-oxidant effects of atorvastatin in treating atherosclerosis associated with hyperlipidemia.

*Keywords: Atorvastatin; coagulation; oxidative stress; antioxidants; atherosclerosis.*

## 1. INTRODUCTION

Statin therapy is proved to be effective and remains the first-choice treatment for high blood cholesterol levels. It reduces the risk of coronary heart disease (CHD) in both primary and secondary dyslipidemia [1-3]. Atorvastatin is the most widely used statin. It acts through inhibiting the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase [4]. Its effectiveness in lowering total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) has been demonstrated in the previous studies [4,5].

High doses of statins are needed for aggressive lipid lowering but these were found to have a greater risk of causing adverse effects on liver and muscles [6]. However, despite the consistent effects of low-density lipoprotein-lowering medications in reducing cardiovascular morbidity and mortality, the reductions in clinical events remain modest, ranging from 25 to 35% [7]. Although serum lipids serve a crucial role in atherogenesis, atherosclerosis develops in many people without hypercholesterolemia and continues to progress in peoples with pharmacologically controlled lipids. Other mechanisms therefore must operate along with hyperlipidemia to promote atherosclerosis [7]. Recently, because of the pleiotropic effects of statins including maintenance of endothelial function, reduction of inflammatory response, attenuation of smooth muscle cells proliferation, reduction of oxidative stress and stabilization of plaque, the importance of other target sites other than LDL-cholesterol lowering have been suggested [8].

Statins have various effects on haemostasis and thrombosis. Although most studies support a predominantly antithrombotic action of the statins, some opposite or neutral effects have been reported [9]. Studies focusing on the effect of statins upon fibrinogen levels have demonstrated inconsistent results: while some studies reported no change [10] or a decrease in fibrinogen levels during treatment with various statins [11], others found significant increases [12]. Although simvastatin was found to shift the fibrinolytic balance towards increased fibrinolytic activity, not all statins affect fibrinolysis to the same degree and comparative studies have demonstrated some differences [13-15]. Other in vivo effects common to statins include a reduction of platelet aggregation [16]. Independent of cholesterol levels, atorvastatin has been shown to decrease platelet activation in mice [17].

This study aims to investigate the antioxidant effects of atorvastatin in treating cases of atherosclerosis associated with hyperlipidemia. This study also aims to evaluate the behaviour of coagulation parameters in response to atorvastatin therapy.

## 2. METHODOLOGY

### 2.1 Preparation of Animals

The experimental procedures and animal uses related to this study were approved by the Scientific and Ethical Committee of the College of Medicine, University of Kufa, according to the guidelines for the care and use of laboratory animals in scientific research. A total of 40 New Zealand White Male Rabbits (*Oryctolagus cuniculus*), aged 3–4 months and weighing 2.0 to 2.4 kg, were used in this study. The animals were placed in Kufa Medical College's animal house. The rabbits were housed in an isolated room, in a group caging system, at controlled temperature (25±2 ◦C) and ambient humidity. Lights were maintained on a 12-h light/dark cycle. The animals were given free access to water and libitum.

### 2.2 Design of the Study

After two weeks acclimatization period, a group of 8 rabbits (Group I) were randomly selected and sacrificed at the start of the experiment where baseline values of the study parameters were measured. Another 8 rabbits were selected and maintained on standard chow diet (4% fat, 18% protein, 60% carbohydrate, and 4% fibers) throughout the experiment (12 weeks) and served as a normal diet control (Group II). The rest 24 rabbits were fed on an atherogenic diet for 8 weeks to induce atherogensis. At the end of $8^{th}$ week, a group of 8 atherogenic rabbits (Group III) were separated and sacrificed and served as an atherogenic-baseline group. Then the remaining 16 atherogenic rabbits were randomly allocated into two groups, eight animals each; first group received atherogenic diet only for the next four weeks and served as an atherogenic control (Group IV). In addition to atherogenic diet, the other group received atorvastatin for the next four weeks (Group V).

### 2.3 Animal Model of Atherosclerosis

Induction of hyperlipidemia and subsequent development of atherosclerosis were carried out by feeding the rabbits an atherogenic diet (4% cholesterol-enriched diet made by addition of cholesterol powder to chow pellets) for 8 weeks [18].

### 2.4 Preparation of Atorvastatin

Atorvastatin was used in a dose of 5mg/kg/day orally [19]. A 20 mg tablet (Avas-20, Micro Labs, Batch No. AVFH0035, India) was suspended in carboxymethylcellulose to produce 5% solution and doses were prepared from this stock solution and given to the rabbits according to body weight once daily through stomach tube.

### 2.5 Preparation and Collection of the Samples

At the end of the experiments, food was withheld for 16-18 hr and all animals were sacrificed. The chest was opened by thoracotomy. 5 ml of blood were collected from the heart directly using disposable syringe. The blood sample was divided into 3 parts:

- One ml of the blood was placed in a tube that contained sodium citrate, as an anticoagulant, in 9:1 ratio (9 volumes of blood: 1 volume of anticoagulant). The

plasma was prepared via centrifugation at 2500 rpm for 10 minutes for determination of fibrinogen, prothrombin time (PT) and activated partial thromboplastin time (aPTT) [20].

- 0.5 ml of blood was placed in a tube containing EDTA, as anticoagulant, in 9:1 ratio (9 volumes of blood: 1 volume of anticoagulant).This part was used for platelets counting.
- The last 3.5 ml of blood was placed in plane tube and left to stand for 30 minutes. The serum was prepared via centrifugation at 3000 rpm for 10 minutes. 2 ml of serum frozen at (-80ºC) and subsequently used for determination of serum lipids, oxidation parameters: superoxide dismutase (SOD) [21], serum glutathione (GSH) [22], serum malondialdehyde (MDA) [23] and uric acid. The serum kept frozen unless immediately analyzed.

## 2.6 Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) version 13.0 and Microsoft Excel (Office2007, Microsoft). Data were expressed as mean ± SEM. Post-hoc test was used to compare the mean values within each group at different time using LSD technique. Analysis of Variance (ANOVA) was used for the multiple comparison among all groups. The statistical significance, direction and strength of linear correlation between 2 quantitative variables were measured by Pearson's linear correlation coefficient. A significance differences were set at p value equal or less than the 0.05. Power analysis using a high power coefficient & effect size indicated that the number of samples included in the analysis were sufficient.

## 3. RESULTS

## 3.1 Effect of Atherogenic Diet on Serum Lipids and Coagulation Parameters

Feeding an atherogenic diet to rabbits for eight weeks resulted in significant changes in serum lipid profile, as compared to baseline group. TC, TG, VLDL-C and LDL-C were significantly increased ($p< 0.001$) while HDL-C was significantly decreased ($p< 0.001$) Table 1.

**Table 1. Changes in serum lipids and coagulation parameters of rabbits fed on atherogenic diet for 8 weeks, (N=8). The data expressed as means ± SEM**

| Parameters | Baseline values | After 8 weeks of atherogenic diet | P-value |
|---|---|---|---|
| TC (mg/dl) | 86.5±3.354 | 546.875±9.33 | *P* < .001 |
| TG (mg/dl) | 94.125±4.231 | 149.5±2.405 | *P* < .001 |
| HDL-C (mg/dl) | 38.375±2.938 | 19.375±0.532 | *P* < .001 |
| VLDL-C( mg/dl) | 18.825±0.846 | 29.9±0.481 | *P* < .001 |
| LDL-C (mg/dl) | 29.3±3.226 | 496.35±9.818 | *P* < .001 |
| Plasma fibrinogen (mg/dl) | 150.875±4.06 | 442.125±6.145 | *P* < .001 |
| PT (second) | 11.27 5±0.343 | 10.587±0.232 | *P*= .08 |
| PTT (second) | 21.737±0.563 | 21.2±0.496 | *P* = .12 |
| Platelets counts (x109/L) | 168.625±3.348 | 173.625±3.99 | *P* = .09 |

Rabbits fed on atherogenic diet for 8 weeks showed a non significant shortening of PT and aPTT, as compared to baseline group. While atherogenic diet produced significant elevation in

plasma fibrinogen levels (*P*<.001). There was also a non significant change in platelets counts, Table 1.

### 3.2 Effect of Atherogenic Diet on Oxidation Parameters

In comparison with baseline group, feeding an atherogenic diet to rabbits for eight weeks resulted in significant changes in oxidation parameters (serum MDA level, SOD activity and GSH) level. there were significant rise in serum MDA levels and SOD activity (*P* < .001) associated with a significant decrease in GSH levels at (*P* < .001), Table 2.

**Table 2. Changes in serum oxidation parameters of rabbits fed on atherogenic diet for 8 weeks, (N=8), the data expressed as means ± SEM**

| Parameters | Baseline | After 8 weeks of atherogenic diet | P-value |
|---|---|---|---|
| MDA (µmol/L) | 1.622±0.052 | 3.803±0.164 | *P* < .001 |
| SOD (U/ml) | 1.009±0.048 | 2.013±0.034 | *P* < .001 |
| GSH (µmol/L) | 3.772±0.069 | 2.282±0.07 | *P* < .001 |

### 3.3 Effect of Atherogenic Diet on Serum Uric Acid

Serum uric acid levels were significantly elevated in the sera of rabbits fed on atherogenic diet for eight weeks, as compared to baseline group (4.3± 0.15 vs. 2.641± 0.152), (*P* < .001), Fig. 1.

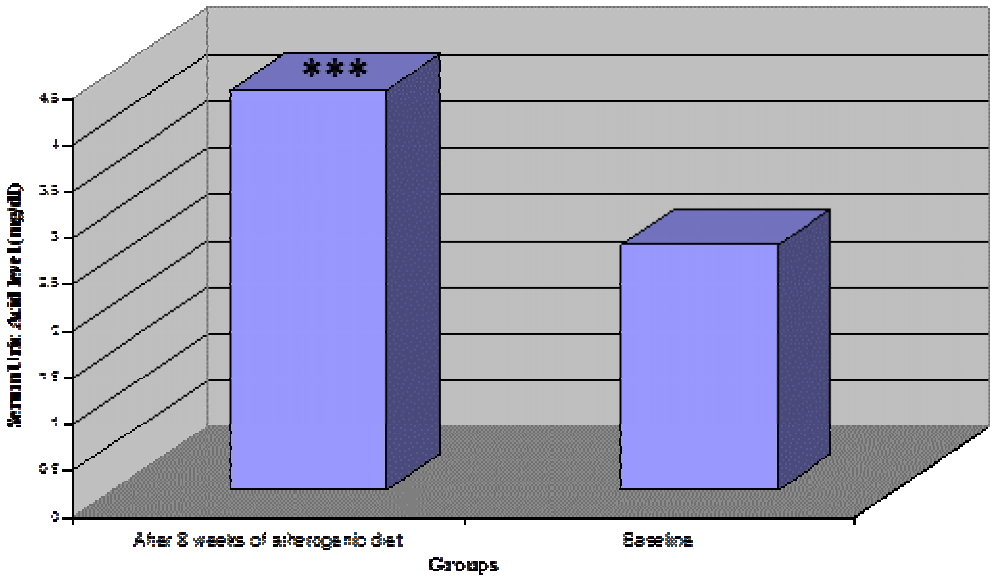


**Fig. 1. Changes in serum uric acid (mg/dl) or rabbits fed on atherogenic diet for 8 weeks compared to the baseline. The data expressed as means ± SEM, *P*<.001**

### 3.4 Effect of 4 Weeks Drug Treatment on Coagulation Parameters

The atorvastatin treated group showed a significant reduction in plasma fibrinogen levels (*P* < .001), prolongation of PT (*P* < .001) and aPTT (*P* < .001) , while there was non significant changes in the platelet's counts (*P* = .078) Table 3.

**Table 3. Effects of 4 weeks atorvastatin (5 mg/kg) treatment on coagulation parameters, (N= 8); the data are expressed as means ± SEM**

| Parameters | Atherogenic Control (Group IV) | Atorvastatin Treated (Group V) | *P*-value |
|---|---|---|---|
| Plasma fibrinogen (mg/dl) | 442.125±6.145 | 270.5±10.471 | *P* < .001 |
| PT (second) | 10.587±0.232 | 17.175±0.385 | *P* < .001 |
| aPTT (second) | 21.2±0.496 | 32.01±1.2 | *P* < .001 |
| platelets counts (x$10^9$/L) | 173.625±3.99 | 172.62±3.585 | *P* = .78 |

### 3.5 Effect of 4 Weeks Drug Treatment on Oxidation Parameters

The atorvastatin treated group showed a significant reduction in serum MDA levels (p< 0.001), accompanied with a significant decrease in serum SOD activity (*P* < .001) and a significant increase in serum GSH levels (*P*= .01) Table 4.

**Table 4. Effects of 4 weeks atorvastatin (5 mg/kg) treatment on oxidation parameters, (N= 8); the data are expressed as means ± SEM**

| Parameters | Atherogenic Control (Group IV) | Atorvastatin Treated (Group V) | *P* -Value |
|---|---|---|---|
| MDA (µmol/L) | 3.803±0.164 | 1.975±0.118 | *P* < .001 |
| SOD (U/ml) | 2.013±0.034 | 1.775±0.112 | *P* < .001 |
| GSH (µmol/L) | 2.282±0.07 | 2.845±0.059 | *P* = .01 |

### 3.6 Effect of 4 Weeks Drug Treatment on Serum Uric Acid

Serum uric acid was significantly reduced in the atorvastatin treated group (4.937±0.256) compared to the atherogenic group (3.820±0.215) at (*P*= .05).

## 4. DISCUSSION

In the present study, the plasma fibrinogen levels were significantly increased in hyperlipidemic rabbits. Moreover, cholesterol fed rabbits showed no significant changes in PT, aPTT and platelet count. In fact there was a shortening in PT and aPTT but it did not reach a statistical significance. They found that hyperlipidemia caused a significant rise in plasma fibrinogen and enhanced blood clotting. A possible explanation for these findings is that hyperlipidemia may accelerate the rate of prothrombin activation and enhance inflammatory process thereby increase fibrinogen which is acute phase reactant [24-26].

The cholesterol fed rabbits developed hyperlipidemia which subsequently promoted oxidative stress as evident by the significant changes in serum oxidation parameters namely MDA (lipid peroxidation product), and antioxidants SOD and GSH. Serum MDA level was

significantly higher in hyperlipidemic than in normolipidemic rabbits. Additionally, there was strong positive relationship between atherogenic index and serum MDA. Similar findings were reported by Hakimoglu et al. [27]. MDA level is widely utilized as a marker of lipid peroxidation and its measurement gives indirect evidence for LDL oxidation and is important in predicting free radical-induced injury. Thus the increase in MDA level is an indication of elevated oxidative stress condition. Therefore the observed elevation in serum MDA level may be attributed to hyperlipidemia that enhances the process of lipid peroxidation [28-30].

The measurement of antioxidant concentration or activity has been performed to estimate the amount of oxidative stress. The current study observed that serum SOD activity was significantly increased in hyperlipidemic rabbits as compared to normolipidemic animals. Moreover, there was strong positive relationship between atherogenic index and serum SOD activity. These findings agree with Meilhac et al. [31] who reported that serum SOD activity was increased in hyperlipidemia. While Pandya et al. [32] observed that SOD activity was not significantly changed in hyperlipidemia. The observed increase in SOD activity is an adaptive mechanism to oxidative stress. An increased vascular superoxide formation has been demonstrated in hyperlipidemia. High levels of serum SOD activity indicated the presence of high level of superoxide anion, which represents an indicator to high degree of oxidative stress, therefore increased amounts of substrate of superoxide results in a stimulation to increase synthesis of serum SOD in order to provide protection [33].

In the present study, it has been found that serum GSH level of hyperlipidemic rabbits was significantly decreased in comparison to that of normolipidemic animals. A strong inverse relationship was observed between atherogenic index and serum GSH. These findings are in agreement with our previous study [34]. It has been found that hyperlipidemia increased lipid hydroxyperoxides. GSH, in addition to its direct antioxidative effect, has been reported also to detoxify the lipid hydroperoxides protecting the cells against the damage induced by oxidized LDL. Therefore, consumption of the antioxidant GSH is a possible explanation for the decreased serum GSH level [35].

The relationship between SUA levels and oxidative stress-caused disorders including hyperlipidemia and atherosclerosis is not clear yet. In this setting, enhanced xanthine oxidase activity resulted in raised SUA concentrations, and increased hydrogen peroxide synthesis, which stimulates further liberation of free radicals. On the other hand, uric acid reacts with peroxynitrite to form a stable nitric oxide donor and thereby lowers the risk of peroxynitrite-mediated oxidative injury [36,37]. However, the situation is much complex and the beneficial or detrimental effects of raised SUA concentrations depend on the degree of xanthine oxidase activity, which is ordinarily quiescent in health but is characteristically up-regulated in high risk groups. Furthermore, high SUA concentrations could promote cardiovascular risk through induction of endothelial dysfunction and excessive platelet aggregability, by decreasing the production of nitric oxide (NO) in the vascular endothelial cells, which might predispose to thrombosis and atherosclerotic plaque progression [36,37].

Concerning coagulation parameters, this study showed that treatment of hyperlipidemic rabbits with atorvastatin resulted in significant fall in plasma fibrinogen level. These findings agree with those reported by Joukhadar et al. [38]. While Black, [39] reported no change Nair et al. [40] observed a significant increase in plasma fibrinogen levels during treatment with atorvastatin [40]. The observed reduction in plasma fibrinogen level might be attributed to the lipid lowering and anti-inflammatory effects of atorvastatin. As statins block the synthesis of mevalonate, the precursor of cholesterol and isoprenoids, they lower plasma cholesterol and interrupt proinflammatory signaling leading to hypocholesterolemic and anti-

inflammatory effects thereby inhibiting the hepatic synthesis of fibrinogen [39].

Moreover, this study demonstrated that atorvastatin significantly prolonged PT and aPTT. Regarding platelets count, no significant changes were observed by atorvastatin treatment. The postulated potential mechanisms by which atorvastatin exerts an antithrombotic action could include decreased expression and activity of tissue factor in the endothelium, inhibition of platelet activation and modulation the major anticoagulant system, the protein C pathway, which inactivates factor FVa and FVIIIa and shuts down thrombin generation [41].

Atorvastatin also showed to be beneficial in reducing serum MDA, SOD and increases serum GSH in hyperlipidemic rabbits. These observations suggest that atorvastatin may exert antioxidant effects that protect against hyperlipidemia-mediated oxidative stress [42]. Passi et al., 2003 observed that statin therapy might lead to a reduced antioxidant capacity of lymphocytes, and probably of tissues such as liver [43]. However, this reduction did not appear to induce a significant oxidative stress in blood. A pleiotropic cholesterol-independent effect of statins may suggest a potential explanation for the inhibition of NAD(P)H oxidase activity and for the up-regulation of eNOS expression. Thus it may be assumed that reduced production and increased elimination of reactive oxygen species (ROS) may participate in the antioxidant effects of statins [44]. In this study atorvastatin treatment resulted in a significant reduction of SUA levels. The possible explanation for the reduction in SUA levels by atorvastatin may involve augmentation in its urinary excretion [45-48].

## 5. CONCLUSION

This study illustrated the beneficial anti-oxidative effects of atorvastatin in treating atherosclerosis associated with hyperlipidemia.

## CONSENT

Not applicable.

## ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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