ANTIOXIDANT EFFECTS OF LOVASTATIN ON COPPER-MEDIATED IN-VITRO OXIDATIVE MODIFICATION OF LDL IN INFLAMMATION INDUCED HYPERLIPIDEMIA IN RATS

Amir Khan1*, Shozab Jawed2, Mahesa Nand3, Lakhvinder Singh3, Rishab Jain and Munish Thakur4
1Department of Biotechnology & Biochemistry, Division of Life Science, Sardar Bhagwan Singh Post Graduate Institute (SBSPGI) of Biomedical Sciences & Research Balawala, Dehradun- 248 161 UK, India
2Department of Biotechnology, Beehive College of Advance Studies, Dehradun, UK, India
3Department of bio-medical Sciences, Dolphin Post Graduate Institute of Bio-medical & Natural Sciences, Dehradun, UK, India
4Department of Biotechnology, Seth Jai Parkash Mukand Lal Institute of Engineering & Technology, (Chota Bans) Radaur, Yamuna Nagar (Haryana)
5Department of Pharmaceutical Chemistry, UCST, Dehradun, UK, India

Article Received on: 20/02/12 Revised on: 17/03/12 Approved for publication: 21/04/12

ABSTRACT
Cardiovascular diseases (CVD) are the main cause of disability and premature dead worldwide. Epidemiological studies have suggested a link between atherosclerosis and inflammation. Atherosclerosis is a multifaceted diseases process with several different well defined risks factors, such as hypercholesterolemia, hypertension and diabetes. The present study was carried out to investigate the efficacy of antioxidant agent lovastatin. The study comprises the antioxidant status of Lovastatin by analyzing all the parameters in plasma, TL, TC, TG, VLDL-C, LDL-C, non-HDL-C, MDA and in-vitro oxidizability of LDL in absence or presence of Lovastatin. All the plasma lipids parameters, TL, TC, TG, VLDL-C, LDL-C, non-HDL-C and MDA levels were significantly increased in inflammation induced hyperlipidemic (IIH-C) rats. After 4-weeks administration of Lovastatin (1mg/ml) to IIH-C rats were useful in the prevention and treatment of inflammation induced hyperlipidemia, CVD and atherosclerosis. Lovastatin significantly reduced the overall oxidative burden and effectively ameliorated the above altered parameters.

Keywords: Cardiovascular diseases (CVD), Atherosclerosis, Hypercholesterolemia, Hypertension and Inflammation.

INTRODUCTION
Cardiovascular disease (CVD) is the main cause of disability and premature death worldwide, and is projected to remain the leading cause of death. CVD is common in the general population, affecting the majority of adults. Hence, this disease greatly contributes to the rising costs of health care in the world. It is a major public-health challenge, especially in low and middle income countries. Excessive dietary lipids and cholesterol are the major factor of relevance for the development of hypertriglyceridemia and hypercholesterolemia, two important cardiovascular risk factors. Hyperlipidemia with accompanying increase in peripheral inflammation is a risk factor for stroke. Abnormalities in lipid profiles, folate metabolism and other traditional risk factors (e.g., diabetes mellitus and hypertension) play a rather peripheral role and serve to amplify the atherosclerotic process initiated by persistence of infection and inflammation. Several clinical and epidemiological studies indicate that diabetes mellitus (DM) is an independent risk factor for CVD in both men and women, which increases the CVD risk by two-to six-fold relative to non diabetic subjects. Infection and inflammation are accompanied by cytokine-induced alterations in lipid and lipoprotein metabolism. Of note, inflammatory cytokines are increased and play a pathogenic role in a variety of very common disorders, such as diabetes, obesity, metabolic syndrome, hypertension, chronic heart failure, chronic renal failure, and atherosclerosis. Moreover, recent epidemiological studies have strongly suggested that disorders that lead to systemic inflammation increases the risk of developing CVD. Studies have shown that patients with autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, or psoriasis have an increased risk of CAD. Furthermore, a number of clinical disorders that are well recognized to increase the risk of CVD, such as diabetes, chronic renal failure and obesity, are now recognized to induce systemic inflammation. Both infection and inflammation induce the systemic host response known as acute phase response (APR), and produce many abnormalities that could increase the risk of developing atherosclerosis including alterations in lipid and Lipoprotein metabolism, which is often, mediated by cytokines, particularly highly sensitive C-reactive protein, TNF-α, IL-1 and IL-6. Hyperlipidemia or hyperlipoproteinemia is the condition of abnormally elevated levels of any or all lipids and/or lipoproteins in the blood. It is the most common form of dyslipidemia (which also includes any decreased lipid levels). Lipids (fat-soluble molecules) are transported in a protein capsule, and the size of that capsule, or lipoprotein, determines its density. Lipid-lowering drugs such as statins have also been shown to antagonize inflammation. Lovastatin is a member of the drug class of statins, used for lowering cholesterol (hypolipidemic agent) in those with hypercholesterolemia and so preventing cardiovascular disease. Lovastatin is a naturally occurring drug found in food such as oyster mushrooms (Mevador (Merek & Co.) in the United States Gunde-Cimerman N, Cimerman A. Mar 1995) and red yeast rice. Lovastatin, a natural product with...
a powerful inhibitory effect on HMG-CoA reductase, were discovered in the 1970s, and taken into clinical development as potential drugs for lowering LDL cholesterol\textsuperscript{12,13}.

As discussed above, the role of inflammation in atherosclerosis has burgeoned. In this study we investigate the efficacy of antioxidant agent lovastatin by analyzing all the parameters in plasma, TC, VLDL-C, LDL-C, HDL-C and its sub fraction( HDL\textsubscript{2}-C, HDL\textsubscript{3}-C), Conjugated cine, MDA and as well as \textit{in vitro} oxidizability of Low Density Lipoprotein.

**MATERIAL AND METHODS**

**Chemicals:** 1-Chloro 2, 4-Dinitrobenzene was purchased from Central drug house, Pvt. Ltd. (India). All other chemicals used for this study were of analytical grade and obtained from HIMEDIA (India), Sisco (India), Ashirwad (India), Sigma-Aldrich (USA), Miles (USA), Acros (USA) and Lovastin drug was supplied as a gift from Saimira Inmoform Pvt. Ltd. Chennai, India.

**Estimation:** Plasma triglyceride\textsuperscript{14}, Plasma Cholesterol\textsuperscript{15} Plasma VLDL-C\textsuperscript{16}, Fractionation of Plasma lipoprotein such as LDL\textsuperscript{17,18}, HDL and its sub fractions-HDL\textsubscript{2}, HDL\textsubscript{3}, Plasma FRAP\textsuperscript{19}, \textit{ex vivo} and \textit{in vitro} Cu\textsuperscript{2+}-mediated LDL oxidation\textsuperscript{20,21}.

**Experimental Design:** The experimental (IAEC no-bc1962) study was approved by the Dolphin Institute of Biomedical and Natural Sciences, Dehradun, Uttarakhand, where the study was conducted. Healthy male albino rats, weighing about 150-180g were purchased from Indian Veterinary Research Institute, (IVRI) Bareilly (India), were maintained to animal house environmental condition prior to the experiment. For the present study, animals were divided into following 3 groups:-NC (normal control), IIH-C (inflammation induced hyperlipidemic control), and IIH-LT (inflammation induced hyperlipidemic Lovastatin treated).

**Diet/Drug Administration:** The rats were given pelleted rat chow. Maintenance and treatment of all the animals was done in accordance with the principles of Institutional Animal Ethics Committee constituted as per the directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Six rats in IIH-LT group were given 1.0 mg Lovastatin/rat/day, through gastric intubation for 4 weeks.

**Induction of Inflammation:** Inflammation was induced in IIH-C and IIH-LT by the subcutaneous injection of turpentine (0.5ml/rat) in the dorsolumbar region and left for five hours.

**Collection of Blood and Plasma:** For the estimation of different parameters, overnight fasted rats in each group were anesthetized and blood drawn from cardiac puncture, and were collected in heparinated tube. Plasma was separated from blood by centrifugation at 2500 rpm for 30 min.

**Statistical evaluation:** This was done by employing two-tailed student t-test as describe by Bennet and Franklin (1967)\textsuperscript{21}. P values less than 0.02 were considered significant.

**RESULTS**

**Impacts of Lovastatin on average body weight in each group of rats:** Table 1 depicts the average body weight (g) of N-C, IIH-C, IIH-LT was167g, 166g and 170g, whereas, the average body weight of N-C, IIH-C, IIH-LT rats showed a significant gain of 35%, 24% and 39% respectively after 4 weeks of treatment. These results demonstrate that in inflammation induced hyperlipidemic Lovastatin treated rats (IIH-LT) the gain in body weight after 4 weeks was significantly higher than N-C rats.

**Table 1. Average body weight in each group of rats before and after 4 weeks of Lovastatin treatment**

<table>
<thead>
<tr>
<th>Group</th>
<th>Before treatment</th>
<th>After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-C</td>
<td>167.12±1.65</td>
<td>226.75±14.62</td>
</tr>
<tr>
<td>IIH-C</td>
<td>166.74±2.92</td>
<td>208.13±16.13</td>
</tr>
<tr>
<td>IIH-LT</td>
<td>170.21±6.96</td>
<td>238.21±12.16</td>
</tr>
</tbody>
</table>

*Values are mean ± SD from 6 rats in each group, N-C, normal control; IIH-C, inflammation induced hyperlipidemic rats; IIH-LT, fed 1 mg Lovastatin/rat/day for 4 weeks. Significantly different from N-C at \(p<0.001\), Significantly different from IIH-C at \(p<0.001\).

**Effects of Lovastatin on plasma total lipid (TL), triglycerides (TG) and total cholesterol (TC) in inflammation induced hyperlipidemic rats after 4 weeks of treatment:** As seen in Fig 1, all the plasma lipids parameters were significantly increased in Inflammation induced hyperlipidemic (IIH-C) rats, when compared to N-C values. Total lipids (TL), triglycerides (TG) and total cholesterol (TC) significantly increased from 385, 48, and 74 mg/dl in N-C to 497, 98, and 141 mg/dl, respectively, in IIH-C group. After 4 weeks of Lovastatin treatment, levels of TL, TG, and TC were significantly decreased by 6.9 %, 42 %, and 37 %, respectively, when compared to corresponding N-C values. These results demonstrate that 4-week treatment of inflammation induced hyperlipidemic rats with 1.0 mg Lovastatin mediated a significant reduction in above lipid parameters.

**Effects of Lovastatin on the plasma lipoprotein lipids and on the ratios of LDL-C/HDL-C and HDL-C/TC:** As seen in Fig 2, plasma VLDL-C, LDL-C and non-HDL-cholesterol (non-HDL-C) levels were significantly increased from 8.9 mg/dl, 49 mg/dl and 59 mg/dl in N-C to 18 mg/dl (104%), 107 mg/dl (118 %) and 127 mg/dl (114 %) respectively in IIH-C. After 4 weeks of Lovastatin treatment, both VLDL-C, LDL-C and non-HDL-C levels showed a significant reduction 44 %, 50 % and 47 %, respectively, in IIH-LT. Whereas HDL-C, HDL\textsubscript{2}-C and HDL\textsubscript{3}-C levels were decreased from 17, 5 and 9 mg/dl in IIH-C to 15 mg/dl (11%
LT fed 1 mg Lovastatin/rat/day for 4 weeks, significantly different from N.

Impacts of Lovastatin on plasma lipoprotein lipids, in inflammation induced hyperlipidemic rats after 4 weeks of treatment. Values are mean (mg/dl) ± SD from pooled plasma of 6 rats in each group, N-C, normal control; IIH-C, Inflammation induced hyperlipidemic rats; IIH-LT fed 1 mg Lovastatin/rat/day for 4 weeks. Significantly different from N-C at *p<0.001 and †p<0.05. Significantly different from IIH-C at ‡p<0.001.

Table 2. Impacts of Lovastatin on the ratio of LDL-C/HDL-C, HDL-C/TC, in inflammation induced hyperlipidemic rats after 4 weeks of treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>IIH-C</th>
<th>IIH-LT</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-C/HDL-C</td>
<td>2.87±0.003*</td>
<td>7.13±0.012*</td>
<td>2.20±0.005</td>
</tr>
<tr>
<td></td>
<td>(148.43 %)*</td>
<td>(-69.14 %)*</td>
<td></td>
</tr>
<tr>
<td>HDL-C/TC</td>
<td>0.228±0.023*</td>
<td>0.105±0.002*</td>
<td>0.269±0.016*</td>
</tr>
<tr>
<td></td>
<td>(+53.39 %)*</td>
<td>(-156.19 %)*</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean (mg/dl) ± SD from pooled plasma of 6 rats in each group, N-C, normal control; IIH-C, Inflammation induced hyperlipidemic rats; IIH-LT fed 1 mg Lovastatin/rat/day for 4 weeks. Significantly different from N-C at *p<0.001. Significantly different from IIH-C at ‡p<0.001.

Impacts of Lovastatin on plasma total antioxidants and lipid peroxidation products: Fig 3 depicts the antioxidant impact of Lovastatin on plasma concentrations of total antioxidants, conjugated diene, lipid hydroperoxide, and MDA in inflammation induced hyperlipidemic rats. In IIH-C rats, plasma total antioxidants level was reduced from a control value of 50 to 36 (27%) µmole/dl. Treatment of IIH-LT rats with Lovastatin for 4 weeks resulted in a significant increase of total antioxidants levels by 15 % when compared to IIH-C value. The oxidative stress induced in IIH-C rats significantly enhanced plasma lipid peroxidation products, such as conjugated diene, lipid hydroperoxide and MDA. Formation of conjugated diene, lipid hydroperoxide and MDA in plasma was increased from 8.48, 1.12 and 1.18 in N-C to 12.35 (45 %), 1.89 (68 %) and 2.98 (152 %) µmole/dl, respectively, in IIH-C. After Lovastatin treatment, in IIH-LT, a significant decrease of 15 %, 33 % and 33 % was seen in the formation of conjugated diene, lipid hydroperoxide and MDA, respectively, when compared to corresponding values in IIH-C rats. These results demonstrate that in IIH-C rats, due to increase in oxidative stress, total antioxidants level was decreased, whereas, concentration of plasma conjugated diene, lipid hydroperoxide and MDA were significantly increased. Lovastatin treatment significantly restored the total antioxidants level and blocked the increase in plasma conjugated diene, lipid hydroperoxide and MDA to a level close to corresponding normal values.

Impacts of Lovastatin on the ex vivo and in vitro Cu²⁺ mediated LDL Oxidation, conjugated diene formation, lag phase and total MDA release: Table 3 depicts the ex vivo base line diene conjugation (BDC) levels of LDL in inflammation induced hyperlipidemic rats (IIH-C) was increased by 54 % respectively, in comparison to the corresponding N-C values. Feeding of Lovastatin to inflammation induced hyperlipidemic rats partially blocked the in vivo oxidation of LDL and reduced their BDC levels by 20% respectively in comparison to the corresponding IIH-C values. As expected, the lag phase time of LDL oxidation was reduced from 90 min in N-C to 58 min in IIH-C. Treatment of IIH-LT rats with Lovastatin restored the lag phase time of LDL oxidation to 68 min. On the other hand the ex vivo base line levels of MDA in LDL was significantly increased by 55 % in (IIH-C) rats, when compared to corresponding values in normal control(N-C) rats. Lovastatin treatment significantly blocked the in vivo increase in the formation of MDA of LDL in inflammation induced
CD and MDA formation were significantly increased when compared to NC values, after 4 weeks of Lovastatin treatment, both values are significantly blocked.

### DISCUSSION

The present study demonstrates the extensive proatherogenic changes, that occurred as a part of the host response to turpentine (acute localized sterile inflammation) administration, on a variety of parameters, like, plasma and lipoprotein lipids in plasma, liver lipid peroxidation products, \textit{ex vivo} and \textit{in vitro} oxidizability of LDL, erythrocytes MDA release; erythrocytes, liver and plasma total antioxidant. Pretreatment of stressed rats with Lovastatin significantly reduced the overall oxidative burden and effectively ameliorated the above altered parameters, thus, indicating a potent atheroprotective effect of Lovastatin. Our results demonstrate a significant increase in plasma total lipids and TG in turpentine (IIH-C) stressed rats. A similar increase in serum TG in LPS treated hamsters or rats were previously reported\(^{22}\). In another report an increase in plasma TG level was seen during inflammation, induced by turpentine oil in pigs\(^{23}\). The increase in plasma TG levels is apparently due to an increase in VLDL which can be the result of either an increase in serum TC level in LPS treated hamsters or rats were previously reported\(^{22}\). The lag phase defined as the interval between the intercept of the tangent of the slope of the curve with the time expressed in minutes. Maximal in vitro oxidation of LDL was achieved after 12h of incubation with CuSO\(_4\) in each group.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Conjugate Dine Formation(^{a})</th>
<th>MDA Content(^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Maximal^2</td>
</tr>
<tr>
<td>N-C</td>
<td>174.51</td>
<td>1038.32</td>
</tr>
<tr>
<td>IIH-C</td>
<td>270.31(^{+54.89%})</td>
<td>1425.47(^{+57.29%})</td>
</tr>
<tr>
<td>IIH-LT</td>
<td>215.43(^{-20.30%})</td>
<td>1002.24(^{-29.70%})</td>
</tr>
</tbody>
</table>

\(^{a}\)Values are mean ±SD from pooled plasma of 6 rats in each group. conjugated diene values are expressed as nmole malondialdehyde equivalents/mg protein. Basal conjugated diene values represent the status of oxidized LDL \textit{in-vivo}. \(^{b}\)The lag phase defined as the interval between the intercept of the tangent of the slope of the curve with the time expressed in minutes. Maximal in vitro oxidation of LDL was achieved after 12h of incubation with CuSO\(_4\) in each group. \(^{c}\)Percent increase with respect to basal value in N-C, \(^{d}\)Percent decrease with respect to basal value in IIH-C. \(^{e}\)Percent decrease with respect to lag phase value in N-C, \(^{f}\)Percent increase with respect to maximal value in N-C. \(^{g}\)Percent decrease with respect to maximal value in IIH-C. Significantly different from N-C at \(^p<0.001\).

Mechanism wise, as previously shown in HepG2 cells, as well as in normolipidemic and hyperlipidemic rats, tocotrienols reduce cholesterol synthesis by suppressing HMG-CoA reductase activity, which in turn is reduced by a decline in its protein mass\(^{29}\). The decline in protein mass may be achieved by inhibition of HMG-CoA reductase synthesis and/or enhanced degradation. Consistent with \textit{in vivo} results in rats\(^{25}\), \(\gamma\)-tocotrienol has been shown to mediate the suppression of enzymatic activity and protein mass of HMG-CoA reductase in HepG2 cells through decreased synthesis (57 % of control) and enhanced degradation (2.4-fold versus control) of the enzyme. In addition, \(\gamma\)-tocotrienol was shown to up regulate LDL receptor in mammalian cells and may be implicated in part for the reduction of apoB-100 protein in \textit{in vivo} \(^{26}\). Thus, tocotrienols reduce cholesterol formation in mammalian cells by suppressing HMG-CoA reductase activity through two actions: decreasing the efficiency of translation of HMG-CoA reductase mRNA and increasing the controlled degradation of HMG-CoA reductase protein, post-transcriptionally. In addition, another report indicates that \(\gamma\)-tocotrienol influences apoB secretion by both cotranslational and posttranslational processes involving a decreased rate of apoB translocation and accelerated degradation of apoB in HepG2 cells. This activity correlated with a decrease in free and esterified cholesterol\(^{30}\). Taken together, the information indicates an association between the suppression of hepatic cholesterol synthesis and apoB secretion, and the observed lowering of apoB and LDL-C levels in animal and human models\(^{3}\). In our result, show that the \textit{ex vivo} base line diene conjugation (BDC) levels of LDL in Inflammation induced hyperlipidemic (IIH-C) rats was increased by 54 % respectively, in comparison to the corresponding N-C values. Feeding of Lovastatin to Inflammation induced hyperlipidemic rats partially blocked the \textit{in vivo} oxidation of LDL and reduced their BDC levels by 20 % respectively in comparison to the corresponding IIHC values. As expected, the lag phase time of LDL oxidation was reduced from 90 min in N-C to 58 min in IIHC. Treatment of IIH-LT rats with Lovastatin restored the lag phase time of LDL oxidation to 68 min. It has been established that LDL-C/HDL-C and HDL-
C/T ratios are good predictors for the presence and severity of CAD\textsuperscript{2,24}. LDL-C/HDL-C and HDL-C/T ratios were calculated from the data presented in Table 2 and 3. LDL-C/HDL-C ratio was significantly increased from 2.87 in N-C to 7.13 (148 %) in IIH-C group, when compared to ratio in N-C. After 4 weeks of treatment, the increase in LDL-C/HDL-C ratio was significantly prevented and decreased to 2.20 in IIH-LT, which is close to normal control value. On the other hand, HDL-C/T ratio was significantly decreased from 0.228 in N-C to 0.105 (53 %) in IIH-C group. Lovastatin treatment to these rats significantly prevented the increase in HDL-C/T ratios and fully restored them to a ratio value similar to normal control. In addition, the ratios related to HDL-C in Lovastatin treated rats were positively modulated and restored similar to normal control value, indicating normalization of cholesterol levels associated with the above lipoproteins Oral pretreatment of rats with Lovastatin for 28 days significantly prevented the turpentine induced adverse effects and ameliorated the levels of all the evaluated parameters. Our results strongly suggest that the alleviation of inflammatory conditions is due to potent lipid lowering and free radical scavenging properties of Lovastatin and, thus, can be useful in the therapy of systemic inflammatory process which might induce atherosclerosis. Based on these findings, the anti-inflammatory potential of Lovastatin looks promising and more comprehensive studies should be undertaken to determine their actual mode of action. In conclusion, considering the strong hyperlipidemic/athero-protective and antioxidant, and possibly anti-inflammatory actions of Lovastatin, intake of Lovastatin may be useful in the prevention and treatment of infection/inflammation induced hyperlipidemia and atherosclerosis.

ACKNOWLEDGMENT

The authors like to acknowledge University Grant Commission (UGC), New Delhi (India), for financial support. This study was carried out at the Department of Biochemistry, J N Medical College, Aligarh Muslim University, Aligarh, India. The authors like to thank Dr. Z. H. Beg, Dr. Shakir Khan, Nasir Khan for giving guidance from time to time and Dr. Asif Ali, Chairman, for providing facilities to carry out research work.

REFERENCES

atherogeni

Source of support: Nil, Conflict of interest: None Declared