Ameliorative role of atorvastatin on methionine-induced hyperhomocysteinemia and hematological changes in albino rats

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Methionine (1g/kg, po) administration to pathogenic control rats for 30 days significantly increased the levels of homocysteine, total cholesterol (TC), low density lipoprotein (LDL-C), very low density lipoprotein (VLDL-C) and triglycerides (TGs) and decreased the levels of high density lipoprotein (HDL-C) in serum. Hematological observations of the peripheral blood smears of pathogenic rats fed with methionine also showed crenation of RBCs cell membrane and significant increase in total leukocyte count, differential leukocyte count and platelet counts with significant decrease in the mean hemoglobin levels as compared to vehicle control rats. Administration of atorvastatin (0.2 mg/kg/po) to hyperhomocysteinemic rats significantly decreased the levels of homocysteine, TC, TGs, LDL-C and VLDL-C and increased the levels of HDL-C in serum. The present results provide clear evidence that oral treatment with atorvastatin exhibit homocysteine and lipid lowering activity and also reversal of hematological changes induced by methionine in albino rats.

Keywords: Atorvastatin, Lipid profile, Methionine, Hyperhomocysteinemia

A moderate increase in plasma homocysteine (Hcy, both bound and free) is considered an independent risk factor for premature cardiovascular disease1. High plasma concentration of Hcy is also considered to be an indicator of cardiovascular morbidity and mortality2. Evidence now indicates that hyperhomocysteinemia, which occurs in approximately 5-7% of the general population, is an important, independent risk factor for atherosclerosis and thrombotic disease3. Further, up to 40% of patients diagnosed with premature coronary artery disease, peripheral vascular disease or recurrent venous thrombosis have hyperhomocysteinemia4. The mechanisms in homocysteine (Hcy)-induced vascular disease have been actively investigated using different experimental models and have provided important insights into the understanding of the role of Hcy in cardiovascular diseases. Homocysteine is a sulfur containing amino acid which is formed during the metabolism of amino acid methionine5. Epidemiological evidence has demonstrated that hyperhomocysteinemia is an independent risk factor for development of coronary, cerebrovascular, peripheral arterial occlusive disease and induces hepatic cholesterol biosynthesis and lipid accumulation via activation of transcription factors6. Hyperhomocysteinemia can be induced experimentally by dietary manipulation with methionine7,8. Methionine is the only dietary source of homocysteine, which disrupts and interfere endothelial integrity9. Excessive intake of methionine causes typical hematological changes; excess promotes methemoglobin accumulation and Heinz-body formation in erythrocytes and causes morphological changes in erythrocystic membrane which leads to hemolytic anemia and to morphological changes10,11. In the present study, methionine supplementation is taken as an experimental model to induce hyperhomocysteinemia in rats.

Bath et al.12 reported increased involvement by monocytes in hypercholesterolemia-induced atherogenesis. Rasouli et al.13 found that the total leukocyte count and its subgroups are associated with the presence and severity of coronary artery disease, but the associations were not independent.
Furthermore, Durand et al.\textsuperscript{14} reported that acute methionine load-induced hyperhomocysteinemia enhances thromboxane biosynthesis, and macrophage-derived tissue factor activity in Wistar albino rats. A moderate elevation of plasma homocysteine is a risk factor for atherosclerosis and arterial and venous thrombosis responsible for coronary heart disease and ischemic stroke incidence and cardiovascular disease mortality.

Arteriosclerosis and other lipid disorders induced-hematological changes are evidently shown in clinical studies. Statin administration has been shown to modulate gene expression in peripheral blood mononuclear cells \textit{in vitro}\textsuperscript{15}. Stalker et al.\textsuperscript{16} showed that intraperitoneal administration of 0.5 and 1.25 mg/kg rosvastatin, 18 h prior to the study, dose dependently and significantly attenuated leukocyte rolling and adherence in the rat mesenteric microvasculature superfused with 0.5µ/ml thrombin\textsuperscript{16}. Also, Ikeda et al.\textsuperscript{17} found that rosuvastatin significantly attenuates polymorphonuclear cells-induced cardiac contractile dysfunctions in the isolated perfused rat heart.

Statins lower cholesterol levels in people at risk of cardiovascular disease because of hypercholesterolemia. As atherosclerosis is considered to be an inflammatory disease Bickel et al.\textsuperscript{18} showed a significant relation of markers of inflammation (C-reactive proteins, fibrinogen, Von Willebrand factor, leukocyte count) and HMG-CoA-reductase inhibitors (statins) therapy in the primary and secondary prevention of coronary artery disease and atherosclerosis. However, less is known about the effect of statin therapy on homocysteine. Luftjohann et al.\textsuperscript{19} reported a significant decrease in Hcy plasma levels after high doses of simvastatin (80 mg daily) in patients with hypercholesterolemia, for 24 weeks, suggesting a possible contribution to the reduction in cardiovascular events seen with high doses of simvastatin. Millionis et al.\textsuperscript{20} performed trials and compared the effects of atorvastatin (40 mg/ day), simvastatin (40 mg/ day) and fenofibrates on serum homocysteine levels in patients with hyperlipidemia. However, their result showed a little change in serum homocysteine levels in statin group. The down-regulating effect of atorvastatin on homocysteine formation \textit{in vitro} indicates that statins may prevent homocysteine accumulation in the blood via immunosuppression\textsuperscript{21}. On the contrary, the other clinical studies failed to detect any changes in Hcy after statin therapy\textsuperscript{22,23}. There are no experimental studies reported so far, investigating the effect of atorvastatin therapy on homocysteine.

Therefore, the present experimental study has been undertaken to investigate the hypothesis that oral administration of atorvastatin for 30 days would alleviate the circulating levels of homocysteine and attenuate the augmented hematological changes in a model of methionine-induced hyperhomocysteinemia in albino rats.

### Materials and Methods

**Animals**—The study was approved by Institutional Animal Ethics Committee (IAEC) of Hamdard University, New Delhi, which is registered with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, (Registration no. and date of Registration: 173/CPCSEA, dated 28th January, 2000). Wistar rats of either sex (150 - 200 g) procured from the Central Animal House Facility, Jamia Hamdard, New Delhi and acclimatized under standard laboratory conditions at 25° ± 2°C, 50 ± 15% RH and normal photoperiod (12 : 12 light dark cycle) for 7 days, were used for the experiment. Commercial rat pellet diet (manufactured by Nav Maharastra Chakan Oil Mills Ltd, Delhi, India) and water \textit{ad libitum} were given to animals. Adequate measures were taken to minimize pain or discomfort, and the experiments were conducted in accordance with international standards on animal welfare as well as being compliant with local and national regulations.

**Drugs and chemicals**—Atorvastatin was a gift sample from Ranbaxy Labs Pvt. Ltd., Gurgaon, Haryana, India. L-Methionine was purchased from SRL, Mumbai, India. All other chemicals used were of analytical grade. Double distilled water was used for all biochemical assays.

**Experimental design**—After acclimatization, all the animals were randomly divided into 4 groups of 8 animals each and treated as follows: Group I (vehicle control): rats received only 1% Tween-80 in normal saline (2 ml/kg body weight; po) for 30 days; Group II (pathogenic control): rats treated with methionine (1 g/kg body weight; po) for 30 days; Group III (Atorvastatin treated): rats co-treated with atorvastatin (0.2 mg/kg body weight; po) and methionine (1 g/kg body weight; po) for 30 days; Group IV (Atorvastatin per se): rats received only atorvastatin (0.2 mg/kg body weight; po) for 30 days. Co-treatment with drug in experimental animals is
taken as a therapeutic measure to prevent the occurrence and progression of disease complications. Hence, in the present study, co-treatment with the test drug was planned, along with methionine (1 g/kg body weight; p.o.) for 30 days, which is clinically significant as co-treatment with the drug under investigation, if found beneficial will prevent the development and progression of vascular diseases associated with elevated homocysteine concentrations. This study might suggest that statins may be therapeutically useful for the treatment of coronary heart diseases. Similar treatment strategies were followed in other research studies\textsuperscript{24,25}.

The dose of atorvastatin (0.2 mg/kg body weight; p.o.) was selected based on earlier studies in which atorvastatin has shown protection against the lipemic-oxidative disorder and acting as hypcholesterolemic, hepatoprotective agent and improve cardiovascular function through modulation of oxidative stress, NO and Hcy in rats\textsuperscript{26,27}.

After completion of all treatments, animals were evaluated for various biochemical studies in serum and blood.

Biochemical analysis—The blood was collected from the retro-orbital plexus of all the groups of overnight fasted rats using microcapillary tube on 31\textsuperscript{st} day. Serum was separated for biochemical estimations of homocysteine\textsuperscript{28}, HDL-C\textsuperscript{29}, total cholesterol\textsuperscript{30}, and triglyceride levels\textsuperscript{31}.

Hematological studies—The whole blood was used for estimation of hemoglobin levels and for the mounting of blood smears slides to investigate the hematological changes in rats viz, platelet count, total leucocyte count and lymphocyte count by using standard techniques\textsuperscript{32}. The stained slides were studied under the low and high power objectives of the microscope for the study of morphology of white blood cells and differential leucocyte count (DLC). The slides were placed on a fixed stage and two drops of cedar wood oil were placed on the stained smear at a point about 2 cm from the start of the film.

Statistical analysis—Statistical analysis was carried out using Graphpad Prism 3.0 (Graphpad software; San Diego, CA). All data were expressed as mean ± S.E. Groups of data were compared with an analysis of variance followed by Dunnett t-test. Values were considered statistically significant when \(P<0.05\).

Results and Discussion

Elevated homocysteine level is considered as a rather common, independent, and possibly causal risk factor for cardiovascular diseases\textsuperscript{33}. It is estimated that 5-7\% of the general population has mild to moderate hyperhomocysteinemia\textsuperscript{34} and are at an increased risk of developing coronary atherosclerosis and vascular thrombosis in later life. Individuals with mild hyperhomocysteinemia also have a increased prevalence of carotid artery stenosis, premature peripheral and cerebral vascular atherosclerosis\textsuperscript{35}. Epidemiological data have generally showed a strong association between plasma homocysteine and the premature development of atherosclerotic cardiovascular disease\textsuperscript{36}.

Multiple interventional trials demonstrated that HMG-CoA reductase inhibitors (statins) effectively reduce serum cholesterol levels and cardiovascular events (morbidity and mortality\textsuperscript{37}). Nevertheless, many of the pleitropic effects of statins in preventing recurrent coronary heart disease events have been debated\textsuperscript{38}, but it is specifically these effects that may offer the greatest potential benefit to patients with chronic heart failure. Some of the pleitropic effects of statin that may benefit patients with chronic heart failure are decrease LV mass, decrease LV fibrosis, decrease inflammation, decrease immune activation, alter metalloproteinase activity, decrease oxidative stress, increase arterial compliance, decrease thrombosis etc\textsuperscript{39}. The present study was designed to investigate the effect of atorvastatin treatment in the methionine-induced hyperhomocysteinemia and associated hematological changes in albino rats.

L-methionine administration (1 g/kg body weight orally for 30 days) along with normal diet resulted in a 2.5-folds-increase in serum Hcy levels (Table 1), suggesting a detrimental effect of excess dietary methionine which may have practical significance in humans consuming large amounts of foods high in methionine (such as animal proteins). Methionine-rich meals have been reported to cause slight increase in plasma Hcy\textsuperscript{40}. Further, L-methionine administration induced a significant (\(P < 0.01\)) increase in TC, TGs, LDL-C and VLDL-C levels and decrease in HDL-C levels in serum in pathogenic control group rats as compared to vehicle control group (Table 1). Also, a significant correlation was found between the biochemical parameters and the duration of methionine-induced pathological conditions. This increase in the homocysteine levels with methionine administration corroborated the findings of Kapoor et al\textsuperscript{41} and Zulli et al\textsuperscript{41}. These changes may be attributed to the better cholesterol absorption favored by methionine supplementation. LDL-C elevation in
hyperhomocysteinemic rats may be attributed to the reduction in the number of LDL receptor or reduced LDL binding to its receptor in rats. Changes in LDL-receptor contribute to the elevation in serum cholesterol levels induced by methionine. Another risk factor for developing atherosclerosis is the reduction in HDL-C level which attributed to its central function in the reverse of cholesterol transport, a process whereby excess cell cholesterol is taken up and processed by HDL particles for further delivery to the liver for metabolism. Moreover, increased TG and decreased HDL-C levels in hyperhomocysteinemic rats may be attributed to decreased activity of lipoprotein lipase.

Atherogenic index indicates the deposition of foam cells or plaque or fatty infiltration or lipids in heart, coronaries, aorta, liver and kidneys. The higher the atherogenic index, the higher is the risk of the above organs for damage. Therefore in the present study, an increase in atherogenic index was found indicating atherosclerotic vascular damage.

Methionine supplementation produced significant alteration in hematological parameters. An increase of 1.3-folds in mean platelets counts and total leukocyte counts (TLC) was observed. A significant increase in polymorphonuclear cells (PMN), lymphocyte counts and monocyte counts (P<0.01) as well as a significant (P<0.01) decrease in blood hemoglobin levels was observed in methionine-treated group as compared to the vehicle control rats (Table 2). All these alterations in blood cell counts especially increased platelet count may predispose atherosclerotic complications and further leave heart vulnerable to vascular damage. An elevated total white blood cell (WBC) count is a risk factor for atherosclerotic vascular disease. WBC-derived macrophages and other phagocytes are believed to contribute to vascular injury and atherosclerotic progression. Previous in vivo and in vitro studies suggest that homocysteine changes the endothelium from a nonthrombogenic to a thrombogenic phenotype. Homocysteine, thus, has adverse effects on endothelial function, stimulates smooth muscle proliferation and is a procoagulant, thereby increasing risks of major vascular events.

Oral treatment with atorvastatin (0.2 mg/kg body weight) for 30 days significantly (P<0.01) reduced the increased levels of serum homocysteine, TC, TGs, LDL-C and VLDL-C and increased the HDL-C levels as compared to pathogenic control group. Further, the mean platelet counts, total leukocyte count (TLC), polymorphonuclear cells (PMN i.e. neutrophils, eosinophils and basophils) and lymphocyte count were significantly (P<0.01) increased in methionine-treated group as compared to the vehicle control rats (Table 1).

### Table 1—Effect of atorvastatin on serum homocysteine (µ mol/L), TC (mg/dl), HDL-C (mg/dl), TGs (mg/dl), LDL-C (mg/dl), VLDL-C (mg/dl) levels and atherogenic index of albino rats

[Values are mean ± SE from 8 animals in each group]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Homocysteine</th>
<th>TC</th>
<th>HDL-C</th>
<th>TGs</th>
<th>LDL-C</th>
<th>VLDL-C</th>
<th>Atherogenic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Vehicle control</td>
<td>8.75±0.250</td>
<td>113.77±3.647</td>
<td>43.15±2.732</td>
<td>71.42±3.210</td>
<td>59.94±1.00</td>
<td>14.28±0.641</td>
<td>2.785±0.16</td>
</tr>
<tr>
<td>II Methionine treated</td>
<td>22.55±1.852</td>
<td>189.87±4.864</td>
<td>17.77±1.539</td>
<td>197.70±5.932</td>
<td>132.55±5.712</td>
<td>39.53±1.185</td>
<td>10.660±0.77</td>
</tr>
<tr>
<td>III Atorvastatin treated</td>
<td>16.86±0.726</td>
<td>135.19±2.876</td>
<td>32.99±1.654</td>
<td>115.65±4.375</td>
<td>79.04±3.466</td>
<td>23.12±0.874</td>
<td>4.172±0.23</td>
</tr>
<tr>
<td>IV Atorvastatin per se</td>
<td>8.75±0.366</td>
<td>112.83±2.506</td>
<td>44.63±1.993</td>
<td>54.17±3.147</td>
<td>57.37±3.043</td>
<td>10.82±0.628</td>
<td>2.568±0.14</td>
</tr>
</tbody>
</table>

P values: <0.01 when compared with * vehicle control, 1 methionine treated group; m > 0.05

### Table 2—Effect of atorvastatin on hemoglobin (g/dl) levels, platelets (Lacs/cu mm), TLC (thousands/cu mm), PMNs cells(%), lymphocytes (%) and monocytes (%) of albino rats

[Values are mean ± SE from 8 animals in each group]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hb</th>
<th>Platelets</th>
<th>TLC</th>
<th>PMNs Cells</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Vehicle control</td>
<td>14.35±0.445</td>
<td>0.85±0.025</td>
<td>6800±0.87</td>
<td>09±1.000</td>
<td>82±0.500</td>
<td>05±0.100</td>
</tr>
<tr>
<td>II Methionine treated</td>
<td>12.37±0.252</td>
<td>1.10±0.021</td>
<td>8900±0.77</td>
<td>13±1.3148</td>
<td>95±1.250</td>
<td>06±1.308</td>
</tr>
<tr>
<td>III Atorvastatin treated</td>
<td>14.77±0.332</td>
<td>0.88±0.012</td>
<td>7675±0.21</td>
<td>09±0.478</td>
<td>79±0.843</td>
<td>04±0.8894</td>
</tr>
<tr>
<td>IV Atorvastatin per se</td>
<td>13.96±0.240</td>
<td>0.76±0.029</td>
<td>5366±0.26</td>
<td>08±0.33</td>
<td>79±0.881</td>
<td>05±0.9911</td>
</tr>
</tbody>
</table>

P values: <0.01 when compared with * vehicle control, 1 methionine treated group; m > 0.05
When methionine-induced hyperhomocysteinemic rats were treated with atorvastatin (0.2 mg/kg body weight, po), there was a significant ($P<0.01$) decrease in blood platelet counts, TLC and PMN cells and lymphocytic count and a significant increase ($P<0.01$) in blood hemoglobin (Hb) levels as compared to pathogenic control group, which is a significant finding of the study, not reported earlier (Table 2).

Fig. 1— (A & B) Photomicrographs of vehicle control rat showing lymphocyte with uniformly stained oval nucleus and a thin surrounding rim of cytoplasm and a small group of blood platelets is seen in the lower portion of image. Normal polymorphonuclear cells (PMNs) and erythrocytes with clearly brought out normal cell membrane and cytoplasm; (C & D) Photomicrographs of pathogenic control rat treated with methionine showing red blood cells crenation and other morphological changes in erythrocytic membrane. Lymphocyte with uniformly stained nucleus showing marked inflammatory infiltrate with oedema. A small group of aggregated platelets is seen in the lower portion of the image. Polymorphonuclear leukocyte with multi-lobed nucleus; (E & F) Photomicrographs of atorvastatin treated rat showing decrease in crenation of erythrocytic membrane and lymphocyte with uniformly stained oval. Polymorphonuclear leukocyte with bi-lobed nucleus; (G & H) Photomicrographs of atorvastatin per se rat showing lymphocyte with uniformly stained oval nucleus and a thin surrounding rim of cytoplasm and erythrocytes with clearly brought out normal cell membrane and cytoplasm. Polymorphonuclear leukocyte with multi-lobed nucleus (x 400).
Further, there were no significant changes in the monocytes count in methionine treated as well as atorvastatin treated rats. However, atorvastatin per se treatment in rats produced no significant changes in levels of homocysteine and lipids, as well as hematological parameters. Dudman et al. observed that leucocyte-mediated changes in endothelial integrity and function may lead to the vascular disease seen in individuals with elevated plasma homocysteine.

The results of biochemical observations were supplemented by hematological examination of rat’s blood smears of all the groups. Multiple peripheral smears stained with Leishman’s stain were examined with particular attention to morphological alterations in the two predominant leukocytic groups, i.e. lymphocytes and polymorphonuclear leucocytes (PMN). No alterations of morphological appearance of either type of leucocytes could be observed in any of the groups examined. The distribution patterns of platelets in all the slides were examined and were found to be normal.

Photomicrograph of vehicle control group revealed a normal architecture with regular morphology of various blood cells viz. erythrocytes, lymphocytes, polymorphonuclear cells and platelets distributed and stained uniformly (Fig. 1A and 1B). Further, there was crenation of RBCs (shrinkage of red blood cells giving notched appearance to the cell profile) cell membrane in methionine-treated rats indicative of hemolytic anemia (Fig. 1C and 1D). It has been reported by Klavins and Kinney and Benevenga and Steele reported that excessive intake of methionine causes typical hematological changes; excess promotes Heinz-body formation in erythrocytes and causes morphological changes in erythrocytic membrane which leads to hemolytic anemia and morphological changes. However, atorvastatin treatment along with methionine could not fully protect the RBCs membrane and crenation of few RBC (Fig. 1E and 1F). Similarly, there were no changes in RBCs membrane in atorvastatin per se (Fig. 1G and 1H).

Conclusions

The present study is first to report an effect of atorvastatin on hyperhomocysteinemia associated hematological changes induced by methionine as observed by reduction in raised homocysteine levels, restoration of altered lipid profile and hematological blood counts as well as damaged morphological hematological perturbations. However, further studies like peripheral leukocytic transmigration, platelet aggregation will be needed to warrant the potential of atorvastatin supplementation as a novel and safe tool to reduce cardiovascular risk and hematological changes produced with methionine-induced hyperhomocysteinemia. Atorvastatin therapy may be used to reduce homocysteine concentration in patients with coronary artery disease. The possible beneficial effect of such treatment on coronary risk, including effects on cardiac morbidity and mortality, will require a long-term, prospective randomized placebo controlled trial. The appropriate use of a statin therapy with maximum improvements across the lipid profile will provide effective treatment of dyslipidemia and may increase benefits for CVD reduction.

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