Anti-atherosclerotic effect of atorvastatin and clopidogrel alone and in combination in rats

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Atherosclerosis is a disease affecting arterial blood vessels due to the accumulation of macrophage white blood cells and low density lipoproteins. Effects of atorvastatin, a recently introduced lipid lowering statin was studied alone and in combination with clopidogrel in high fat diet fed atherosclerotic rats orally. Results showed significant reduction in total serum cholesterol and malondialdehyde levels and significant improvement in urine creatinine levels. Aortic cross sections of rats treated with clopidogrel alone showed reversal of atherosclerotic calcification. The same effect was observed with the combined treatment of clopidogrel and atorvastatin. Only atorvastatin treatment did not show any histological atheroprotective effect. Atorvastatin and clopidogrel alone and in combination have offered significant atheroprotective effect. No specific advantage was seen with combined treatment of atorvastatin and clopidogrel, moreover the advantages seen with independent drug administration also reduced with combined treatment.

Keywords: Atherosclerosis, Atorvastatin, Clopidogrel, LDL

Myocardial and cerebral infarctions are the main clinical syndromes resulting from atherosclerosis and are the leading causes of death all over the world¹. Atherosclerosis refers to fatty deposits formed on the inner lining of the blood vessels. It is a multifactorial disease induced by the effects of various risk factors on appropriate genetic backgrounds. It is characterized by vascular areas containing mononuclear and proliferation of smooth muscle cells resulting in hardening and thickening of the arterial walls². The high concentration of cholesterol, particularly LDL-cholesterol is one of the principal risk factors. The atherosclerotic lesions contain large number of immune cells and T-cells. Further, the disease is associated with systemic immune responses and signs of inflammation³. During recent years, experiments in gene-targeted mice have provided mechanistic evidence in support of the hypothesis that immune mechanisms are involved in the atherosclerosis. The HMG-CoA reductase inhibitors constitute the most powerful class of lipid lowering drugs widely used in medical practice. During the past several years, additional actions of statins in addition to cholesterol lowering effect have been identified which includes anti-inflammatory and immunomodulatory properties and plaque stabilization in atherosclerosis⁴. Clopidogrel, a new thienopyridine derivative similar to ticlopidine, is an inhibitor of platelet aggregation induced by adenosine diphosphate. Since hyperlipidemia, inflammation and immune modulations play a key role in the development of atherosclerosis and lipid lowering by statins and anti-aggregation drug therapy have become the basis of any anti-atherosclerotic prophylaxis either as primary or secondary prophylaxis⁵, the present study has been carried out to evaluate the anti-atherosclerotic effects of atorvastatin and clopidogrel alone and in combination in rats.

Materials and Methods

Animals—Albino Wistar rats (25), 6-8 weeks old of either sex supplied by Chakraborty Enterprises, Kolkata, weighing 170±30 g, were maintained under controlled environment (25±2°C) and provided standard animal food pellet (Ratan Brothers laboratory animal feed, India) and water ad libitum. Animal experiments were conducted strictly according to INSA-Ethical guidelines for use of

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animals in scientific research and animals were sanctioned and issued after the approval of the Animal Experimentation Ethics Committee of the Institute.

**Materials**—Standard commercial pellet diet (Ratan Brothers, Hyderabad), cholesterol extra pure (Merck and BDH products), calcium (Shecal 250 mg), vitamin D$_3$ (Arachitol – 3L IU) and saturated fatty oil (Vanaspathy) were used.

**Chemicals and drug administration**—Atorvastatin (HMG-CoA reductase inhibitor) a gift sample from Sun Pharmaceutical Industries Limited, Mumbai, was suspended in 1% gum acacia solution and administered (10 mg/kg) daily orally for 6 weeks. Clopidogrel (ADP-receptor antagonist) was a gift sample from Cipla Pharmaceutical Industries Limited, Mumbai, and suspended in 1% gum acacia solution and administered (50 mg/kg) daily orally for 6 weeks.

**Induction of atherosclerosis**—Although the cause and pathogenesis of atherosclerosis still remains largely unresolved, it is generally agreed that correlation exists between high blood cholesterol and cardiovascular diseases$^6$. Antischkow for the first time succeeded in inducing atherosclerosis in rabbits by feeding cholesterol containing diet$^7$. Development of atherosclerosis in rabbits usually takes at least 60 days of feeding atherogenic diet$^8$. Rat is said to be resistant to such dietary manipulations for the development of atherosclerosis, but with supplementation of very high doses of vitamin-D$_3$ along with atherogenic diet, success has been achieved in developing atherosclerosis in rats in a short period of 5 days$^9$. The atherogenic diet (AD) consisting of 2 g of cholesterol and 8 g of saturated fat and 100 mg calcium were added to 90 g of powdered standard commercial pellet diet and thoroughly mixed. The rats were fed with high fat diet along with weekly challenge of oral vitamin-D$_3$ for one month per oral route$^{10}$.

**Experimental protocol**—Except group-I, all the four groups i.e. group-II to group-V were fed with the atherogenic diet along with weekly challenge of vitamin-D$_3$ orally (3, 20,000 IU) in 1.5 ml of olive oil. The group-I rats were fed with the standard commercial pellet diet. After induction of atherosclerosis as confirmed by histological studies, the treatment schedule was started. During the treatment period, rats of all the groups were fed with normal standard pellet diet. Group-II rats served as an atherogenic control. Group-III rats were treated orally with both doses of atorvastatin (10 mg/kg) and clopidogrel (50 mg/kg), group-IV rats were treated orally with atorvastatin (10 mg/kg), and group-V rats were treated orally with clopidogrel (50 mg/kg). The serum total cholesterol, malondialdehyde levels and urine creatinine levels were estimated before and after the treatment, i.e. 45 days. After the 45 days treatment, all the groups of rats were subjected to histological examination.

**Biochemical evaluations**—The serum total-cholesterol levels were estimated by using monozyme cholesterol and HDL-cholesterol kit$^{11,12}$. The levels of total cholesterol in serum are expressed as µg/dL. Serum lipid peroxidation estimation was done by the use of thiobarbituric acid assay method$^{13-15}$. The levels of lipid peroxides in serum are expressed as nmol/mL malondialdehyde. Urine creatinine estimation was carried out using Dr. Reddy’s creatinine kit, which is based on the alkaline picate method$^{16}$ and the levels of creatinine in urine are expressed as mg/dL/24 hr.

**Histological evaluation**—For histological evaluation of the atherosclerosis, rats were anaesthetized with sodium pentobarbital (120 mg/kg/ip). Blood was withdrawn from the heart into a heparinized syringe. Serum total cholesterol and malondialdehyde levels were estimated. Rats were sacrificed by using high doses of sodium pentobarbital and the aortas were fixed in 10% buffered formalin. The aortas were cut transversely at the arch and mid-thoracic level$^{17}$. They were fixed in bouin’s fluid for 24 hr and process for routine histopathological examination by passing through graded alcohols. Sections (6-8 µm thick) were taken with the help of microtome, mounted on glass slides and processed for histological studies with haematoxylin/eosin staining.

**Statistical analysis**—All variables are expressed as mean±SD. Group differences of variables were compared by using one way ANOVA followed by post-hoc (Newman-Keuls Multiple Comparison Test). For all analyses, a $P<0.05$ was considered statistically significant. All analyses were performed by using Graph Pad Prism 4 software.

**Results**

Effects of atorvastatin and clopidogrel on various biochemical parameters were shown in Table 1. Serum total cholesterol levels were found to be significantly increased in rats fed with atherogenic
A significant reduction in serum total cholesterol levels was observed with both the drugs used i.e. atorvastatin (10 mg/kg) and clopidogrel (50 mg/kg) alone (73.15 mg%) and in combination (76.12 mg%) respectively. Both atorvastatin and clopidogrel showed similar percent reduction with respect to serum total cholesterol levels. There was no advantage observed with combined treatment. A significant reduction in lipid peroxidation was observed with both the drugs atorvastatin and clopidogrel alone (0.12 nmol/ml) (0.13 nmol/ml) and in combination (0.15 nmol/ml) respectively. The degree of reduction was more or less same with both the drugs, but with the combined treatment, there was less reduction in the lipid peroxidation than the individual drugs. A significant increase was observed in urinary excretion of creatinine in both experimental groups treated with atorvastatin and clopidogrel alone (0.12 nmol/ml) (+1.13 nmol/ml) and in combination (0.15 nmol/ml) respectively. The degree of percent increase in urinary excretion of creatinine was more with clopidogrel when compared to atorvastatin. No significant increase was observed with combined treatment. Aortic cross sections from rats fed with atherogenic diet exhibited well defined calcification, which is an indication in advanced complicated plaques (Fig. 2). With clopidogrel (50 mg/kg) treatment aortic cross sections were observed normal and the atherosclerosis was reversed (Fig. 5). The same reversal was observed with the combination of atorvastatin and clopidogrel (Fig. 3). But with atorvastatin treatment alone (10 mg/kg) focal areas of foamy cells are seen in sub-intimal region, no reversal of calcification was observed (Fig. 4).

**Discussion**

Atherosclerosis is a slowly progressing, inflammatory, proliferative disease in which various cells such as macrophage, endothelial and smooth muscle cells (SMC) are involved. Serum total cholesterol levels were found to be significantly increased in rats fed with atherogenic diet for 4 weeks. Hypercholesterolemia plays a major role in atherogenesis and it is an important risk factor for atherosclerosis. Patients with hypercholesterolemia are at an increased risk of coronary heart disease. Lowering elevated cholesterol levels has been shown to effectively reduce the risk of coronary events. Lipid lowering reduces disease progression, prevents myocardial infarction and other hard end points and prolongs survival. Elevated cholesterol levels can be controlled through drugs or natural supplements such as fiber, niacin etc. In the present study, with both the drugs used i.e. atorvastatin (10 mg/kg) and clopidogrel (50 mg/kg) alone and in combination, a significant percent reduction in serum total cholesterol levels was observed. Both atorvastatin and

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal Group</th>
<th>Total cholesterol (mg/dL)</th>
<th>Urine creatinine (mg/dL)</th>
<th>Malondialdehyde (n.mol/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After 45 days</td>
<td>Before</td>
</tr>
<tr>
<td>I</td>
<td>Normal Control</td>
<td>55.51 ± 1.78</td>
<td>55.51 ± 1.70</td>
<td>26.65 ± 1.42</td>
</tr>
<tr>
<td>II</td>
<td>Atherogenic Control</td>
<td>175.46 ± 5.48</td>
<td>170.55 ±1.29</td>
<td>14.90 ± 2.01</td>
</tr>
<tr>
<td>III</td>
<td>Atorvastatin (10 mg/kg) + Clopidogrel (50 mg/kg)</td>
<td>161.09 ± 9.43</td>
<td>76.12 ± 2.55 (-52.74)</td>
<td>19.73 ± 1.98</td>
</tr>
<tr>
<td>IV</td>
<td>Atorvastatin (10 mg/kg)</td>
<td>167.55 ±12.72</td>
<td>73.15 ± 3.09 (-56.34)</td>
<td>16.98 ± 2.69</td>
</tr>
<tr>
<td>V</td>
<td>Clopidogrel (50 mg/kg)</td>
<td>179.92 ± 5.58</td>
<td>74.36 ± 3.42 (-58.66)</td>
<td>14.66 ± 2.14</td>
</tr>
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*P < 0.05 statistically significant compared to respective control.
Figs 1–5—Transverse sections of rat aorta. 1: normal control; 2: atherogenic control; 3: treated with atorvastatin (10 mg/kg) and clopidogrel (50 mg/kg); 4: treated with atorvastatin (10 mg/kg); 5: treated with clopidogrel (50 mg/kg)

clopidogrel have more or less same percent reduction with respect to serum total cholesterol levels. There
was no advantage observed with combined treatment. Atorvastatin, a recently introduced statin produces pronounced lipid lowering via HMG-CoA reductase inhibition. Atorvastatin leads to greater decreases in LDL-cholesterol, total cholesterol, apolipoprotein B and triglyceride levels than other statins. Percent reduction of serum total cholesterol observed with atorvastatin in the present study is in agreement with the previous reports. Surprisingly, in the absence of sufficient evidence in the present study, clopidogrel has also shown significant serum total cholesterol reduction. This aspect needs further studies and evaluation.

Oxidative stress may cause vascular damage and dysfunction and initiate a cascade of events that ultimately lead to atherosclerosis. Lipid lowering drugs like atorvastatin and probucol have shown anti-atherogenic action attributed to their anti-oxidant activities also. Hence, in the present study, serum MDA levels were measured in plasma as a parameter for lipid peroxidation before and after the treatment period in various experimental groups. A significant reduction in the lipid peroxidation was observed with both the drugs atorvastatin and clopidogrel. The degree of reduction was more or less same with both the drugs, but with the combined treatment, there was less reduction in the lipid peroxidation.

Elevated serum creatinine levels could be associated with atherosclerotic renovascular disease which correlates with early development of atherosclerosis. With elevated serum creatinine levels, urinary excretion of creatinine is less in atherosclerotic condition. Hence, in the present study, urinary excretion of creatinine was estimated periodically during the treatment period. A significant increase was observed in urinary excretion of creatinine in both experimental groups treated with atorvastatin or clopidogrel. The degree of increase in urinary excretion of creatinine was more with clopidogrel than with atorvastatin. No significant increase was observed with combined treatment.

Hypercholesterolemia has been reported to cause endothelial cell dysfunction, as evidenced by an increase in endothelial cell turnover in cholesterol fed rabbits and swine and increased permeability of the endothelium in cholesterol-fed rabbits. Aortic cross sections from rats fed with atherogenic diet exhibited well defined calcification, which is an indication of advanced complicated plaques. With clopidogrel (50 mg/kg) treatment aortic cross sections were found to be normal and the atherosclerosis was reversed.

**Conclusion**

When compared to atorvastatin, clopidogrel treatment was found to be more atheroprotective. This is supported by the same degree of reduction in serum cholesterol and malondialdehyde levels and more increase in urinary creatinine levels. Histological studies also revealed that atherogenic calcification was reversed. No significant advantage was seen with the combined treatment of atorvastatin and clopidogrel. Moreover the advantage seen with independent drug administration was similar when compared with combined treatment. This needs further investigation to understand the reasons.

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