Atherosclerosis/Arteriosclerosis is a disease of large and medium-sized muscular arteries and is characterized by endothelial dysfunction, vascular inflammation, and the buildup of lipids, cholesterol, calcium and cellular debris within the intima of the vessel wall. This buildup results in plaque formation, vascular remodeling, acute and chronic luminal obstruction, abnormalities of blood flow and diminished oxygen supply to target organs.

In the United States, approximately 1.5 million myocardial infarctions occur annually, and 12.4 million people are affected by coronary heart diseases as a consequence of atherosclerosis. In India, myocardial infarction (MI) is a leading cause of mortality and disability of adults in urban and rural areas, and occurs at a younger age than in western populations. About 30% of all estimated MI mortality occurs at ages 45-59 years, in India versus only 14% in high-income countries. Hence, atherosclerosis is considered as one of the major causes of coronary heart diseases and is a common threat to life, and is usually seen in individuals consuming high cholesterol and saturated fats in their diets. In animal studies it is also shown that raising dietary cholesterol alone can increase atherosclerosis susceptibility. Earlier studies showed that best suited animal model for induction of atherosclerosis were genetically atherogenesis prone strain, Watanabe heritable hyperlipidemic (WHHL) rabbits for screening of drugs against atherosclerosis during drug development. Since WHHL rabbits are difficult to maintain under Indian conditions and are expensive, in the present study New Zealand white rabbits were selected as a suitable, cost effective animal model to develop atherosclerosis by feeding cholesterol. Though diet-induced hypercholesterolemia in New Zealand white rabbits are widely used model system to study atherosclerosis, the present study has been taken up to evaluate atherosclerosis by various biochemical and histopathological parameters using different special staining methods, which can be used as an additional tool for confirming the development of atherosclerosis in New Zealand white rabbits.

Materials and Methods

Induced New Zealand white rabbits (12) of either sex weighing between 1.5-2.0 kg were divided into two groups of 6 each. The animals were maintained on a natural light-dark cycle at 22°C ± 2°C, fed ad libitum with commercial pellet diet (Lipton India Ltd, Mumbai) and had free access to water. All the animals received humane care according to criteria outlined in the “Guide for the care and use of laboratory animals” prepared by the National Academy of Sciences and published by the National Institutes of Health. The local ethics committee approved the protocol.

One group of animals received water (10 ml/kg body weight, po) as vehicle for 4 weeks and served as normal control (NC). The other group served as high

Indian Journal of Experimental Biology
Vol. 44, March 2006, pp. 203-208
cholesterol-fed group animals (HC), and received cholesterol (200 mg/kg, body weight) in coconut oil, po, for 12 weeks. At the end of the study period, blood was collected from the marginal ear vein with or without anticoagulant after overnight fasting. Blood and plasma were subjected for the estimation of clotting time (by routine capillary method) and prothrombin time (PTT) (Brain Thromboplastin Kit, Span Diagnostics Ltd.) respectively. Serum was subjected for the estimation of total cholesterol (TC)\(^8\text{--}^{10}\), triglycerides (TG)\(^1\), high density lipoprotein (HDL) CPT Diagnostics kit\(^12,13\), (Boerhinger Mannheim kits), apolipoproteins A and B (Central Lab, Bangalore, India), and lipid peroxide levels by thiobarbituric acid reaction. Lipid peroxidation was expressed as malondialdehyde (MDA) nano moles/ml of serum\(^14,15\). Serum low density lipoprotein (LDL), very low density lipoprotein (VLDL) were calculated from the lipid profiles.

Liver chemistry—After blood collection the animals were sacrificed under ether anesthesia, and the livers were collected for the estimation of cholesterol and aorta for histopathological evaluation. Liver pieces were homogenized in saline and lipids were extracted by soaking overnight in 3:1 chloroform-methanol solution. The organic layer was separated and dried under nitrogen after which lipids were dissolved in isopropyl alcohol, and subjected for the estimation of cholesterol as mentioned above. Cholesterol content was expressed in mg/g tissue.

Gross pathology—A portion of the descending thoracic aorta was stained with oil-o-red and observed under stereozome microscope.

Histopathology—Small tissue pieces of aortic arch were collected in neutral buffered formalin for routine histoprocessing by paraffin embedding technique\(^16\) and also pieces of thoracic aorta in formal calcium for frozen microtomy\(^17\). Paraffin sections of aorta were stained with Haemotoxylin & Eosin (H&E) and Verhoff’s Vangeison’s to demonstrate the accumulation of foam cells, plaque formation and fibrous tissue proliferation replacing elastic fibers in the intima respectively. Frozen sections were stained by Schultz reaction and oil-o-red to demonstrate cholesterol and lipid droplets respectively in the intima.

Statistical analysis—The biochemical values were expressed as Mean ± SEM and analyzed statistically using unpaired Student’s \(t\) test. The minimal level of significance was fixed at \(P<0.05\). The analysis was performed using Graph Pad Prism software package (Version 4.0).

Results and Discussion

Serum chemistry—High cholesterol fed animals showed significantly elevated levels of serum TC, TG, LDL, VLDL and also an increase in the LDL / HDL ratio. The present findings are in concurrence with several epidemiological studies, which have stated that high cholesterol diet is known to increase serum TC, TG, LDL and VLDL fractions and also increase in the LDL/HDL ratio, which influence the early development of atherosclerosis with an increased risk of cardiovascular disease\(^18,19\).

Apolipoprotein values depicted that there was a decrease in Apo A and an increase in Apo B levels in the HC group compared to NC group. Apo A is primarily found in HDL particles. It serves the function of preventing the accumulation of cholesterol-loaded macrophages, which deposit on the arterial wall as foam cells.

Apo B, the sole protein of LDL serves the function of solubilising cholesterol within the LDL complex, which in turn increases the transport capacity of LDL for subsequent deposit on the arterial wall. Hence, in the present study, the alterations in the Apo A and Apo B levels clearly indicated their roles in the induction of atherosclerosis in the HC group\(^19,20\).

The prothrombin and clotting time were reduced in a mild to moderate degree in the HC group thereby indicating that there was accelerated fibrin deposition leading to increased blood clot formation and in turn the development of thrombi, which is a predisposing factor for induction of atherosclerosis\(^21\).

In the present study, increase in the levels of serum lipid peroxides in the HC group as compared to NC was observed in the lipid peroxidation assay. Lipoproteins once accumulated in the intima undergo oxidative modification at both the lipid and protein moieties, escaping plasma antioxidants, which are
absorbed by the monocyte and turn into foam cells in the vascular subendothelial cavity, triggering local inflammatory response responsible for signaling atherogenesis in the aorta and eventually atherosclerosis²².

The liver TC levels were significantly increased in the HC group as compared to the NC group. Liver cellular cholesterol negatively regulates the expression of LDL receptors. So, increase in the liver TC levels leads to increased and unregulated uptake of intimal LDL-derived cholesterol by the macrophages, which eventually leads to enhanced foam cell formation and subsequent steps leading to atherogenesis²³ (Table 1).

**Gross pathology**—Tissues of the HC group stained positive for oil-o-red staining on the internal surface of aorta, as shown in Figs 1 and 2, in the NC and HC groups respectively²⁴-²⁶.

**Histopathology**—The tissues in HC group showed well formed, thick, atheromatous plaques on the intimal surface of aorta (H&E), presence of foam cells with increased amounts of cholesterol and lipid deposition in the intimal and subintimal areas of the aorta (Schultz reaction and oil-o-red). There was a

Figs 7-10—(7) Section of aorta in normal control (frozen section; oil-o-red; 4×); (8) Section of aorta showing dense accumulation of foamy cell mass representing lipids (orange red) (arrows) in plaque on the intimal surface (frozen section; oil-o-red; 4×); (9) Section of aorta showing continuity of elastic fibers (black stained) in normal control (Verhoff’s van Geison’s; 4×); and (10) Section of aorta showing accumulation of foamy cell mass (A) along with severe degree of infiltration, disruption and replacement of elastic fibers (black stained, B) by fibrous tissue proliferates (pink stained, C) (Verhoff’s vangeison’s; 4×)
marked replacement fibrosis of aorta observed in the HC group (Verhoff’s Vangieison’s). The relevant aspects are demonstrated in Figs 3-10.

There are various theories to describe the mechanism of development of atherosclerosis but the most attractive hypothesis proposed has been that atherosclerosis begins because the innermost layer of the artery, the endothelium, becomes damaged. The earliest detectable events in lesion formation are intimal retention of LDL, monocyte adhesion to and migration across endothelial cells, their differentiation to macrophages, and accumulation of lipoprotein-derived cholesterol to form ‘foam’ cells. Ongoing lipoprotein infiltration and foam cell development lead to growth of the lesion. As the disease progresses, development of large acellular lipid core (which includes cholesterol crystals), and infiltration and proliferation of fibrous tissue replacing elastic fibers in the intima contribute to lesion volume. Minor disruption to the endothelium can stimulate platelet deposition, while major thrombus formation occurs when the plaque surface ruptures and the underlying procoagulant tissue is exposed.

To support the above hypothesis in the present study, various factors like foam cells, atheromatous plaques, lipids, cholesterol, and replacement fibrosis in the aorta have been clearly described in both gross and histopathological findings by various special staining techniques.

### Table 1—Effect of high cholesterol treatment on blood and liver biochemical profiles in rabbits

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control (NC)</th>
<th>High cholesterol (HC)</th>
</tr>
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<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>427.22 ± 90.85</td>
<td>924.11 ± 128.53*</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>86 ± 19.71</td>
<td>134.89 ± 33.45</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>370.17 ± 89.50</td>
<td>834.09 ± 124.56*</td>
</tr>
<tr>
<td>VLDL (mg/dl)</td>
<td>17.20 ± 3.94</td>
<td>26.98 ± 6.69</td>
</tr>
<tr>
<td>LDL/HDL ratio</td>
<td>9.29</td>
<td>13.23</td>
</tr>
<tr>
<td>Apolipoprotein A (mg/dl)</td>
<td>24.04 ± 6.101</td>
<td>15.911 ± 1.623</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dl)</td>
<td>0.45 ± 0.079</td>
<td>1.991 ± 0.352*</td>
</tr>
<tr>
<td>Serum lipid peroxides MDA (nano mole /ml)</td>
<td>0.343 ± 0.530</td>
<td>0.516 ± 0.022*</td>
</tr>
<tr>
<td>Prothrombin time (sec)</td>
<td>16.17 ± 1.89</td>
<td>14.71 ± 0.81</td>
</tr>
<tr>
<td>Clotting time (sec)</td>
<td>300 ± 16.20</td>
<td>146.14 ± 18.36*</td>
</tr>
<tr>
<td>Liver TC (mg/g)</td>
<td>7.90 ± 1.18</td>
<td>56.87 ± 6.35*</td>
</tr>
</tbody>
</table>

*P< 0.05 as compared to NC

### Conclusion

Considering all the above parameters, it can be concluded that there was successful induction of atherosclerosis. Various studies indicated that a genetically atherogenesis prone strain was a suitable model for studies on atherosclerosis, but findings in the present study demonstrate that New Zealand white rabbits can also be used as suitable animal model for studies on experimentally-induced atherosclerosis as evidenced by various biochemical and histopathological parameters.

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