

Full Length Research Paper

A comparative study on the atheroprotective potential of heparin and atorvastatin in hypercholesterolemic rats

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Accepted 01 May, 2008

The present study is to evaluate the effect of standard drug heparin and atorvastatin on the atherogenic disturbances in hypercholesterolemic atherogenic animals. Six groups of male Wistar rats were employed in this study, of which three groups received atherogenic CCT diet (rat chow supplemented with 4% cholesterol, 1% cholic acid and 0.5% thiouracil) for 17 days. Two groups were treated with standard heparin (200 units/kg/day, s.c) and atorvastatin (1.34 mg/kg/day, oral gavage) respectively commencing from 10th day of the experimental period with the remaining group served as control. Another 3 groups received normal rat chow diet during the entire experimental period. One group received only CCT diet while the other two groups received heparin and atorvastatin respectively. Increase in the plasma levels of cholesterol, triglycerides, high density lipoprotein, very low density lipoprotein and low density lipoprotein induced by CCT diets were normalized by heparin and statin treatment. The CCT diet induced abnormal rise with the plasma hepatic marker enzymes alkaline phosphatase, alanine transaminases and aspartate transaminases, plasma urea, glucose and creatinine levels were restored to normal with the treated groups. The CCT diet induced lecithin cholesterol acyltransferase (LCAT) and lipoprotein lipase (LPL) activities were restored to normal in the treated groups. Standard heparin and atorvastatin intervention minimized the atherogenic diet induced histopathological lesions in liver, kidney and aortic tissues.

Key words: CCT diet, Atherosclerosis, standard heparin, atorvastatin, LCAT, LPL.

INTRODUCTION

Heparin is a heterogeneous mixture of highly sulfated glycosaminoglycan with strong negative surface charge. The revelation that heparin exerts functions more than just the anticoagulant effect has been a major impulsion

for exploring the drug for its multi-faceted biological properties and functions at the cellular and the molecular levels. The high and low-molecular-weight heparins (5,000 to 30,000) are glycosaminoglycans consisting of chains of alternating residues of D-glucosamine and a uronic acid, either glucuronic acid or iduronic acid (Johnson and Mulloy, 1976; Deepa and Varalakshmi, 2003). The Glycosaminoglycan (GAG) heparin has been the most commercially and therapeutically important sulfated polysaccharide. For the first 30 years of its clinical use, not much was known about its structure except that it was composed of glucosamine and uronic acid with heavy sulfate substitution (Petitou et al., 2003).

The use of heparin has also been suggested in the management of atherosclerosis (Jaques, 1987; Engelberg, 1988; Shulman, 1990), besides its regular use as

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Abbreviations: CCT, cholesterol-cholic acid-thiouracil; LCAT, lecithin cholesterol acyltransferase; LPL, lipoprotein lipase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CPC, cetyl pyridinium chloride; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; TGL, triglycerides; ALP, alkaline phosphatase; AST, aspartate transaminases; ALT, alanine transaminases.

an anticoagulant in acute thrombotic episodes. Atherosclerosis is a generalized degenerative vascular disease occurring in all age groups and among all races (Kanchan and Balbir, 2002). This disease affects most of the major systemic blood vessels such as the coronary, renal, femoral, carotid arteries and aorta. Pathologically it is characterized by progressive narrowing or stenosis of the vessel lumen, while functionally there occurs reduced blood flow, tissue or organ ischemia and loss of normal vessel elastic properties. Endothelial damage has been indicated as the first event in atherogenesis and progression (Ross, 1986, 1993). The atherosclerosis has been called a chronic inflammatory disease (Editorial, 1992), in which macrophages activated by oxidized LDL assume a pivotal role in vascular damage and atheroma formation (Ragazzi and Chinellato, 1995).

Statins were developed to reduce serum cholesterol levels, which are strongly associated with coronary atherosclerotic disease (Klag et al., 1993). It has been generally assumed that cholesterol reduction by statins is the predominant, if not the only, mechanism underlying their beneficial effects in cardiovascular disease. However, recent investigators have challenged this concept and have suggested that the beneficial effect of statins may extend to mechanisms beyond cholesterol reduction. Recent numerous clinical studies suggest direct, endothelium-dependent effects of statins (Packard, 1998; Rauch et al., 2000; Sacks et al., 1996; Schwartz et al., 2001).

Atherosclerosis is a proliferative disease of the arterial intima, in which the principal components of the lesion are smooth muscle cell (SMC), connective tissue components which are synthesized and secreted by the SMC and variable amounts of intra and extracellular lipids (Handley., 1988). The commercial standard heparin and atorvastatin were primarily indicated for clinical use in lipid lowering effects and validation of the property along with commercial standard drugs.

An atherogenic diet experiment has been followed for seventeen days, to study the detrimental changes in the liver and aortic tissues in experimental hypercholesterolemic atherosclerosis. The high plasma cholesterol concentrations have been associated with atherogenesis (Peric-Golia and Peric-Golia, 1983). Biochemical analyses such as lipid profile, LCAT and LPL activities and histological studies in hypercholesterolemic induced rats were carried out to explore the potential therapeutic activity of both standard drugs.

MATERIALS AND METHODS

Experimental animals and diets

Adult male albino rats of Wistar strain (140 ± 10 g) were purchased from Central Animal House, Rajah Muthiah Medical College, Annamalai University, India. They were housed (6 per cage) in plastic cages (47 x 34 x 18 cm) under standard conditions of tem-

perature ($23 \pm 1^\circ$ C), relative humidity (55 ± 10 %), 12 h light and 12 h dark cycle and given food and water *ad libitum*.

They were divided in to six groups of six rats in each group. Group I served as control. Rats in group II, V and VI were fed with atherogenic diet comprising of the normal rat chow supplemented with 4% cholesterol, 1% cholic acid and 0.5% thiouracil (CCT diet) for 17 days. Group V and VI received standard commercial heparin s.c (200 units/kg/day, Heparin sodium EB, s.d. fine-chem limited, Mumbai) and atorvastatin (1.34 mg/kg/day, oral gavage, Cadila healthcare limited, Marketed by Zydus medica) treatment respectively commencing ten days after the start of the CCT diet. Group III and IV rats received standard heparin and atorvastatin alone on the same day as group V and VI. At the end of total experimental period of 17 days, after an overnight fast, blood was collected in heparinized tubes after which the animals were anesthetized and sacrificed by cervical dislocation. Sections of liver, kidney and aortic tissues were set aside for histological processing.

Biochemical estimations in blood

Plasma albumin was estimated using Bayer Diagnostic kit by BCG method (Grande et al., 1964). Blood glucose was estimated using Crest biosystems kit by enzymatic glucose oxidase peroxidase (GOD-POD) method (Trinder, 1969). Creatinine was estimated by Jaffe's method using erzb kit (Owen et al., 1954). Urea was estimated by Autozyme enzymatic method using urease accurex kit (Webster, 1977). All the values were expressed as mg/dl. Plasma total protein was estimated using Erba kit by Biuret method (Tietz, 1986). The levels were expressed as mg/dl.

Estimation of lipid profiles

The total cholesterol in plasma was estimated by cholesterol oxidase enzymatic method using Agappe Diagnostic kit (Siedel et al., 1981). Plasma triglyceride (TGL) was estimated by Smart lab Autoanalyser using enzymatic-GPO method (Rifai et al., 1999). High density lipoprotein (HDL) was estimated by selective precipitation followed by cholesterol oxidase enzymatic method using HDL-cholesterol phosphotungstic acid of Erba diagnostic Mannheim gmbh kit (Burstein et al., 1970). Very low density lipoprotein (VLDL) was calculated using the formula $TGL/5$ and Low density lipoprotein (LDL) was calculated using Friedewalds et al. (1972) formula, all the values were expressed in mg/dl.

The activity of plasma ALT and AST were estimated by IFFC method using Reckon diagnostics kit Bergmeyer et al., (1986). ALP was determined by Crest biosystem kit (Bowers and McCommb, 1972). The activity of the enzymes was expressed in IU/l of plasma.

Lecithin: cholesterol acyltransferase (LCAT) activity assay

LCAT activity was measured by the method of Legraud et al. (1979) with the modification of Hitz et al. (1983). The incubated mixture contained 0.6 ml HDL rich plasma as substrate and 0.6 ml of enzyme. From that 0.4 ml was transferred to another tube and the reaction was stopped by adding 1 ml isopropanol. This gives the cholesterol level present at zero time. Remaining 0.8 ml was processed separately and the reaction was stopped at 90 and 180 minutes. The remain mixture was collected from various time periods and 2 ml of acetone and 1 ml of digitonin (0.5% in 50% ethanol) was added. The cholesterol content at zero time, 90 min and 180 min were estimated as described by Leffler and McDougald, (1963). Values were expressed as mg of cholesterol esterified/ ml plasma/hour.

Table 1. Effect of standard heparin and standard drug atorvastatin on plasma lipid components and hepatic marker enzymes in early phase atherogenesis.

Parameters	Group I Control	Group II CCT diet	Group III Control + Standard heparin	Group IV Control + Standard atorvastatin	Group V CCT diet + Standard heparin	Group VI CCT diet + Standard atorvastatin	Significance
Cholesterol (mg/dl)	61.50±10.84	153.63±11.72	61.78±10.86	59.85±10.20	76.25±12.35	72.71±11.03	a, b, c, d $p<0.05$
TGL (mg/dl)	37.63±11.73	54.66±11.02	35.65±8.56	34.29±7.85	34.86±8.02	32.33±7.08	a, b, c, d $p<0.05$
HDL (mg/dl)	25.63±5.60	27.50±6.09	25.96±5.42	26.71±4.86	26.0±7.07	26.83±5.38	a, b $p<0.001$ c, d $p<0.05$
VLDL (mg/dl)	7.52±2.18	10.93±3.32	7.26±2.63	7.88±3.26	6.97±2.63	6.46±2.43	a, b, c, d $p<0.05$
LDL (mg/dl)	65.08±33.60	128.20±31.51	64.12±30.45	64.79±31.54	59.33±14.20	53.78±7.10	a, b, c, d $p<0.001$
ALP (U/L)	324.8±64.12	391.5±62.42	318.6±58.64	326.78±34.28	294.1±4.54	298.20±5.70	a, b, c, d $p<0.05$
ALT (U/L)	41.16±7.02	80.20±9.42	44.46±10.34	47.62±11.34	51.00±12.61	58.60±8.39	a, b, c, d $p<0.05$
AST (U/L)	303.1±58.77	437.6±71.56	302.8±57.84	306.5±55.86	315.4±53.12	324.5±59.50	a, b, c, d $p<0.001$

Values are expressed as means ± S.D. for six animals in each group.
a= Group I Vs II; b= Group II Vs III, IV, V, VI; c= Group III Vs V; d= Group IV Vs VI

Table 2. Biochemical evaluation of CCT diet induced atherosclerosis and the role of standard heparin and standard drug atorvastatin

Blood parameters	Group I Control	Group II CCT diet	Group III Control + Standard heparin	Group IV Control + Standard atorvastatin	Group V CCT diet + Standard heparin	Group VI CCT diet + Standardatorvastatin	Significance
Urea (mg/dl)	53.33±7.93	59.83±8.84	49.46±7.16	47.95±7.02	51.33 ± 8.94	50.50±7.68	a, b, c, d $p<0.05$
Glucose (mg/dl)	51.66±10.80	53.66±9.73	51.12±9.86	50.46±7.86	47.07 ± 8.04	49.45±8.25	N.S
Albumin (mg/dl)	3.4±1.3	2.8±1.1	3.2±1.2	3.3±1.2	3.5 ± 1.5	3.4±1.2	a, b, c, d $p<0.001$
Total protein (g/dl)	9.0±1.3	8.8±1.6	9.23±1.62	9.82±1.4	9.5 ± 1.05	9.3±1.2	N.S
Creatinine (mg/dl)	1.03±0.41	1.54±0.20	1.22±0.86	1.06±0.58	1.09 ± 0.52	0.98±0.45	a, b, c, d $p<0.05$

Values are expressed as means ± S.D. for six animals in each group. N.S = Not Significant
a= Group I Vs II; b= Group II Vs III, IV, V, VI; c= Group III Vs V; d= Group IV Vs VI

Lipoprotein lipase (LPL)

Lipoprotein lipase was assayed according to the method of Schrecker and Greter (1979). The incubated mixture contained 220 μ l of 7.5 mM triolein, 1.8 mg of BSA and 20 μ l of plasma. 1 ml of 1.5 M NaCl was added in lipoprotein lipase assay. Values were expressed as μ moles/ml of plasma/hour.

Histopathological studies

Immediately after sacrificing the animal, liver, kidney and aortic tissues were collected and fixed in 10% neutral formalin. The washed tissues were dehydrated in descending grades of isopropanol and cleared in xylene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5 μ m thickness and stained with hematoxylin and eosin and then viewed under light microscope for histopathological changes.

Statistical analysis

The results were expressed as mean values \pm S.D. Differences between groups were assessed by one-way analysis of variance (ANOVA), using the SPSS-10 system for Windows.

RESULTS

The plasma cholesterol, triglyceride (TGL) levels were significantly ($p < 0.05$) increased in the CCT diet fed rats, and restored to normal in standard heparin and atorvastatin treated group rats (Table 1). Cholesterol concentration in standard heparin treated (group III) and atorvastatin (group IV) treated groups was significantly reduced compared to the untreated group II ($p < 0.05$). The safety of the present dosage of heparin and atorvastatin is appreciated in group III and IV, where plasma cholesterol and TGL levels remain in the control range. HDL, VLDL and LDL activities were significantly increased in CCT diet group II compared to control group I ($p < 0.001$). In treated groups V and VI, HDL, VLDL and LDL levels were significantly reduced as compared to the CCT diet alone induced rats ($p < 0.001$) (Table 1). Significant increase in activities of plasma ALP, ALT and AST was recorded in group II as compared the control group ($p < 0.05$). The hepatic marker enzymes AST ($p < 0.001$), ALP ($p < 0.05$) and ALT ($p < 0.05$) concentration in standard heparin treated (group V) and atorvastatin (group IV) treated rats was significantly reduced compared to the CCT diet alone induced group II ($p < 0.05$). The safety of the present dosage of heparin and atorvastatin is appreciated in group III and IV, where enzyme activities remain in the control range (Table 1).

The plasma urea and creatinine concentrations were significantly increased in the CCT diet fed group II rats ($p < 0.05$). Heparin and atorvastatin treated groups significantly reduced the plasma urea and creatinine levels ($p < 0.05$) (Table 2). Albumin concentration was significantly decreased in the atherogenic diet fed group II

($p < 0.001$) compared to the control group. There were no significant changes in the level of plasma glucose and total protein among the groups compared (Table 2).

The LCAT activity was significantly increased in the CCT diet fed group ($p < 0.05$) and was restored in the standard heparin and atorvastatin treated groups (Figure 1a). Plasma LPL activity was significantly lowered in the CCT diet induced rats ($p < 0.05$) compared to the control group. Restoration to normal levels of the LPL activity was recorded in groups V and VI ($p < 0.001$) (Figure 1b). The safety of the present dosage of heparin and atorvastatin is appreciated in group III and IV, where LCAT and LPL activities remain in the control range (Figure 1a and 1b).

Lipid lowering effect of atorvastatin was significantly better as compared standard heparin (Cholesterol, TGL, HDL and VLDL ($p < 0.05$), LDL ($p < 0.001$), ALP and ALT ($p < 0.05$), AST ($p < 0.001$) (Table 1), LCAT ($p < 0.05$) and LPL ($p < 0.001$) (Figure 1a and 1b).

Figure 2a (H and E, 20x) shows the fat changes induced by the CCT diet in group II. Figure 2 b, c and d show the normal hepatic architecture, with the parenchymal structure preserved and occasional fat cells in the standard heparin and atorvastatin treated atherogenic diet fed groups, compared to the control group I. Figure 3 a shows mild tubular damage in the renal tissue (H and E, 20x) in group II consequent to the CCT diet. The photomicrograph of renal tissue from the standard heparin and atorvastatin rats shows near normal morphology as comparable with that of control group I (Figure 3 b, c and d). Figure 4a shows that the untreated atherogenic group reveals foam cells which is a significant event in atherogenesis. Foam cells are reduced in the standard heparin and atorvastatin treated groups V and VI (Figure 4b, c and d)

DISCUSSION

Atherosclerosis is a slowly progressing, inflammatory, proliferative disease in which various cells such as macrophages, endothelial and smooth muscle cells (SMC) are involved (Ross, 1999). Even though high doses of heparin act as anticoagulant, low doses have been demonstrated to have anti-atherogenic effects. The heparin also inhibits the platelet derived growth factor mediated the over growth of medial smooth muscle cells which contribute to plaque formation (Shulman, 1990). Hypercholesterolemia has been reported to cause endothelial cell dysfunction, as evidenced by an increase in endothelial cell turnover in cholesterol fed rabbits and swine (Shulman, 1990) and increased permeability of the endothelium in cholesterol – fed rabbits (Stemerman, 1981). The significantly elevated levels of plasma cholesterol in rats fed with CCT diet might damage the endothelial cell membrane lining the large arteries such as aorta and might be the initial events in the aetiology of

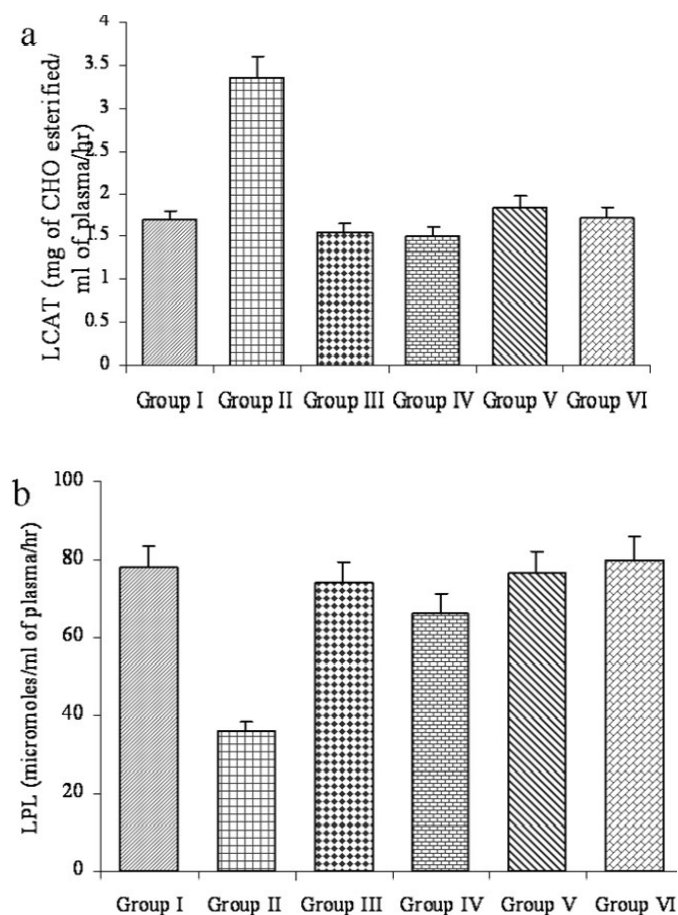


Figure 1. Activities of (a) Lecithin cholesterol acyltransferase (LCAT) and (b) Lipoprotein lipase (LPL) activity.

atherosclerosis (Mirhadi et al., 1991). The blood cholesterol levels were reported to be nearly 2.5 fold higher in the untreated atherogenic group when compared with that of the control and the plasma TGL levels was raised to 1.5 fold in the untreated atherogenic group. In heparin and statin treated animals plasma cholesterol and TGL levels were restored to near control values.

HDL is considered to be a beneficial lipoprotein and has a negative effect on atherogenesis. Risk of coronary heart disease increases progressively with higher levels of low density lipoprotein (LDL) cholesterol while higher levels of high density lipoprotein (HDL) cholesterol reduce the risk significantly (Penumathsa et al., 2007). Cholesterol accumulation in aortas of cholesterol fed guinea pigs was shown to positively correlate with all LDL components as well as plasma VLDL esterified cholesterol (Sable and Sicart, 1983; Sparks et al., 1986). Heparin helps in scavenging cholesterol from the extra hepatic tissues in the presence of lecithin cholesterol transferase (LCAT) and brings it to the liver. Plasma HDL concentration was significantly high in rats that received CCT

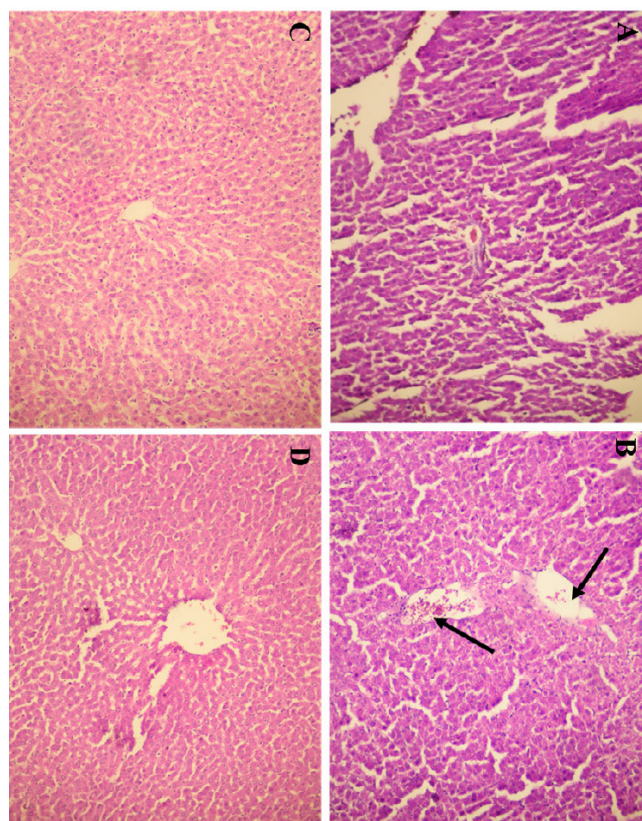


Figure 2. Histopathology of liver in treated and untreated rats. (a) group I – normal parenchymal structure (H and E, 20x); (b) CCT diet induced group (H and E, 20x), reveals severe fatty changes in the liver cells. (c) and (d) CCT diet with standard drug heparin and atorvastatin treated group respectively, (H and E, 20x), normal parenchymal structure with very occasional fat cells, almost like normal hepatocytes.

diet alone than the control rats, where as it was more or less similar to that of control rats in drug treated animals. The higher HDL levels in atherogenic diet fed rat can be attributed to increased plasma LCAT activity as a consequence of hypercholesterolemia (Lehmann et al., 2001).

There was a significant rise in hepatic marker enzyme (ALP, ALT ($p < 0.05$) and AST ($p < 0.001$) activities in the atherogenic diet group (Deepa and Varalakshmi, 2004; Naik and Sheth, 1978). CCT diet induced liver damage causing accumulation of interleukin-6 release ALP and adenosine for protection from ischemic injury (Deepa and Varalakshmi, 2003). Significant rise in plasma urea levels was induced by the CCT diet. The albumin component of plasma proteins was markedly decreased in the rats as observed in earlier studies (Bertani et al., 1982). The restoration of albumin levels by heparin and atorvastatin may therefore be due to restoration of normal liver function and decrease in albumin extravasation in response to inflammation.

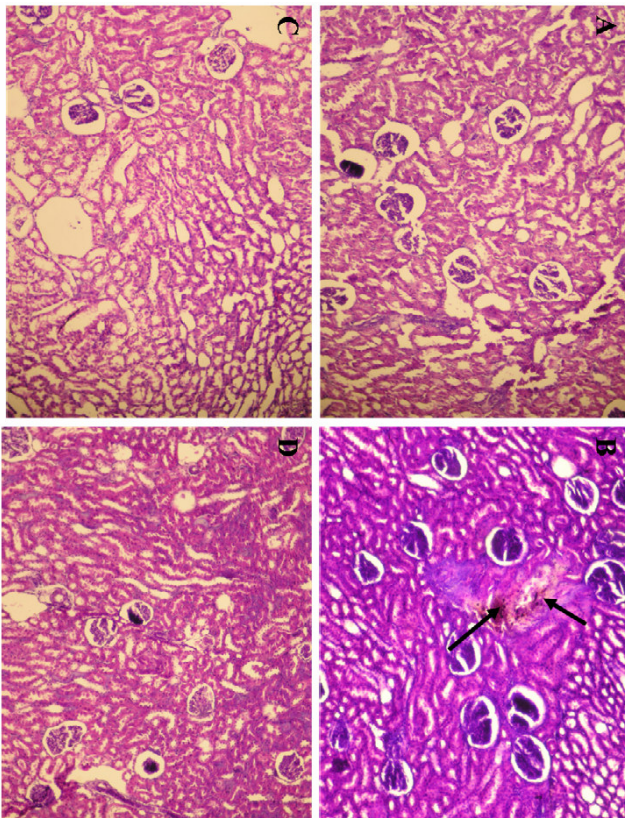


Figure 3. Histopathology of kidney in treated and untreated rats. (a) group I – normal renal architecture (H and E, 20x); (b) CCT diet induced group (H and E, 20x), shows mild renal tubular damage; (c) and (d) CCT diet with standard drug heparin and atorvastatin treated group respectively, (H and E, 20x), presents normal renal histology.

Fielding et al. (1972) suggested that HDL and LCAT might play concerted roles in transporting cholesterol from peripheral tissues. Glomset (1968) showed that addition of LCAT to the system led to an increase in HDL unesterified cholesterol and esterified cholesterol levels representing greater total cholesterol uptake. Once cholesterol is incorporated into HDL, it is esterified by LCAT. Two different activities have been described for LCAT; α -LCAT activity corresponds to cholesterol esterification in HDL, and β -LCAT activity indicates cholesterol esterification in VLDL/LDL (Carlson and Holmquist., 1985; Franco et al., 2003). Our results showed that LCAT activity was increased in Wistar rats using external HDL for cholesterol esterification, which is indicative of high LCAT concentration. The decreased LPL activity in plasma and the increased TGL levels were observed in the CCT diet group. An inverse correlation between serum triglycerides and LPL activity has been reported by Persson et al. (1966). The standard heparin and atorvastatin treatment restored TGL levels.

In earlier studies, it is shown that (1) depletion of hepa-

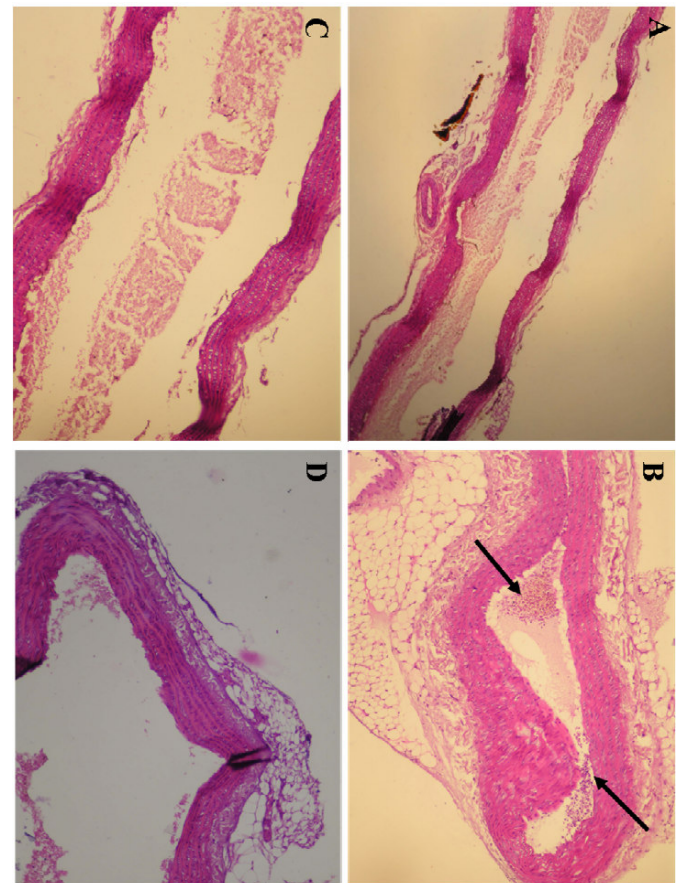


Figure 4. Histopathology of aorta in treated and untreated rats. (a) Control shows normal histology (H and E, 20x); (b) CCT diet induced group (H and E, 20x) shows appearance of foam cells. (c) and (d) CCT diet with standard drug heparin and atorvastatin treated group respectively, (H and E, 20x) shows disappearance of foam cells as in normal aortic histology.

rin-like anionic molecules predisposes to the development of atherosclerosis, because such areas have an increased susceptibility to endothelial injury, increased permeability and an enhanced infiltration of cholesterol (Hansson et al., 1979); (2) exogenous heparin corrects the depletion of normal negative charge of the endothelial surface in vascular injury, and thereby restores normal function (Engelberg, 1984, 1988); (3) numerous anti-inflammatory actions of heparin substantiate its protective role in atherosclerosis, because many inflammatory factors are involved in all stages of this process (Engelberg, 1996; Nelson et al., 1993). Administration of heparin as long term therapy has been suggested for atherosclerosis (Jaques, 1987; Engelberg, 1988). Intermittent heparin by subcutaneous or intravenous routes provides effective anti-atherogenic therapy. Intrapulmonary administration of heparin has been reported to be effective as alternative treatment for atherosclerosis when prolonged therapy is required (Ragazzi and Chinellato., 1995).

In the present study, the standard heparin rendered protection against early harmful changes in the liver and aortic tissues consequent to the CCT atherogenic diet and the results were comparable with that of atorvastatin treatment. The affiliated administration of atorvastatin, a second-generation lipophilic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, suppresses tissue factor expression without affecting plasma cholesterol levels (Jones et al., 1998). Aikawa et al., (1999 and 2001) have recently reported that the enhanced expression of tissue factor in atherosclerotic lesions of hypercholesterolemic rabbits is reversed by lowering the plasma cholesterol levels, either by dietary means or by cerivastatin treatment. These findings provided new insights regarding the potential mechanisms by which lipid lowering reduces the clinical complications of atherosclerosis. In the present study, we showed that atorvastatin and standard heparin may exert direct nonlipid-related antithrombotic effects at the level of the arterial intima by reducing lipid levels in experimental atherosclerotic lesions of cholesterol-fed rat without concomitant changes in serum cholesterol levels. So, our results clearly demonstrated that the commercial atorvastatin may be more effective in hypercholesterolemic induced rat than heparin. In general, HMG-CoA reductase inhibitors have been reported either to reduce (Ishida et al., 1990; Shiomi and Ito, 1999) or to leave unaltered (Boger et al., 1997; Bandoh et al., 2000) the plasma cholesterol levels in hypercholesterolemic rats. Further biochemical and histological investigations elucidated the protective role of standard drugs against atherogenesis (Murata et al., 1975; 1978; Izuka and Murata., 1972). In conclusion, this study showed the lipid lowering effect of standard heparin and atorvastatin in hypercholesterolemic rat.

ACKNOWLEDGEMENT

The authors are thankful to Prof. T. Balasubramanian, Director, CAS in Marine Biology, Annamalai University, Parangipettai, Tamilnadu, India for providing all facilities.

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