Anti-Oxidant Activity of Enalapril maleate in Hypercholesterolemic Rabbits

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ABSTRACT
The present study was to evaluate the anti-oxidant activity of Enalapril maleate in hypercholesterolemic rabbits, which developed atherosclerosis. Enalapril maleate is a class of Angiotensin Converting Enzyme Inhibitors. Normal healthy rabbits of either sex were divided in three groups. Each group contained twenty-five animals, all of which received commercial diet and water ad-libitum throughout the experimental period. The experiment was carried for a period of sixteen weeks. Group I animals received normal saline (5 ml/kg P.O) for a period of sixteen weeks. Group II received 500 mg cholesterol in 5 ml starch mucilage P.O daily for 16 weeks- hypercholesterolemic group. Group III received 500 mg cholesterol suspended in 5 ml starch mucilage and Enalapril maleate (10 mg/kg b.w PO daily) for 16 weeks. Various biochemical estimations like total serum cholesterol, serum HDL cholesterol levels, TBARS (Thiobarbituric acid reactive substances), glutathione, catalase were estimated. Results showed that decrease in total serum cholesterol levels, TBARS levels and increase in HDL cholesterol levels can play a part in anti-atherosclerotic action of Enalapril maleate. Catalase and glutathione which detoxify free radicals are not involved in the anti-atherosclerotic effect of the drug.

Key words:- Hypertension, Atherosclerosis, Enalapril Maleate

INTRODUCTION
Angiotensin converting enzyme (ACE) inhibitors are an important class of antihypertensive drugs. There has been a link between hypertension and atherosclerosis. It is observed that there is formation of atherosclerotic plaques in hypertensive patients because angiotensin II helps in atherogenesis. Atherosclerosis causes narrowing or blockage of the coronary arteries which in turn lead to coronary heart disease. It is the leading cause of death for both men and women and accounts for approximately 600,000 deaths every year. Over 3 million deaths owing to cardiovascular diseases every year, India is set to be the heart disease capital of the world. The main reasons for this epidemic is lifestyle changes such as sedentary jobs, improvement in socioeconomic status leading unhealthy diets rich in fats, high stress jobs and addictions like smoking and tobacco chewing. ACE inhibitors reduce vasoconstriction, increase the bioactivity of NO and can inhibit vascular superoxide production. These drugs have achieved widespread usage in the treatment of cardiovascular and renal disease. Oxidative stress also plays an important role in the pathogenesis of hypertension and atherosclerosis which occurs mainly due to free radicals. Free radicals are independent chemical species with one or more unpaired electrons. These include oxyradicals. The term reactive oxygen species (ROS) is now generally preferred because singlet oxygen, hydrogen peroxide, hypochlorous acid, peroxide, hydroperoxide and epoxide metabolites of endogenous lipids contain chemically reactive oxygen containing functional groups and behave as radicals. The free nature of the electrons in free radicals makes them able to combine readily with electrons within a tissue in order to achieve a more stable paired electron status. Once a free radical interacts with a tissue, many changes can occur. These changes can be defined in terms of injury or other more general responses. The term “Oxidative Stress” was created to denote a disturbance in the pro-oxidant and antioxidant balance in favour of the former, leading to potential damage. The prevalence of oxygen in biological systems means that oxygen-centered radicals are the most common type found. Among these are the superoxide radicals, hydrogen peroxide, hydroxyl radical and hydroperoxyl radical. Transition metal ions are...
important in the production of radical species. The sources of free radicals include the activated phagocytic cells, mitochondrial electron transport system, microsomal electron transport system, soluble oxidase enzymes, by auto-oxidation of endogenous or exogenous substrates and transition metals. The free radicals are highly reactive, short lived exist in low concentration and can act as oxidizing and reducing agents. Cellular components damaged by free radicals include lipids, proteins, carbohydrates, nucleic acids, and membrane potential, increased permeability to H* and other ions, and eventual rupture leading to release of cell and organelle contents such as lysosomal hydrolytic enzymes. Some end products of metal ion-dependent lipid peroxide fragmentation are also cytotoxic e.g. malonaldehyde 4,5 dihydroxy, Decenal and 4-hydroxynonenal. Disrupted tissues often undergo lipid peroxidation more quickly than healthy ones, especially after mechanical disruption.

Disease or toxin leads to cell damage or death due to increased lipid peroxidation. However, there are human conditions in which lipid peroxidation does appear to play a major role in the pathology e.g. in atherosclerosis. Lipid peroxides present in the low-density lipoproteins (LDL) present in atherosclerosis could conceivably contribute to the initial endothelial cell damage that is thought to start off the whole process, for example, by worsening hemodynamic damage. As an example, studies in vitro have shown that linoleic acid hydroperoxide increases the permeability of endothelial cell monolayers to macromolecules. Further, the products formed in peroxidized LDL [35] such as lysorphosphatidyl choline, might act as chemotactic factors for blood monocytes, encouraging their recruitment into an atherosclerotic lesion. Low (submicromolar) concentrations of peroxides also accelerate cyclooxygenase and lipoxygenase - catalyzed reactions in endothelium and in any platelets present, leading to enhanced formation of eicosanoids. Oxidized LDL [36] may also stimulate the production of eicosanoids by macrophages. It might be supposed, therefore, that elevated blood lipid concentrations could lead to elevated blood lipid peroxide concentrations, contributing to endothelial injury and accelerating the whole process of atherosclerosis [37,38].

The other parameters responsible for atherosclerosis is hypercholesterolemia [39] which means there is elevated blood cholesterol levels. Cholesterol is the main constituent responsible for hypercholesterolemia. It is an insoluble lipid containing a steroid nucleus. Being on essential component of cell membranes (required for transmembrane transport) and serum lipoproteins (required for the transport of triglycerides), is also a precursor of Bile acids (which are required for fat absorption); adrenal steroids (hydrocortisone, aldosterone) and sex hormones (estrogens and androgens).

The definition of hypercholesterolemia has been changing rapidly in recent years. A variety of National and International panels have formulated guidelines for the interpretation of plasma
cholesterol concentrations \(^{[40]}\). The most influential of these is the National Cholesterol Education Panel. Three categories of blood cholesterol levels are recognized-Desirable blood cholesterol : < 200 mg/dl (lowest risk of coronary artery disease),Borderline high blood cholesterol : 200 - 239 mg/dl (absolute risk in patients by cholesterol depends on other risk factors), High blood cholesterol : 240 mg/dl (greater risk of coronary artery disease).

Enalapril maleate is an important Angiotensin converting enzyme (ACE) inhibitor \(^{[41]}\). It is a peptidyl dipeptidase which catalyses the conversion of angiotensin II to the pressor substance angiotensin II. After absorption, enalapril is hydrolysed to enalaprilat, which inhibits ACE. Inhibition of ACE results in decreased plasma angiotensin II, which leads to increased plasma renin activity and decreased aldosterone secretion. ACE is identical to kininase II. Thus enalapril maleate may also block the degradation of bradykinin, a potent vasodepressor peptide. The onset of action of oral enalapril is gradual and smooth; it begins within one hour and its effects usually continue for 24 hours. The cardio-protective properties of enalapril include retardation of the development of symptomatic heart failure in asymptomatic patients \(^{[42]}\) with left ventricular dysfunction \(^{[43]}\) and prevention of coronary ischaemic events \(^{[44]}\) in patients with left ventricular dysfunction, specifically reduction in the incidence of myocardial infarction \(^{[45-46]}\) and reduction in hospitalization for unstable angina pectoris. Excretion of enalaprilat is primarily renal. It is taken mainly as tablets. Since absorption is not affected by food, the tablets may be given before, during or after meals. Minimum dose is 2.5 mg and maximum dose is 40 mg. The dosage is adjusted according to the needs of the patient. In the management of symptomatic heart failure, diuretics are also given.

Enalapril maleate has been demonstrated to be generally well tolerated. However dizziness and headache are the more commonly reported adverse effects \(^{[47]}\). Fatigue is reported in 2-3% of patients. Other adverse effects occurred in less than 2% of patients and included hypotension, orthostatic hypotension, syncope, nausea, diarrhoea, muscle cramps and cough. Skin rash was reported in 1.2% of patients and taste disturbances in 0.5% of patients. Less frequently renal dysfunction, renal failure and oliguria have been reported.

**MATERIALS AND METHODS**

**Selection of Animals:** Normal healthy rabbits of either sex weighing between 1.2 to 2 kg were divided into three groups. Each group contained twenty five animals at random, all of which received commercial diet and water *ad libitum* throughout the experimental period. The experiment was carried out for a period of sixteen weeks. The animals were handled as per guidelines of committee for the purpose of control and supervision on experimental animals(CPCSEA), Govt of India. Animals were acclimatized to laboratory conditions before testing. The use of experimental animals was approved by Institutional Animal Ethics Committee (IAEC). Blood samples for analyzing biochemical parameters-total serum cholesterol levels and serum HDL cholesterol levels were withdrawn from marginal ear vein after an overnight fasting. Animals were sacrificed for biochemical parameters-catalase levels, TBARS levels and glutathione levels. Enalapral maleate was obtained from Pharmax Corporation Ltd. Cholesterol was obtained from Ubichem Ltd England. Stains alcoholic eosin was obtained from Qualigens fine chemical, Bombay and Hematoxylin was obtained from BDH Bombay India. Animals -Albino Rabbits were obtained from Lucky Zoological Delhi.Rabbit Chow was obtained from Hindustan Lever Ltd. Bombay.

**Experimental Protocol:**  
Group I : Animals of this Group received normal saline (5 ml/kg (P.O) and a standard rabbit diet for a period of 16 weeks. They were treated as Normal Control Group.  
Group II: Animals of this Group received 500 mg cholesterol suspended in 5 ml starch mucilage P.O for 16 weeks and were treated as Hypercholesterolemic Group.  
Group III: Animals of this Group received 500 mg cholesterol suspended in 5 ml starch mucilage and Enalapril Maleate (10 mg/kg body wt P.O daily) for 16 weeks. They were treated as Drug Treated Group. The oral feeding was carried out by intubation. Various biochemical investigations were carried out at 0,4,8,12 and 16 weeks. At the end, the histopathological studies were also carried. Various parameters studied during the experiment were:-  
A) Biochemical estimations: Total serum cholesterol levels, serum HDL cholesterol levels, TBARS levels, glutathione levels and catalase levels.  
B) Histopathological studies: Study of aorta.

**Estimation of Total serum cholesterol:**  
Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. In the second reaction cholesterol oxidase converts cholesterol to cholest 4-en-3-one and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide...
oxidatively couples with 4-aminocoumpyrine and phenol to produce red quinoneimine dye which has absorbance maximum at 510 nm. (500 – 530). The intensity of the red colour is proportional to the amount of total cholesterol in the specimen. The working solution contained buffer pH 6.8 in the conc of 50 mmol/l, cholesterol oxidase 100 IU/l, cholesterol esterase 150 IU/l, peroxidase 500 IU/l, 4 amino antipyrine 0.5 mmol/l and phenol in the conc of 10 mmol/l. Blood was collected in clear dry test tube. Fasting blood was preferred for cholesterol assay. The reaction time was 10 min at 37°C and wavelength was 510 nm (500-530nm), sample volume was 10µl. Reagent volume was 1.0ml and standard concentration was 200mg %. Calculation was done as per procedure [48,49].

Estimation of serum HDL–cholesterol. In the first step, LDL, VLDL & Chylomicrons are eliminated under specific conditions. In the second step, special reagents break up HDL and make HDL-cholesterol available for measurement. The working solution has two reagents R1 and R2. R1 contained chromogen, cholesterol oxidase and buffer whereas R2 has 4 amino antipyrine and buffer. Blood was collected in a clear and dry container. The reaction time was 5+5 minutes and wavelength was 578nm. Sample volume was 10µl and reagent volume was (0.750ml + 0.250ml). The mixtures were incubated for 5 minutes at 37°C [100].

Estimation of Thiobarbituric Acid Reactive Substances (TBARS): Aldehydes are always produced when lipid hydroperoxides breakdown in biological systems and it is necessary to measure these compounds both as an index of the extent of lipid peroxidation and as an aid to elucidate the role of aldehydes as causative agents in certain pathological conditions like atherosclerosis. Malondialdehyde (MDA) is the most abundant aldehyde resulting from lipid peroxidation and its determination by thiobarbituric acid (TBA) is one of the most common assays in lipid peroxidation studies. In the TBA test reaction, one molecule of MDA reacts with two molecules of TBA with the production of a pink pigment having an absorption maximum at 532-535 nm [31].

Aortas were rinsed and placed in an ice-cold 0.1 M potassium buffer with 1 mM EDTA. The fat and perivascular connective tissue were carefully excised. The vessels were weighed, minced and homogenized in the buffer for 3 minutes in an icebath with a commercial homogenator. After homogenization, the homogenate was taken to a final concentration of 1:2 v/v with 2.3% potassium chloride solution. Added 0.2 ml of 8.1% sodium lauryl sulphate solution. Added 1.5 mM of 20% acetic acid solution, and adjusted to pH 3.5 with 0.25 M HCL and 1 m NaOH. Added 1.5 ml of aqueous solution of 0.8% thiobarbituric acid (TBA), and heated the mixture in water bath for 60 minutes at 95°C. Added 1 ml of distilled water and 5 ml of n-butanol plus pyridine (15:1v/v). The mixture was shaken vigorously and centrifuged at 3000-5000 r.p.m for 10 minutes. The absorbance of the organic layer was measured spectrophotometrically, at 532 nm using 1.53 x10⁻⁵ M⁻¹ cm⁻¹ as molar extinction coefficient. The values were expressed as µmoles TBARS/mg wet tissue.

Estimation of Glutathione Direct determination of sulphhydryls in tissue extracts using Ellmans reagent (DTNB,5,5'-dithiobis (2-nitrobenzoic acid) gives a rapid approximation of Glutathione (GSH) content. Glutathione and Ellmans reagent gives Glutathione disulphide or Glutathione reductase and 2 nitro 5 thiobenzoic acid. The formation of TNB is followed spectrophotometrically at 412 nm. The homogenate obtained in TBARS estimation was again centrifuged at 1000 r.p.m for 3 minutes at 4°C to remove fibrous material. 0.5 ml of the supernatant was taken and 0.02 ml of the sulphosalicylic acid was added to it and again centrifuged at 3000-5000 r.p.m for 15 minutes. Then about 0.4 ml of the supernatant fraction was taken for analysis. 2 ml of potassium phosphate buffer containing 1 mM EDTA was added to the supernatant fraction. To this was added, 0.1 ml of 0.3 M DTNB. The absorbance was measured at 412 nm using 13.6x10⁻³ M⁻¹ cm⁻¹ as molar extinction coefficient. The values were expressed as µmoles/mg wet tissue [52,53].

Estimation of catalase Catalase activity can be measured by following either the decomposition of H₂O₂ or the liberation of O₂. The method of choice for biological material is the UV spectrophotometric method. In the ultraviolet range H₂O₂ shows a continuous increase in absorption with decreasing wavelength. The decomposition of H₂O₂ can be followed directly by the decrease in extinction at 240 nm. The difference in extinction per unit time is a measure of the catalase activity.

The supernatant obtained in glutathione estimation was again centrifuged at 3000 r.p.m for 10 minutes at 4°C to remove cellular debris. The supernatant was then adjusted to 1% by adding Triton-X-100 and incubated at room temperature for 30 minutes (1 ml of supernatant+ 9 ml of Triton-X-100). Two solutions were taken one as a blank and other containing the sample solution. The total volume taken was 3 ml. The sample solution contained 50 mM potassium
phosphate buffer pH 7; 14 mM hydrogen peroxide and 0.1 ml of the appropriate supernatant sample which was added at the end to start the reaction. The blank solution was taken without the supernatant sample [54,55].

Statistical Analysis: The results were expressed as mean ± standard error of mean. The results were statistically analyzed using students “t” test.

Histopathological Studies [56]: First of all, the tissues were fixed by denaturing proteins and cross linking them to provide a solid structure in place of delicate membranes, using formalin(10%). The water present in the tissue was replaced using increasing concentrations of ethyl alcohol. The final absolute alcohol was then replaced by wax in which it is easily soluble. The tissue block was soaked in paraffin wax at a temperature above its melting point. The wax was cooled and this block of tissue was taken for sectioning. Fixation was done in 10% formalin for 24-48 hrs. Dehydration was done in 70% ethyl alcohol for 18 hrs. Embedding was done in molten paraffin wax(50°C) for 2 hrs and blocks were casted in paraffin wax and sections were cut at 5 um. The sections were floated on water on slides to remove wrinkles and then dried. Hydration was done in xylene for 2 min, in 70% ethyl alcohol for 2 minutes and several rinses were given in distilled water for 2 minutes. Staining was done using hematoxylin for 3 minutes, then rinsed in distilled water, in 1% eosin and in 90% alcohol for 1 minute. Mounting was done using ethyl alcohol 100% and xylene for 1 minute. The slides were covered with mounting medium and coverslip.

RESULTS AND DISCUSSION

The work undertaken for the present study is to evaluate anti-oxidant activity of Enalapril Maleate. This could be one of the mechanisms by which it can act as an antiatherosclerotic agent. Enalapril maleate belongs to a class of Angiotensin Converting Enzyme (ACE) inhibitors. These are highly specific drugs that have been successfully used in the treatment of cardiovascular diseases especially in congestive heart failure and hypertension. Several experimental studies have also shown an antiatherosclerotic effect of ACE inhibitors. Captopril lowers blood pressure [58] which reduces arterial permeability and decrease lipoprotein entry into the intima or even possibly enhance cholesterol removal from there. It reduces the effect of certain proliferative stimuli on smooth muscle cells and their migration. Ramipril, releases EDRF factor like nitric oxide, and elevates HDL cholesterol levels. Besides these, there are some other mechanisms that can be studied, to evaluate the anti-atherosclerotic action of ACE inhibitors, e.g. effect on lipid peroxides [59].

The antiatherosclerotic effect of enalapril maleate (ACE inhibitor) has been elucidated by evaluating those biochemical factors which are involved in the pathogenesis of atherosclerosis such as total cholesterol, HDL cholesterol [60,61] lipid peroxidation products like TBARS (Thiobarbituric Acid Reactive Substances). Furthermore, attempts were made to assess the status of biochemical defenses such as catalase and glutathione. Hypercholesterolemia was induced experimentally in rabbits by feeding cholesterol. Albino rabbits are highly susceptible and cholesterol feeding induced a sharp rise in serum total cholesterol levels. Rise in total cholesterol is comparatively less in rabbits those also received simultaneously enalapril maleate along with cholesterol (Fig 1).

One of the most important observations of the present study is that of serum HDL-cholesterol levels. Simultaneous administration of enalapril maleate along with high cholesterol diet changed HDL - cholesterol values, found to be decreased in only high cholesterol group. It has been reported that HDL-cholesterol has a protective effect against atherosclerosis. HDL particles trap excess cholesterol from cellular membranes by esterification and transfer the esterified cholesterol to triglyceride - rich lipoproteins, which are subsequently removed by hepatic receptors. This reverse cholesterol transport from peripheral cells to the liver counteracts the deposition of cholesterol at sites where an excess cholesterol load produces atherosclerosis. HDL cholesterol levels would signify a high rate of reverse cholesterol transport and thus reduces the incidence of atherosclerosis (Fig 2).

Continuous feeding of high cholesterol results a significant increase in TBARS (Thio-barbituric Acid Reactive Substances). TBARS levels serve as an index of lipid peroxidation in biological systems. Lipid peroxides contribute to the initial endothelial cell damage that starts off the whole process e.g. by worsening haemodynamic change. Elevating lipid concentrations could lead to elevated lipid peroxide concentrations, contributing to endothelial injury and accelerating the whole process of atherogenesis. Simultaneous administration of enalapril maleate with high cholesterol shows comparatively less rise in TBARS level, than in the group only receiving high cholesterol diet(Fig 3). There is significant decrease in the catalase level in response to continuous feeding of high cholesterol diet. Simultaneous administration of enalapril maleate
with cholesterol does significantly change the level of catalase (Fig 4). Glutathione levels also decreased significantly in response to continuous feeding of high cholesterol as well as with simultaneous administration of enalapril maleate (Fig 5). Catalase reduces hydrogen peroxide to water and oxygen. Similarly, glutathione also reduces hydrogen peroxide to water through glutathione peroxidase. Hydrogen peroxide is responsible for the oxidation of lipids which is a crucial event in the pathophysiology of atherosclerosis. So, in order to detoxify the free radicals the body cells develop a defense mechanism to fight back the damage caused by these radicals. The defense mechanisms present in the body also include the catalase and glutathione system.

Histopathological examination of thoracic aorta shows, injury to the endothelium, proliferation of smooth muscle cells and cholesterol clefs, more mononucleated cells adhering to the endothelium in animals fed with cholesterol diet. Lesser changes were observed in rabbits treated with the drug-enalapril maleate (Fig 6-9). The drug-enalapril maleate may act by multiple mechanisms, namely antihypertensive effect which may also contributing to its antiatherosclerotic action. A lower blood pressure decreases the wall pressure which might be reducing the arterial permeability and decreasing lipoprotein entry into the intima or even possibly enhance cholesterol removal from there. It has been suggested that a significant portion of the hypotensive effect is also mediated by preventing the degradation of endogenous bradykinin. There is now evidence that ACE inhibitors enhance the basal synthesis of EDRF (Nitric Oxide) from cultured endothelial cells and potentiate its release upon stimulation with bradykinin. Nitric Oxide has also been shown to prevent proliferation of vascular smooth muscle cells and thus, it is interesting to speculate that the enhancement of NO production contributes to the effectiveness of ACE inhibitors not only as antihypertensive, but also by reversing the vascular changes that occur in hypertension and atherosclerosis.

CONCLUSION AND FUTURE OUTLOOK

The antioxidant activity of ACE Inhibitors seems to be responsible for its anti-atherosclerotic activity. There is need to explore other mechanisms for its anti-atherosclerotic activity as it is considered as a potent antihypertensive drug. It can reduce the complications associated with cardiovascular diseases.
Fig 3: Effect of Enalapril maleate on TBARS levels (µmoles/mg wet tissue) in hypercholesterolemic rabbits

Fig 4: Effect of Enalapril maleate on Catalase levels (units/mg protein) in hypercholesterolemic rabbits

Fig 5: Effect of Enalapril maleate on Glutathione levels (µmoles/mg protein) in hypercholesterolemic rabbits
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