Introduction/Review of Literature
Cardiovascular disease (CVD) is the main cause of disability and premature death worldwide, and is projected to remain the leading cause of death. An estimated 17.5 million people died from this disease in 2005, representing 30% of all global deaths. Of these deaths, 7.6 million were due to coronary heart disease (CHD) and 5.7 million because of stroke. If immediate and proper attention is not paid, by 2015 an estimated 20 million people will die from CVD, including stroke (WHO, 2005). Between 1990 and 2020 these diseases are expected to increase by 120% for women and 137% for men in developing countries as compared with 30–60% in developed countries (Murray and Lopez, 1997). Hence, this disease greatly contributes to the rising costs of health care in the world. It is a major public-health challenge, especially in low and middle income countries, where 80% of these deaths occur. South Asian (people who originate from India, Srilanka, Bangladesh and Pakistan) migrants to the United Kingdom, South Africa, Singapore and North America experience 1.5-4.0 times higher CHD mortality compared with indigenous populations (Enas et al., 1992). It has been projected that by the year 2010, 60% of the world’s patients with heart disease will be in India (Gaziano et al., 2006). Epidemiological studies show that there are at present 29.8 million patients with CHD in this country (Indrayan, 2004). Age standardized CVD death rates (per 100,000) in middle-aged subjects (30–69 years) are low in developed countries such as Canada (120) and Britain (180) and high in developing countries Brazil (320), China (280), Pakistan (400), Nigeria (410), Russia (680) and India (405) (WHO, 2005). Moreover, in India about 50% of CHD-related deaths occur in people younger than 70 years compared with only 22% in the West. In developing countries 94% of deaths from stroke occur in people aged, 70 years in contrast to 6% in developed countries (Gaziano et al., 2006). In the Indian urban population, the prevalence of hypertension and CVD is 3-4 times higher than in rural subjects. Coronary risk factors such as hypertension and high cholesterol levels were more prevalent in the urban subjects (Padmavati, 1962; Gupta and Malhotra, 1975; Chadha et al., 1990; Reddy et al., 2002). The WHO estimates that, over the next 10 years, India will lose 237 billion USD due to heart disease, stroke, and diabetes (WHO, 2005).
1.1 Hypercholesterolemia and CHD

It is well known that high levels of plasma cholesterol contribute to the development of atherosclerosis and thereby enhance the risk for coronary heart disease (CHD). This effect usually is mediated through the plasma low density lipoproteins (LDL), which appear to be the most important atherogenic lipoprotein. In addition, elevated LDL-cholesterol and decreased high density lipoprotein (HDL) cholesterol plasma levels have been independently associated with an increased risk for premature CHD in man (Barr et al., 1951; Miller and Miller, 1975; Rhoads et al., 1976). Subjects with strikingly elevated LDL cholesterol concentrations, such as patients with homozygous familial hypercholesterolemia in the untreated state, frequently develop severe CHD by age 15 yr, and are often dead by age 35 yr (Khachadurian and Uthman, 1973). Individuals with familial hyperalphalipoproteinemia (elevated HDL levels) appear to experience enhanced longevity (Glueck et al., 1975). Therefore, the relationship between high levels of LDL and atherosclerotic disease is illustrated best in the genetic disorder called familial hypercholesterolemia (Goldstein and Brown, 1983) but it is also revealed by differences in LDL concentrations between or within populations that have different levels of plasma LDL. Although genetic factors undoubtedly affect the plasma concentrations of LDL, dietary factors also play a role.

1.1.1 Regulation of LDL metabolism

The basic steps in the metabolism of LDL are outlined in Fig. 1.1. Panel (a). The liver secretes a triglyceride-rich lipoprotein called very low density lipoprotein (VLDL). As this lipoprotein passes into the peripheral circulation, it comes into contact with an enzyme, lipoprotein lipase, which is located on the surface of capillary endothelial cells. This enzyme hydrolyzes the triglycerides leaving a particle called a VLDL remnant, which returns to the circulation. VLDL remnants can have two fates. They can either be taken up directly by the liver or they can be degraded further to LDL. The LDL are the major cholesterol carrying lipoproteins of plasma. They are rich in cholesterol esters and contain very little triglyceride. They filter readily into the arterial wall and thereby can promote the development of atherosclerosis. Most LDL are removed by the liver (Bilheimer et al., 1984) although lesser quantities are cleared by other tissues. The primary mechanism for
clearance of LDL is the LDL receptor (Goldstein and Brown, 1983). This receptor is a protein located on the surface of cells, particularly hepatocytes. LDL receptors bind circulating LDL and the resulting receptor-LDL complex is internalized into lysosomes of cells where the LDL is degraded. Normally, two-thirds to three-fourths of circulating LDL are removed via the receptor pathway (Kesaniemi et al., 1983); the remainder is cleared by a nonspecific nonreceptor pathway. The activity of LDL receptors thus is the major factor determining the level of the plasma LDL.

1.1.2 Causes of hypercholesterolemia

An elevation of the plasma cholesterol usually is due to an increase in the level of LDL. As the plasma cholesterol level increases, so does the risk for CHD. The National Institutes of Health Consensus Conference on Cholesterol (NIH, 1985) defined hypercholesterolemia as a plasma cholesterol exceeding 240 mg/dl. At this level, the risk for CHD is about twice that of a cholesterol level < 200 mg/dl (Neaton et al., 1984). Approximately 40% of patients with CHD have cholesterol levels > 240 mg/dl (Neaton et al., 1984). Most of the remainder of patients with CHD have cholesterol concentrations in the range of 200-240 mg/dl (NIH, 1980). Without little doubt, cholesterol levels in this range, compared with levels < 200 mg/dl, accelerate the development of atherosclerosis and contribute to the high rate of CHD in populations such as the US. But further, many patients with premature CHD, who have cholesterol levels in the range of 200-240 mg/dl, have other risk factors—smoking, hypertension, obesity, or abnormalities in metabolism of triglycerides, often reflected by reduced concentrations of HDL. Nonetheless, even in this group, moderately high concentrations of plasma cholesterol (and LDL) are a contributing factor to CHD (Neaton et al. 1984). The major cause of a high level of LDL is a reduced activity of LDL receptors (Fig. 1.1. Panel (b)). When the numbers of receptors are decreased, two changes in the metabolism of LDL occur. First, the clearance of LDL itself is decreased; but second, because VLDL remnants also are cleared by LDL receptors, more VLDL remnants also are converted to LDL. Normally, one functional gene for LDL receptors is inherited from each parent and LDL levels are kept relatively low by the activity of both genes. However, in one of 500 people, one gene is abnormal and the number of functioning LDL receptors is only half normal. Concentrations of LDL consequently are roughly doubled by the
Fig. 1.1. **Panel (b): Metabolic consequences of a decreased activity of low density lipoprotein (LDL) receptors.** The fractional clearance rates of both very low density lipoprotein (VLDL) remnants and LDL are reduced. This leads to enhanced conversion of VLDL remnants to LDL (overproduction of LDL). Both changes increase LDL levels. (Adapted from Grundy, S. M. 1987. Disorders of lipids and lipoproteins. In: Stein, J. H. 2nd ed. Internal Medicine. Boston, M. A.: Little, Brown and Company. 302: 2046).
mechanisms shown in Fig. 1.1. Panel (b). This condition is called heterozygous familial hypercholesterolemia (FH). Patients with heterozygous FH are prone to develop tendon xanthomas and to have premature CHD. Although FH is a dramatic form of hypercholesterolemia, most individuals with cholesterol levels > 240 mg/dl do not have this genetic disorder. The causes of such levels have not been determined with certainty but both genetic and dietary factors probably are involved. The majority of patients with hypercholesterolemia seemingly have a reduced activity of LDL receptors even though they do not have a primary defect in the structure of the genes encoding for LDL receptors (Grundy and Vega, 1985). An important discovery was the finding that the synthesis of LDL receptors is under feedback control by amounts of cholesterol in cells (Goldstein and Brown, 1977).

For example, when concentrations of cholesterol in liver cells increase, the formation of LDL receptors is suppressed and plasma levels of LDL rise. Thus, any factors that will increase the liver’s content of cholesterol should suppress the activity of LDL receptors. One cause for an excess of hepatic cholesterol could be an inherited hyperabsorption of cholesterol. Another could be a defect in the conversion of cholesterol into bile acids in the liver. Other genetic defects, which are poorly understood at present, likewise may lead to accumulation of cholesterol in liver cells and thus reduce the activity of LDL receptors.

Still another factor that appears to modulate the activity of receptors is the diet. Two dietary constituents that suppress receptor activity are saturated fatty acids and cholesterol (Fig. 1.1. Panel (c)). Dietary cholesterol suppresses the activity of LDL receptors by the mechanisms described above, i.e. by increasing the hepatic content of cholesterol. The saturated fatty acids appear to act by a similar mechanism. In laboratory animals, they have been shown to inhibit receptor-mediated uptake of LDL (Spady and Dietschy, 1982). The intracellular mechanisms responsible for this action have not been determined. They may act on either the metabolism of cholesterol or on the function of receptors on the surface of cells. Still another dietary factor that can affect the plasma level of LDL is the total caloric intake. When the intake of calories is high, especially in overweight patients, the production of VLDL is stimulated (Egusa et al., 1985). Because VLDL is a precursor of LDL, an overproduction of VLDL can increase the concentration of LDL. This is
Fig. 1.1. Panel (c): The activity of low-density lipoprotein (LDL) receptors appears to be suppressed by both saturated fatty acids and cholesterol in the diet. The consequence is an enhanced conversion of very low density lipoprotein (VLDL) remnants to LDL and a delayed removal of LDL. Both increase the LDL concentration. (Adapted from Grundy, S. M. 1986. Cholesterol and coronary heart disease: a new era. JAMA. 256: 2849-58).
especially true when the activity of LDL receptors has been suppressed by dietary saturated fatty acids and cholesterol.

1.1.3 Diet and epidemiology of CHD

Diet has long been suspected of being a factor in development of CHD. Coronary disease is particularly common in affluent countries and one obvious difference between these countries and poorer nations is the diet. In populations where CHD rates are high, the diets tend to be rich in total fat, saturated fatty acids, and cholesterol. At the same time, plasma cholesterol levels are relatively high in affluent countries and higher levels can be attributed mainly to dietary factors. Certainly obesity and lack of exercise contribute to higher concentrations of cholesterol but dietary saturated fat and cholesterol appear to be even more important determinants of the cholesterol level. A major investigation relating intakes of saturated fatty acids to the plasma cholesterol (and CHD rates) was the Seven Countries Study (Keys, 1970). This was a major epidemiological study that examined the associations among diet, plasma cholesterol, other risk factors, and CHD rates in several different populations within seven countries including southern and northern Europe, the US, and Japan. The dietary factor most closely correlated with higher concentrations of plasma cholesterol and rates of CHD was the level of intake of saturated fatty acids. Many other epidemiological studies are consistent with this result. The high prevalence of CHD in eastern Finland, where intakes of butter products are particularly high, is a striking example of this phenomenon. In the Seven Countries Study (Keys, 1970), the other nutrients of the diet appeared to be less important for determining either the cholesterol level or coronary rates. For example, in Crete, the diet contained 40% of calories as fat, but most of this fat came from olive oil; and CHD rates in Crete were very low. A similarly low rate of CHD was found in Japan where the diet is very low in total fats. Thus, on the basis of epidemiological studies, the only nutrients that have been strongly correlated with CHD rates are dietary saturated fatty acids and possibly dietary cholesterol. The numerous epidemiological surveys thus strongly implicate intakes of saturated fatty acids in the causation of CHD. These findings have led many investigators and agencies to recommend that high-risk populations reduce their intakes of saturated fatty acids. With this recommendation usually goes the suggestion that dietary cholesterol be reduced.
1.2 Cholesterol-Induced Oxidative Stress-Linked Hypercholesterolemia and CHD

Hypercholesterolemia is one of the most important risk factors for atherosclerosis and subsequent cardiovascular disease (Steinberg, 2002). Diets high in fat, saturated fat, and cholesterol have been shown to cause significant elevations in plasma cholesterol, while diets low in these constituents cause a decrease in cholesterol levels in man (Schaefer et al., 1981). Feeding animals with cholesterol has often been used to elevate serum or tissue cholesterol levels to study the etiology of hypercholesterolemia-related metabolic disturbances (Bocan, 1998). The rabbit is susceptible to the development of atherosclerosis, whereas rats and mice are considered to be resistant to induction of hypercholesterolemia by cholesterol feeding (Stehbens, 1986). However, hypercholesterolemia can be produced in rats by feeding cholic acid along with a cholesterol rich diet, but this treatment does not result in atherosclerotic lesions in the rat (Beynen et al., 1986). Studies in animals (Boccio et al., 1990; Smith et al., 1985; Thiery and Seidel, 1987; Tsai and Kelley, 1978; Uysal, 1986; Uysal et al., 1988) and humans (Uzel et al., 1985; Yalcin et al., 1989) have shown that there is a close relationship between lipid peroxidation and hypercholesterolemia and/or hypercholesterolemic atherosclerosis. Oxysterols represent one of the primary proatherogenic components of a cholesterol rich diet (Staprans et al., 1998; Rong et al., 1998; Vine et al., 1998). Cholesterol per se has little atherogenic or cytotoxic effect, as shown by venous infusion (Imai and Nakamura, 1980; Peng et al., 1985) and in vitro studies, when compared with oxysterols (Taylor et al., 1979; Peng et al., 1991). The barrier function of endothelial cells in culture was not significantly affected when exposed to cholesterol-enriched low density lipoproteins (LDL), whereas triol (cholestane-3β, 5α, 6β-triol)-enriched LDL resulted in a significant loss of barrier function (Boissonneault et al., 1991) indicating that oxysterols rather than cholesterol promoted atherogenesis. Rabbits fed a concentrate of oxysterols by gastric gavage showed arterial wall damage within 24 hr, while atheromatous lesions developed with prolonged feeding (Imai et al., 1976). Exogenous hypercholesterolemia causes fat deposition in the liver and depletion of the hepatocyte population; it can also cause malfunctioning of the liver, which apparently follows microvesicular stenosis due to the intracellular accumulation of lipids (Gupta et al., 1976; Assy et al., 2000).
The liver is an essential organ in the removal of excess cholesterol from the blood circulation for excretion into the bile (Glomset, 1980; Brown and Goldstein, 1986). The liver regulates its intra-hepatic cholesterol homeostasis, by maintaining an appropriate balance between the regulatory free cholesterol and the more inert cholesterol ester pool. The liver consists of different cell types: parenchymal, endothelial, and Kupffer cells that may exert different functions in the hepatic flux of cholesterol. Parenchymal liver cells are located between bile canaliculi and blood sinuses, and mediate chylomicron and VLDL remnant and HDL cholesterol ester removal from the blood circulation (Out et al., 2004; Pieters et al., 1991). Additionally, parenchymal liver cells perform the biliary efflux of cholesterol and its conversion products, bile acids (Frimmer and Ziegler, 1988). Hepatic endothelial cells line the blood sinuses, where they function in the removal of modified lipoproteins from the blood circulation and mediate their natural barrier function. Kupffer cells are tissue macrophages located within the blood sinuses of the liver. They mainly function in the removal of bacteria and in the clearance of modified lipoproteins from the circulation (Stenback et al., 2002; Rijke and Berkel, 1994). In addition, feeding cholesterol rich diets induces free radical production, followed by oxidative stress and hypercholesterolemia (Stehbens 1986; Bulur et al., 1995). Oxidative stress, which results from impairment of the equilibrium between production of free radicals and antioxidant defense systems, is one of the factors that links hypercholesterolemia with atherogenesis (Halliwell, 1996). Thus, there is evidence that oxidative stress contributes to the development of atherosclerosis in the vascular wall through the formation of reactive oxygen species (ROS) (Shi et al., 2000). In order to protect the tissues from damage caused by ROS, organisms possess enzymatic and non-enzymatic antioxidant systems (Parthasarathy et al., 2000). Protection against ROS and the breakdown products of peroxidized lipids and oxidized proteins is provided by enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (Gpx) and glutathione-S-transferase (GST). Nonenzymatic antioxidants such as reduced glutathione (GSH), vitamins C and E, play a vital role in protecting cells from oxidative stress by participating in various biochemical pathways. Erythrocytes are perforce in an environment in which they are constantly exposed to both extracellular and intracellular sources of ROS. Hypercholesterolemia leads to increased cholesterol accumulation in the
erythrocytes and endothelial cells thereby activating and enhancing them to produce oxygen free radicals (Prasad and Kalra, 1989; Kay, 1991). Thus, erythrocytes are extremely vulnerable to these prooxidant challenges and hypercholesterolemia. Osmotic fragility, the sensitivity of erythrocytes to changes in osmotic pressure, is elevated during oxidative stress and also in various pathological conditions.

1.2.1 Oxidative stress in human erythrocytes

Reactive oxygen species are of increasing interest as agents of pathologic states including atherosclerosis, hypertension, Parkinson's disease, nephropathy, inflammatory arthritis and diabetes (Durak et al., 2001a; Durak et al., 2001b; Cimen et al., 2000; Buyukkocak et al., 2000). In most cells, mitochondria are major source of ROS (Johnson et al., 2005). Despite their lack of mitochondria, ROS are continuously produced in the red cells due to the high O\textsubscript{2} tension in arterial blood and their abundant heme iron content (Baynes, 2005). Various factors lead to generation of oxidizing radicals such as O\textsubscript{2}•−, H\textsubscript{2}O\textsubscript{2}, HO• in erythrocytes (Dumaswala et al., 1999). The source of ROS in erythrocytes is the oxygen carrier protein hemoglobin (Hgb) that undergoes autoxidation to produce O\textsubscript{2}•−. Since the intraerythrocytic concentration of oxygenated Hgb (oxyHgb) is 5 mM, even a small rate of autoxidation can produce substantial levels of ROS. Occasional reduction of O\textsubscript{2} to O\textsubscript{2}•− is accompanied by oxidation of Hgb to met hemoglobin (metHgb), a rustbrown-colored protein that does not bind or transport O\textsubscript{2}. Although oxidative stress may damage the red cell itself, the mass effect of large quantities of ROS leaving the red cell have a tremendous potential to damage other components of the circulation (Johnson et al., 2005). Thus, it is of special interest to determine the extent of this oxidant challenge and ROS balance in the erythrocyte. As a consequence of their physiologic role, erythrocytes are exposed to continuous oxidant stress. Although the normal red cell reducing capacity is greater than 250 times its oxidizing potential several erythrocyte abnormalities have been identified that circumvent or overwhelm the erythrocyte oxidant defense system. Complex aerobic organisms have assured an adequate and continuous flow of oxygen to their tissues, while simultaneously protecting themselves from the inherent toxicity of oxygen. This occurs by two mechanisms: oxygen-carrying proteins, i.e., Hgb, and oxidant defense systems (Scott et al., 1989). Polyunsaturated fatty acids within the membrane, an oxygen rich environment, and iron rich Hgb make reds cells...
susceptible to peroxidative damage (Claster et al., 1984). Reactive oxygen species initiate lipid peroxidation reactions that lead to loss of membrane integrity and cell death (Baynes, 2005). Malondialdehyde (MDA), a highly reactive bifunctional molecule, is an end product of membrane lipid peroxidation. Malondialdehyde has been shown to cross-link erythrocyte phospholipids and proteins. This process results in impairment of the membrane related functions that ultimately leads to diminished survival. MDA accumulation can affect the anion transport and function of the band 3 associated enzymes, glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase (Dumaswala et al., 1999). Several reports have documented that in vitro exposure to oxidants increases erythrocyte membrane instability by damaging protein band 4.1 and forming a defective spectrin-band 4.1-actin tertiary complex. Membrane-bound proteinases, the secondary antioxidant defense mechanism, protect erythrocytes by preferentially degrading oxidatively damaged proteins (Dumaswala et al., 1999). Although many membrane components are possible targets for oxidants, calcium ATPase may be of crucial importance for the survival of red cells. Ca-ATPase contains one or more reactive sulphydryl groups that are susceptible to oxidation with resultant loss of enzyme activity. Because this enzyme is instrumental in maintaining the very steep gradient between extracellular and intracellular calcium, loss of activity is associated with decreased red cell deformability and premature destruction (Shalev et al., 1981).

1.2.2 Formation of ROS from molecular oxygen

As depicted in Fig. 1.2, the addition of one electron to O$_2$ produces the superoxide anion radical (O$_2^•$−) (Al-Omar et al., 2004). Oxygen is implicated as a potential source of oxidative damage and as a mediator of oxidative hemolysis in numerous studies (Scott et al., 1989). At least two sources of O$_2^•$− generation within red cell have been identified. First, oxyHgb autoxidizes at a relatively slow rate to yield metHgb and O$_2^•$− (Giulivi and Daviess, 1990). Second, the oxidation state of hemicrom iron (Fe$^{+3}$) indicates that an electron has been lost during its formation and, therefore, that O$_2^•$− has probably been generated or has been derived from exogenous sources, i.e., drugs (Hebbel et al., 1982). This reactive species is capable of attacking the red cell membrane directly and causing alterations in lipid and protein structure (Claster et al., 1984).

\[
O_2^•− + O_2^•− + 2H^+ \rightarrow H_2O_2 + O_2
\]
Fig. 1.2. Pathways of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and enzymatic antioxidants in the management of oxidative stress in erythrocytes and liver.
As seen from the above equation, dismutation of \( \text{O}_2 \) will readily generate excessive amounts of \( \text{H}_2\text{O}_2 \) (Fig. 1.2) (Hebbel et al., 1982). \( \text{H}_2\text{O}_2 \) can cross cell membranes almost as readily as water while the charged \( \text{O}_2^{•−} \) can cross membranes only via transmembrane anion channels. \( \text{H}_2\text{O}_2 \), is not especially toxic to the cell macromolecules, but it can pass through membranes and this feature is potentially important because the extracellular environment possesses few antioxidant defense mechanisms (Al-Omar et al., 2004). \( \text{O}_2^{•−} \) generating agents may indirectly produce metHgb by generation of \( \text{H}_2\text{O}_2 \), in the course of normal cellular events (Scott et al., 1989; Scott et al., 1991). OxyHgb undergoes a slow autooxidation, producing \( \text{O}_2^{•−} \), which yields \( \text{H}_2\text{O}_2 \). Therefore, Hgb is constantly exposed to an intracellular flux of \( \text{H}_2\text{O}_2 \) as well as to an extracellular flux, due to the high permeability of this metabolite. Exposure of oxyHgb to \( \text{H}_2\text{O}_2 \) leads to oxidative modifications that have been proposed as selective signals for proteolysis in erythrocytes (Giulivi and Davies, 2001). As \( \text{H}_2\text{O}_2 \) concentration is increased, a dose-dependent increase in metHgb, lipid peroxidation, and spectrin-Hgb complexes are seen. Peroxidation, which results in globin cross-linking to any one or all of these interrelated proteins, such as spectrin and band 3, may lead to a decreased deformability, as well as morphologic and surface changes in the erythrocyte. Snyder et al. (1985), demonstrated that \( \text{H}_2\text{O}_2 \) induces a covalent complex of spectrin and Hgb as well as a myriad of cellular changes that include alterations in cell shape, membrane deformability, phospholipid organization, and cell surface characteristics.

\[
\begin{align*}
\text{O}_2^{•−} + \text{H}_2\text{O}_2 &\rightarrow \text{O}_2 + \text{OH}^- + \cdot\text{OH} \quad \text{(Haber–Weiss Reaction)} \\
\text{Fe}^{++} + \text{H}_2\text{O}_2 &\rightarrow \text{Fe}^{+++} + \text{OH}^- + \cdot\text{OH} \quad \text{(Fenton Reaction)}
\end{align*}
\]

Hydrogen peroxide can react with \( \text{O}_2^{•−} \) and ferric or cupric ions to produce \( \cdot\text{OH} \), the most active ROS (Fig. 1.2). This arises from a much higher reduction potential in comparison to other ROS. As a result of its reactivity, the \( \cdot\text{OH} \) does not travel far due to its half-life of a few nanoseconds (Al-Omar et al., 2004; Yan et al., 2005). The mechanism of red cell \( \cdot\text{OH} \) generation is, however, not as straightforward (Hebbel et al., 1982). Due to its charge, \( \text{O}_2^{•−} \) is concentrated in the intracellular compartment. As such, \( \cdot\text{OH} \) is produced predominantly from \( \text{H}_2\text{O}_2 \) by
Haber–Weiss reactions whereas the Fenton reaction is more important extracellularly (Al-Omar et al., 2004).

1.2.3 Iron redox status and oxidative stress in erythrocytes

Iron is the most abundant, important, and essential transition metal in biochemical reactions. A heterogeneous group of proteins contain iron in a variety of molecular forms (Volpe, 1993). Iron not only binds oxygen reversibly but also participates in a number of vital oxidation–reduction reactions. To bind oxygen, heme iron must be maintained in the reduced state. If these mechanisms fail, Hgb becomes non-functional (Telen and Kaufman, 1999). As a result of this process, iron is released from Hgb (or its derivatives) and the release is accompanied by metHgb formation. If erythrocytes are depleted of GSH, the release of iron is accompanied by lipid peroxidation and hemolysis (Comporti et al., 2002). Iron levels in the cell must be delicately balanced, as iron loading leads to free radical damage. Copper and iron cations present in some toxic material can promote ROS formation by Fenton reaction which occurs when excess iron reacts with $H_2O_2$ to generate $^*OH$ (Durak et al., 1999; Church and Pryor, 1985). To achieve appropriate levels of cellular iron and to avoid iron-loading, transport, storage and regulatory proteins have evolved (Dunn et al., 2007). Iron released from its storage macromolecules represents the source of iron-catalyzed oxidative stress, such as lipid, protein and DNA oxidation. It is also believed that such processes occur not only in pathological but also in physiological conditions such as those regulating the signal transduction pathways (Comporti et al., 2002). Because of the abundance of $O_2$ in aqueous media, ferrous ion autoxidation may be an important route for initiation of free radical oxidation. This reaction would result in the formation of Fe–O complexes, named as ferryl or perferryl ions. Due to their high electron affinity, these ions would have reactivities approaching those effects of $^*OH$ (Qian and Buettner, 1999). Ferryl species are strong oxidants for several biomolecules including vitamin E, vitamin C, cholesterol, catecholamines, lipoproteins, and membrane lipids (Giulivi and Davies, 2001).

1.2.4 Cellular antioxidant defense systems against ROS

The human erythrocyte, due its role as $O_2$ and $CO_2$ transporter, is under constant exposure to ROS and oxidative stress. Oxidative stress occurs in cells or tissues when ROS concentration exceeds antioxidant protection (Al-Omar et al.,
2004). Extracellular antioxidant capacity and reduction of extracellular oxidants allows erythrocytes to respond to stress. The mobility of the erythrocyte makes it an ideal antioxidant not only for its own membrane and local environment, but also as an oxidant scavenger throughout the circulation. Although \( O_2 \) can act as an electron acceptor for transmembrane redox reactions in some cell lines and may be a physiological electron acceptor, red cells do not possess this activity. Red cells from newborns, especially premature infants, have previously been shown to be more sensitive to peroxidative damage \textit{in vitro} than adults, due in part to deficiencies of antioxidant capacity (Claster et al., 1984). Oxidant/antioxidant equilibrium can change in the erythrocyte in several diseases. Erythrocytes are exposed to high oxidant stress may result in accelerated peroxidation reactions and cellular aberration (Durak et al., 2001a). Protective mechanisms exist to scavenge and detoxify ROS, block production, or sequester transition metals (Masella et al., 2005). The antioxidant system consists of enzymatic and nonenzymatic pathways in human red cells (Fig. 1.2).

1.2.4.1 Enzymatic antioxidants

Enzymes for preventing oxidative denaturation in erythrocytes include SOD, CAT, Gpx, GSH reductase-dependent regeneration of GSH, and NADH–metHgb reductase (Telen and Kaufman, 1999; Scott et al., 1989).

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2
\]

Superoxide anion is converted to \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) by SOD a ubiquitous metal-containing enzyme (Fig. 1.2) (Al-Omar et al., 2004; McCord and Fridovich, 1969).

\[
2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{Gpx}} 2\text{H}_2\text{O} + \text{GSSG} \]

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} 2\text{H}_2\text{O} + \text{O}_2
\]

Hydrogen peroxide is produced by normal metabolic pathways. Two enzyme systems exist to catalyze \( \text{H}_2\text{O}_2 \) (Fig. 1.2) and are present at high activity in human red cells (Scott et al., 1991). Low levels of \( \text{H}_2\text{O}_2 \) (\( 10^{-9} \text{ M} \)) are removed by GSH to form oxidized glutathione (GSSG) and water, a reaction catalyzed by Gpx (Al-Omar et al., 2004). Because the direct reaction between \( \text{H}_2\text{O}_2 \) and GSH is very slow, Gpx
reduces $H_2O_2$ by oxidizing GSH to GSSG. Cytoplasmic, gastrointestinal and lipophilic enzymes are able to reduce hydroperoxides of complex lipids in membranes. Isoenzymes are also present in red blood cells (Jacobasch, 2000). The catalytic activity of CAT appears to be a special case of peroxidase activity in which the electron donor is a second molecule of $H_2O_2$. The mechanism of CAT action is similar to SOD, wherein one molecule of $H_2O_2$ is reduced to water and the other oxidized to oxygen (Fig. 1.2) (Al-Omar et al., 2004; Gunduz et al., 2004). That CAT and SOD react synergistically to protect each other was observed earlier in hemolysis studies of erythrocytes (Kellogg and Fridovich, 1977). Gaetani et al. (1989), demonstrated that CAT and Gpx are equally active in the detoxification of $H_2O_2$ in normal erythrocytes. Nonetheless, several researchers found that the GSH-dependent activity of Gpx has been generally viewed as the primary defense against $H_2O_2$ in erythrocytes (Scott et al., 1991). The data from the Gpx-deficient red cells clarify the issue of intraerythrocytic Hgb oxidation and $H_2O_2$ generation (Johnson et al., 2005). In conclusion, Gpx are important for dealing with the endogenous $H_2O_2$ produced by Hgb autoxidation, while CAT plays an increasingly important role as the erythrocyte is exposed to increased $H_2O_2$ flux. One might therefore anticipate that elevated SOD activity would be protective against $O_2^{•−}$ generating agents. However, red cells with 5 to 9-fold increased activity showed no enhanced resistance to $O_2^{•−}$ generators. Consequently, $O_2^{•−}$ is more likely to reduce metHgb to regenerate oxyHgb. Indeed, metHgb levels were slightly higher in SOD-loaded erythrocytes, indicating perhaps that $O_2^{•−}$ mediated reduction of metHgb was inhibited. Consequently, it is difficult to distinguish the specific roles of the various antioxidants in protecting the erythrocyte from oxidant stress (Scott et al., 1989). Glutathione-S-transferases play a major role in detoxification of electrophilic xenobiotics such as herbicides, insecticides, chemical carcinogens, and other organic and inorganic environmental pollutants. These enzymes catalyze the conjugation of GSH with exogenous and endogenous toxic compounds or their metabolites, rendering them more water soluble, less toxic, and easier to excrete. In addition, they are responsible for various resistance mechanisms including chemotherapeutic or antibiotic drug resistance (Rhee et al., 2007).
1.2.4.2 Nonenzymatic antioxidants

Endogenous non-enzymatic antioxidants are defined in two phases: lipophylic (vitamin E, carotenoids, ubiquinon, melatonin, etc.) and water soluble (vitamin C, glutathione, uric acid, ceruloplasmin, transferin, haptoglobin, etc.). Three antioxidant vitamins, A, C, and E, provide defense against oxidative damage. Vitamin C acts in the aqueous phase whereas vitamin E acts in the lipid phase as a chain breaking antioxidants. Vitamin C reduces $O_2^{\bullet-}$ and lipid peroxyl radical, but is also a well-known synergistic agent for vitamin E (Baynes, 2005; Bıelski and Cabelli, 1991). It exists as the enolate anion at physiologic pH. Dehydroascorbate, formed by a second reduction or dismutation reaction, is recycled by dehydroascorbate reductase, a GSH dependent enzyme. Dehidroascorbyl radical may also dismutate to ascorbate and dehydroascorbate (Baynes, 2005). It has been shown to play a protective role against peroxidation of erythrocyte membrane lipids and tocopherols by t-butylhydroperoxide, preserving lipids by up to 92% and vitamin E by 50% and 65%, respectively (Kennett and Kuchel, 2003). Vitamin E is the most widely distributed antioxidant in nature. When vitamin E donates an electron to a lipid peroxyl radical, it is converted to free radical stabilized by resonance structure (Smith et al., 2005). The enolate anion reduces $O_2^{\bullet-}$, organic and tocopheroxy radicals, forming a dehidroascorbyl radical. Vitamin C and E work together to inhibit lipid peroxidation reactions in plasma lipoproteins and membranes. Vitamin A, a potent free radical scavenger, is a lipophylic antioxidant (Baynes, 2005). Carotenoids, the precursor of vitamin A, can exert antioxidant effects, as well as quench singlet $O_2$. There is considerable in vitro evidence for interaction of β-carotene with free radicals, for its properties as a chain-breaking antioxidant and in scavenging and quenching singlet oxygen (Miller et al., 1996). In all cell types, γ-glutamylcysteinylglycine is the most important nonenzymatic regulator of intracellular redox homeostasis. Several recent studies have shown that red cells are important as biological carriers of GSH by de novo synthesis and as such appear to provide an important detoxifying system within the circulation (Dumaswala et al., 2001; Sharma et al., 2000). GSH synthesis in erythrocytes is limited by the availability of the substrate amino acids, especially cysteine (Masella et al., 2005; Dumaswala et al., 2001). It is synthesized by two ATP-dependent reactions, catalyzed by γ-glutamylcysteine synthetase and GSH synthetase. Defects
of both enzymes are rare, but in severe cases, hemolytic anaemia and neurological abnormalities occur (Jacobasch, 2000). Glutathione exists either in reduced GSH or oxidized GSSG forms and participates in redox reactions by the reversible oxidation of its active thiol. GSH may covalently bind to proteins through a process called glutathionylation and can act as a coenzyme for various cell defense enzymes (Pompella et al., 2003). It can thus directly scavenge free radicals or act as a substrate for Gpx and GST during the detoxification of \( \text{H}_2\text{O}_2 \), lipid hydroperoxides and electrophilic compounds (Masella et al., 2005). In erythrocytes the major antioxidant is GSH which protects important proteins such as spectrin the oxidation of which can lead to increased membrane stiffness (Carroll et al., 2006). GSH not only supports antioxidant defense, but is also an important sulfhydryl buffer, maintaining –SH groups in Hgb and enzymes in the reduced state (Baynes, 2005). Dumaswala et al. (2001), observed that in vitro augmentation of the erythrocyte endogenous antioxidant reserve, especially GSH, provides protection against cell damage induced by oxidative stress. A higher demand for GSH causes increased degenerative oxidative modifications of proteins or lipids.

1.2.4.3 Exogen antioxidants

Several exogen compounds such as inhibitors of NADPH-Oxidase, allopurinol, and flavonoids have antioxidant properties. It is known that flavonoids are good exogen antioxidants against free radical initiated lipid peroxidation in human red cells and that the antioxidant activity of flavonoids depends significantly on molecular structure and initiation conditions (Hou et al., 2004). Many studies have suggested that flavonoids exhibit biological activities, including anti-allergenic, anti-viral, anti-inflammatory, and vasodilation (Seyoum et al., 2006). The capacity of flavonoids to act as antioxidants in vitro has been the subject of several studies that demonstrate their structure-activity relationships. Most ingested flavonoids are extensively degraded to various phenolic acids, some of which still possess a radical-scavenging ability (Sroka and Cisowski, 2003; Chaudhuri et al., 2007). Absorbed flavonoids and their metabolites may display an in vivo antioxidant activity as demonstrated by increased plasma antioxidant status, the sparing effect on vitamin E of erythrocyte membranes and preservation of erythrocyte membrane polyunsaturated fatty acids (Pietta, 2000; Teixeira et al., 2005).
1.3 Changes in Lipid and Lipoprotein Metabolism in Hypercholesterolemia

Major cardiovascular disease risk factors such as hypercholesterolemia, hypertension, diabetes and smoking are known. Dietary cholesterol is regarded as an important factor in the development of atherosclerosis and subsequent cardiovascular disease. Exogenous hypercholesterolemia causes fat deposition in the liver and depletion of hepatocyte population. It causes the malfunction of liver, which is apparently presented through microvesicularstenosis due to the intracellular accumulation of lipids (Gupta et al., 1976; Assy et al., 2000). Feeding of cholesterol diet produces severe hypercholesterolemic and vascular atherosclerotic lesion and increased oxidative stress in several tissues (Balkan et al., 2002). Recent findings support the hypothesis that oxidative stress is one of the causative factors that link hypercholesterolemia with the pathogenesis of atherosclerosis (Young and McEneny, 2001). In hypercholesterolemia, the cholesterol content of erythrocyte, platelets, polymorphonuclearleucocyte and endothelial cells increases, which is reported to activate these cells and cause the enhanced production of oxygen free radicals leading to tissue injury (Prasad and Kalra, 1993). It is known that the oxidative stress derives from disequilibrium between the oxidant and antioxidant components, which may be of enzymatic and non-enzymatic type. Such disequilibrium may be due to an excessive increase of free radicals and/or reduction of the defensive mechanisms. The free radicals may favor aging through their damaging actions on various substrates, which have fundamental biological functions, and favoring pathological processes by pathogenetic stimulation.

The cholesterol rich diet is able to cause tissue oxidative damage at various levels and contributes significantly to the appearance of endothelial dysfunctions, and to the alterations which induce the arteriosclerotic process (Zou et al., 2003). As a matter of fact, one can observe in hypercholesterolemia an increase of lipid peroxidation products, particularly important is the increase of oxidized LDL (Bentley et al., 2002), there are several reports demonstrating that the oxidation of LDL may trigger many proatherogenic properties in vivo and significant increase in plasma TG, TC, VLDL-C and LDL-C with a decrease in antiatherogenic HDL-C and it’s components HDL₂-C as well as HDL₃-C was reported (Lee et al., 2007; Chenni et al., 2007; Bouderbala et al., 2008). On the other hand, Amold et al. (1992)
showed that feeding 1% cholesterol to rats for 8 weeks did not alter their serum susceptibility to lipid peroxidation compared with rats fed a cholesterol-free diet.

There has been increasing evidence of an association between TG and increased risk of Cardiovascular disease (Austin, 1998), a risk that is especially high in subjects with low HDL-C (Castelli, 1992). Hypertriglyceridemia, due to an increase in VLDL, is commonly observed and is due to both enhanced hepatic production of VLDL and decreased triglyceride clearance (Mackinnon et al., 1985). The stimulation of hepatic VLDL production is due to increased delivery of fatty acids to the liver from adipose tissue, increased hepatic de novo fatty acid synthesis, and decreased fatty acid oxidation in the liver (Khovidhunkit et al., 2004). The increase in hepatic TG-rich particles production is secondary to an increase in re-esterification of plasma fatty acid arising from both enhanced lipolysis and increased fatty acid synthesis in the liver (Hardardottir et al., 1994; Feingold et al., 1989; Grunfeld et al., 1990; Hellerstein et al., 1993; Patton et al., 1986; Feingold et al., 1992; Feingold et al., 1990). Increased lipolysis results in a greater free fatty acid flux to the liver, thereby promoting lipoprotein VLDL secretion (Liu et al., 1998). It has been shown that cholesterol feeding increased secretion of β-VLDL and increased hepatic TG synthesis, which results in the accumulation of TG in the livers of rats and rabbits fed the high cholesterol diet (Mackinnon et al., 1985; Saito and Fillios, 1965). In humans, maintaining of cholesterol level is dependent on coordinated changes in the levels of mRNAs of key genes that are known to regulate cholesterol synthesis and cholesterol uptake from plasma (Horton et al., 2002). Up-regulation of low density lipoprotein receptor (LDLR) and down regulation of HMG-CoA reductase are the key mechanisms to control elevated plasma LDL-C (Marc et al., 2004). LDLR mainly regulates the exogenous cholesterol by removing more than 70% of human LDL-C from the blood circulation (Brown and Goldstein, 1997). Cholesterol synthesis is controlled by the regulation of genes encoded with cholesterol synthetic enzymes, especially the rate limiting enzyme HMG-CoA reductase which is down regulated in response to an increase in the cellular cholesterol concentration (Goldstein and Brown, 1990). A cholesterol rich diet results in increased lipid peroxidation by the induction of free radical production, followed by hypercholesterolemia (Wissler, 1992). Oxidative modification of LDL may be a key early step in the pathogenesis of atherosclerosis (Diaz et al., 1997). Evidence of
oxidized LDL has been obtained from arterial walls of animal models of atherosclerosis and of CHD patients (Haberland et al., 1988; Ylä-Herttuala et al., 1989).

Hypercholesterolemia is associated with a decrease in HDL cholesterol levels (Lee et al., 2007; Chenni et al., 2007; Bouderbala et al., 2008). The mechanism for the decrease in HDL cholesterol levels during hypercholesterolemia has not been firmly established. Nevertheless, a persistently low level of HDL-C in hypercholesterolemia suggests that this change may be undesirable, since data from epidemiologic studies have shown a greater risk of CAD in subjects with low HDL cholesterol levels. The function of HDL is mediated through a variety of apolipoproteins associated with HDL particles. Moreover, HDL metabolism is regulated by several enzymes and transfer proteins that can affect various functions of HDL. During hypercholesterolemia, not only do HDL-C levels decrease, but there are also a wide range of changes in these apolipoproteins, enzymes, and transfer proteins. As a result, there are marked changes in lipid and protein composition of HDL, which can theoretically lead to alterations in function of HDL (Khovidhunkit et al., 2004). Because of the above changes in HDL metabolism, it is postulated that the main functions of HDL, namely its role in protecting LDL against oxidation and reverse cholesterol transport (RCT), may be decreased during the hypercholesterolemia.

Reverse cholesterol transport (RCT) is a pathway by which cholesterol from peripheral cells is transported back to the liver for metabolism and/or excretion (Fielding and Fielding, 1995). This pathway is thought to play a key role in removing cholesterol from cells in atherosclerotic lesions, such as macrophage foam cells. The first step of RCT is cholesterol removal from cells, and two major mechanisms of cholesterol removal have been proposed: an apolipoprotein-mediated mechanism and a diffusion mechanism (Oram and Yokoyama, 1996). In the apolipoprotein-mediated mechanism, cholesterol is removed from plasma membrane of peripheral cells to small, lipid-poor preβ HDL particles. Recent data suggest that this step requires apoAI on HDL and an ATP-binding cassette transporter 1 (ABC1) on the cell surface. Free cholesterol can also move from plasma membrane to HDL by the diffusion mechanism. Lecithin cholesterol acyltransferase (LCAT) plays a key role by converting free cholesterol in HDL into cholesterol ester, thus
maintaining a free cholesterol gradient so that continuous diffusion of free cholesterol from plasma membrane to HDL can occur. Cholesterol ester generated by LCAT then moves into the core of HDL particles, resulting in larger α-HDL. After free cholesterol is esterified, cholesterol ester in HDL can be transported to the liver for elimination via several routes. In the presence of cholesterol ester transfer protein (CETP), cholesterol ester from HDL is transferred to triglyceride-rich lipoproteins in exchange for triglyceride. HDL can also be directly endocytosed into liver parenchymal cells as intact particles. In addition, cholesterol ester in HDL can be exclusively taken up without proteins by a nonendocytotic process called selective uptake of cholesterol ester. Some studies (Haffner et al., 1985; Albers et al., 1982) reported that plasma LCAT enzyme, which esterifies cholesterol on HDL (Franceschini et al., 1991), was significantly reduced in hypercholesterolemia (Bielicki and Forte, 1999).

Serum paraoxonase is a calcium-dependent esterase that is known to catalyze hydrolysis of organophosphates, and is widely distributed among tissues such as liver, kidney, intestine, and also serum, where it is associated with HDL (Mackness et al., 1996). Paraoxonase (PON) specificity towards endogenous serum and tissue substrates is not well characterized and therefore synthetic substrates, such as paraoxon and phenyl acetate are used to monitor the enzyme’s activity. Decrease in arylesterase/paraoxonase activity has been already observed after long-term (12 weeks) or short-term (up to 7 days) feeding of susceptible mice with atherogenic diet (Hedrick et al., 2000; Shih et al., 1996). Serum PON activity was shown to be reduced in patients after MI (Ayub et al., 1999), in patients with familial hypercholesterolemia (Mackness et al., 1991), and in patients with diabetes mellitus (Mackness et al., 1991; Abbott et al., 1995), diseases that are associated with accelerated atherogenesis. One of the mechanisms by which HDL may be antiatherogenic is its ability to protect LDL against oxidation. Several HDL-associated proteins have these antioxidant effects, including platelet-activating factor acetylhidrolase (PAF-AH), PON, ceruloplasmin and transferrin (Navab et al., 1998; Kunitake et al., 1992). Paraoxonase another HDL-associated protein protects LDL from oxidative stress. Paraoxonase, an enzyme initially known to hydrolyze organophosphate pesticides including paraoxon, was later recognized as being able to hydrolyze biologically active phospholipids in oxidized LDL. Therefore, it may
have antiatherogenic functions. In contrast to PAF-AH, which is selective against phospholipids with short acyl chains, PON has a specific activity against oxidized phospholipids with longer acyl chains. Depletion of PON activity results in the loss of antioxidant function of HDL. Addition of PON to these HDL particles restores the protective function of HDL (Castellani et al., 1997). An increased susceptibility to atherosclerosis in PON-deficient mice further supports its important role in preventing lipoprotein oxidation and atherogenesis (Shih et al., 1998). Under normal circumstances, circulating LDL is protected from oxidative stress by HDL-associated enzymes, particularly paraoxonase (PON)/arylesterase activity, which destroy biologically active phospholipids (Mackness et al., 1996). Reduced arylesterase/paraoxonase activity has been reported after feeding atherogenic diet in mice (Hedrick et al., 2000; Shih et al., 1996). In addition, other study showed that hypercholesterolemia was independently associated with reduced PON/arylesterase activity in mice fed with a high fat diet (Santos et al., 2002). Boemi et al. (2001) have demonstrated that lower absolute PON concentrations and activities, higher LDL: PON concentration ratios and a lesser capacity to prevent LDL oxidation are consistent with the hypothesis that reduced serum PON in type 1 patients, similar to type 2 diabetics, could contribute to increased risk of vascular disease. Although PON can offer protection against the toxicity of some organophosphates, its physiological role is still not clear; however, evidence exists for a protective effect of PON against oxidative damage (Mackness et al., 1996). Paraoxonase was suggested to contribute to the antioxidant protection conferred by HDL on LDL oxidation (Mackness et al., 1991; Navab et al., 1996; Kontush and Chapman, 2006). The effect of HDL-associated PON or of purified PON on the LDL oxidation process, including its initiation (conjugated diene formation), propagation (peroxides formation), and decomposition (aldehydes formation) phases could be analyzed by using PON inhibitors. The inhibitory effect of HDL on LDL oxidation was suggested to be related to metal ion chelation, or to peroxidase, like activity.

1.4 Atherogenic Properties of Oxidized LDL and Small Dense LDL

Oxidative modification of lipoproteins is believed to play a central role in the pathogenesis of atherosclerosis (Berliner and Heinecke, 1996; Steinberg, 1997). Because plasma contains several antioxidants (Frei, 1995) and lipoproteins with oxidative damage have been isolated from atherosclerotic lesions, (Berliner and
lipoprotein oxidation generally is considered to occur in the vessel wall. Although lipid oxidation in the vessel wall is thought to occur as a result of a local deficiency of endogenous antioxidants or an excess of free metal ions, only limited data support these hypotheses. Research has shown that human atherosclerotic plaques contain massive amounts of lipid peroxidation products, despite the presence of large quantities of α-tocopherol (vitamin E) and ascorbate (Suarna et al., 1995). Therefore, it is unclear whether oxidized lipoproteins originate in the arterial wall or are produced in the circulation and then enter the intimal space. Although LDL is a major risk factor for CAD, experiments have shown that incubation of macrophages with native LDL does not result in foam cell formation, a characteristic feature of atheromatous lesions (Brown and Goldstein, 1983). However, after chemical modification of LDL, rapid uptake occurs, leading to cholesterol accumulation in the macrophage with subsequent foam cell formation, which suggests that LDL modification is a necessary step in atherogenesis. Oxidized (Ox) LDL can induce foam cell formation, and oxidative modification of LDL is now recognized as an important process that occurs in vivo (Steinberg et al., 1989). Several lines of research support the occurrence of LDL oxidation in vivo (Steinberg and Lewis, 1997). Oxidized apoB-100 epitopes and increased levels of lipid peroxidation products can be detected in LDL extracted from both rabbit and human atherosclerotic lesions (Yla-Herttuala et al., 1989). Immunohistochemical staining of atherosclerotic lesions with specific monoclonal antibodies has demonstrated the presence of Ox LDL (Palinski et al., 1989).

Many cell types are capable of oxidizing LDL, including monocytes, macrophages, neutrophils, endothelial cells, smooth muscle cells and fibroblasts. However, cell types that are involved in the atherosclerotic lesion in which Ox LDL is found, i.e. macrophages, endothelial cells and smooth muscle cells, would seem to be the most likely to contribute to LDL oxidation in vivo. Once initiated, oxidation of LDL is a free radical driven lipid peroxidation chain reaction. Lipid peroxidation is initiated by free radical attack on double bond associated with a polyunsaturated fatty acid (PUFA). This results in the removal of a hydrogen atom from a methylene (CH2) group, the rate of which determines the rate of initiation, a key step in lipid peroxidation. Molecular rearrangement of the resulting unstable carbon radical results in a more stable configuration, a conjugated diene. The conjugated diene
reacts very quickly with molecular oxygen, and the peroxyl radical thus formed is a crucial intermediate (Abuja and Esterbauer, 1995) (Fig. 1.3). A PUFA peroxyl radical in LDL may abstract a hydrogen atom from an adjacent PUFA to form a hydroperoxide and another lipid radical, a reaction which results in chain propagation. Removal of hydrogen atoms by the peroxyl radical from other lipids, including cholesterol, eventually yields oxysterols. Lipid hydroperoxides fragment to shorter-chain aldehydes, including malondialdehyde and 4-hydroxynonenal. These reactive aldehydes in turn may bind to ε-amino groups of apoB-100, giving the protein an increased net negative charge. The classical LDL receptor recognizes a specific domain of positive charges from lysine, arginine and histidine residues on apoB. Alteration of this domain results in failure of binding by the apoB/E receptor and an increase in negative surface charge on apoB-100 results in increased recognition by the scavenger receptor. In vitro studies of LDL oxidation have verified the existence of “lag phase”, during which significant oxidation of LDL can not be detected, prior to the onset of the “propagation phase”, presumably after the endogenous antioxidants have been consumed. A steady increase in the detectable byproducts of oxidation continues, until the substrate, i.e. the PUFAs, has been depleted, and a plateau phase is reached. Assessment of resistance of LDL to oxidation has generally involved in measurement of the duration of the lag phase including other variables, such as the rate of propagation, are also noted.

The increased atherogenecity of LDL is associated with a preponderance of small dense (sd-) LDL subpopulation that is more prone to oxidative modification than large buoyant (lb-) LDL (Chancharme et al., 1999). It has been shown that not only the prevalence of sd-LDL but its concentration was substantially increased in healthy subjects with various types of hyperlipidemia such as hyperLDLcholesterolemia, hypertriglyceridemia, combined hyperlipidemia and chylomicronemia, hyperlipididemia with CHD, type 2 diabetic patients with and without CHD (Hirano et al., 2004). Lipoprotein profiles that are relatively rich in sd-LDL particles are associated with up to a 3-fold greater risk of myocardial infarction (MI) than those mainly consist of lb-LDL particles (Austin et al., 1988; Austin et al., 1990; Stampfer et al., 1996). However, the mass of sd-LDL is independently associated with disease risk (Stampfer et al., 1996; Gardner et al., 1996; Lamarche
Fig. 1.3. Basic reaction sequence of lipid peroxidation. (Adapted from Young, I. S. and McEney, J. 2001. Lipoprotein oxidation and atherosclerosis. Biochemical Society Transactions. 29: 358-362).
et al., 1997; Austin et al., 1988), suggesting that these particles are more directly atherogenic than lb-LDL. Similarly, Quebec cardiovascular study has confirmed that a predominance of sd-LDL is a strong and independent predictor of CHD in the first seven years of follow-up (St-Pierre et al., 2005). Recently, Koba et al. (2006) have reported that the progression of CHD was closely linked not to the LDL particle size, but to the concentration of sd-LDL. It has been suggested that in comparison to lb-LDL, sd-LDL are highly atherogenic as a result of their enhanced susceptibility to oxidative modification, higher penetration in the arterial wall, their lower binding affinity for the LDL-receptor, and prolonged plasma half life (Chapman et al., 1998; Bjornheded et al., 1996). Differences in oxidative susceptibility between lb-LDL and sd-LDL have been attributed to differences in their physicochemical properties. Relative to lb-LDL, sd-LDL have a reduced content of antioxidants (De Graaf et al., 1991; Tribble et al., 1994; Tribble et al., 1995) and free cholesterol (De Graaf et al., 1991; Tribble et al., 1992; Tribble et al., 1994), and are enriched with polyunsaturated fatty acids (De Graaf et al., 1991) and, possibly, hydroperoxides (Sevanian et al., 1996). Antioxidant concentrations and free cholesterol content have been shown to predict differences in the oxidative susceptibility of LDL density subfraction (De Graaf et al., 1991; Tribble et al., 1992; Tribble et al., 1994). Therefore, sd-LDL has been highlighted as a new precise and useful CHD marker (Austin et al., 1995; Hirano et al., 2004; Koba et al., 2006).

1.5 Enzymatic and Nonenzymatic Antioxidant Alterations in Hypercholesterolemia

The effects of diet rich in cholesterol on a variety of diseases, including stroke and CVD, have been well documented. Exogenous hypercholesterolemia causes fat deposition in the liver and depletion of the hepatocyte population (Gupta et al., 1976; Assy et al., 2000). Hypercholesterolemia is a dominant risk factor of atherosclerosis (Deepa and Varalakshimi, 2005) and oxidative stress is one of the causative factors that link hypercholesterolemia owing to higher production of reactive oxygen species (ROS) such as superoxide radical, nitric oxide radical, hydroxide radical and hydrogen peroxide (Lee et al., 2002; Ohara et al., 1993; Zou et al., 2003). This stress results from an imbalance between the production of free radicals and effectiveness of antioxidant defense system (Lum and Roebuck, 2001). An impaired radical scavenger function has been linked to the increased or
decreased activity of enzymatic and non enzymatic scavengers of free radicals. Activities of antioxidant enzymes were measured in the erythrocytes from hypercholesterolemic rats and normocholesterolemic rats. CAT, SOD, Gpx and Gred activities were found lower in the erythrocytes from hypercholesterolemic rats compared with normocholesterolemic rats (Bouderbala et al., 2008). These data were in contrast with the study, in which CAT, SOD and Gpx activities were found unchanged (Mahfouz and Kummerow, 2000). Lee et al. (2007) also observed that the activities of CAT, SOD and GPx as well as GSH contents are significantly decreased in the erythrocytes of hypercholesterolemic rats. ATPases are Membrane enzymes which also targets of free radical attack (Moore et al., 1990; Dwight and Hendry, 1996). Reduced activity of ATPase has been found to coincide with pathological changes of other clinical parameters in coronary heart disease (Zhou et al., 1999). ATPases by oxidative attack has been suggested to involve mechanisms including protein crosslinking (Moore et al., 1990), sulfhydryl group oxidation (Hebbel et al., 1986; Shao et al., 1995) and lipid peroxidation (Hebbel et al., 1986; Thomas and Reed, 1990; Lam et al., 2007). The resultant preservation of membrane structure and function is essential for maintaining membrane fluidity and flexibility as well as ionic balance between the intracellular and extracellular compartments. These processes are crucial for the survival of a cell.

Alteration in the levels of glutathione (GSH) and related antioxidants has been demonstrated to be highly associated with the cholesterol-mediated ROS and free radicals. GSH has been known to be widely distributed in human tissues and participates in an array of cellular defense mechanisms (Meister, 1989). Apart from enzymic antioxidants, non-enzymic antioxidants play a vital role in protecting cells from oxidative changes. GSH is one of the important antioxidant in living systems, which is involved in numerous biochemical pathways within the cells. It also plays a key role in liver detoxification reactions by maintaining the structural integrity of cells and organelle membranes and its ability to form conjugates with reactive xenobiotic metabolites (Klaassen et al., 1985) and depletion of GSH increases the vulnerability to free radical damage (Shan et al., 1990). A previously published report, rats fed high cholesterol diet for 4 weeks exhibited a significant decrease in enzymatic activities of SOD, GPx and Gred of liver (Chenni et al., 2007). Gpx is responsible for most of the decomposition of lipid peroxide in cells and may thus
protect the cell from the deleterious effects of peroxides. It is hypothesized that decrease in Gpx and Gred activities of hypercholesterolemic rats would be that increased levels of superoxide radical ($O_2^{-}$) inactivate Gpx due to a loss in total glutathione which would lead to an enhancement of $H_2O_2$ level which in turn would inactivate SOD. In another report (Ismail et al., 2010), activities of SOD and Gpx were significantly reduced in the liver of rats after administration of atherogenic suspension for 8 weeks when compared to control rats. Lu and Chiang (2001) noted that 1 % cholesterol feeding to rats for 6 weeks led to markedly decreased hepatic CAT, SOD and Gpx activities when compared to normal control rats. On the other hand, Mahfouz and Kummerow (2000) reported that cholesterol feeding resulted in increase in hepatic CAT, SOD and Gpx activities. In another report, in contrast to erythrocytes, hepatic CAT and Gpx activities were significantly higher whereas SOD activity was lower in hypercholesterolemic rats when compared to control normal rats (Lee et al., 2007).

1.6 Management of Cholesterol-Induced Oxidative Stress and Hypercholesterolemia

Synthetic antioxidants are widely used to prevent the oxidation of oils and fats and extend the shelf life of lipid containing foods. In recent years, their use in foods has suffered severe criticism, as consumers are becoming increasingly conscious of the safety of synthetic chemical additives (Ozcan, 2003). Therefore, significant emphasis has recently been placed on the use of natural products that can act as antioxidants either alone or synergistically with other additives (Baratta et al., 1998; Pokorny et al., 1997). In addition, experiments have been performed on supplementation of the diet with natural antioxidant compounds in order to attenuate oxidative stress-induced pathogenesis of diseases. These experiments have also dealt with the use of natural antioxidants to alleviate atherosclerosis induced by lipidemic oxidative stress. The dietary intake of phenolic compounds in red wine (Frankel et al. 1995), green tea (Vinson et al., 2004) and olive oil (Aviram, and Kassem, 1993) could inhibit oxidation of LDL and thereby reduce risk factors for CVD. In recent years, *Nigella sativa* (NS) oil and it’s principal bioactive constituent, thymoquinone (TQ) (Fig. 1.4) have received much attention because of their strong antioxidant
Fig. 1.4. Molecular structures of Thymoquinone and Limonene.
(Houghton et al., 1995; Nagi and Mansour, 2000; Mahgoub, 2003; Kruk et al., 2000; El-Saleh et al., 2004; Ismail et al., 2010) as well as cholesterol lowering property in cholesterol-induced oxidative stress-linked hypercholesterolemic rats (Al-Naqeep et al., 2009; Ismail et al., 2010). In the present study, *Nigella sativa*, an herbaceous plant, belonging to Ranunculacea family, has been used. This plant has been found to be salt tolerant and may be considered a glycophyte (Hajar et al., 1996). It is native to Southwest Asia. It is especially grown in the East Mediterranean countries for its seeds and is cultivated extensively in India and Pakistan. *Nigella sativa* is more commonly known as fennel flower plant. The plant is also known by other names e.g. black cumin (English), black caraway seeds (U.S.A.), habba-tus-sawda (Arabic), kalonji (Urdu and Hindi), shonaiz (Persian), krishnajirika (Sanskrit) and kalajira (Bangali) as a natural remedy for many ailments and as a flavouring agent in bread and pickles. The spicy seeds from this plant have proclaimed medicinal usage dating back to the ancient Egyptians, Greeks and Romans. In his *Canon of Medicine* Avicenna states ‘the black seed act as an expectorant, it stimulates the body’s energy and helps recovery from fatigue and dispiritedness’. An authentic saying of the Prophet (Peace Be Upon Him) about NS seed is quoted as Kalonji is remedy for all diseases except death (Al-Bukhari, 815; Al-Jawziyya and Al-Akili, 1993). NS seed is also mentioned as the curative “black cumin” in the Holy Bible (Isaiah 28: 25-28) and described as *Melanthion* by Hippocrates and Dioscorides and as *Gith* by Pliny (Worthen et al., 1998). The seeds of NS, also known as habbatul-barakah, have long been used in folk medicine in the Middle and Far East as a traditional medicine for a wide range of ailments, including fever, cough, bronchitis, asthma, chronic headache, migraine, dizziness, chest congestion, dysmenorrhoea, obesity, diabetes, paralysis, hemiplagia, back pain, infection, inflammation, rheumatism, hypertension, and gastrointestinal problems such as dyspepsia, flatulence, dysentery, and diarrhea. It has been used as a stimulant, diuretic, emmenagogue, lactagogue, anthelmintic, and carminative (Nadkarni, 1976). *Nigella sativa* (NS) seed has also been used externally where it is applied directly to abscesses, nasal ulcers, orchitis, eczema, and swollen joints. In recent times, a considerable research interest has been devoted worldwide to investigate the NS seeds for their historically alleged medicinal properties. In particular, the NS seed oil extract has been examined and shown to have antiviral,
antibacterial, antifungal, anticestodal, antioxidant, analgesic, antiinflammatory, bronchodilatory, hypotensive, hypolipidemic, antitumor, immunopotentiating and antidiabetic activities.

*Nigella sativa* seeds contain more than 30 % fixed oil, 0.20-0.45 % (wt./wt.) of volatile oil (VO), also known as essential oil, 1-4 % nonsaponifiable fraction (NSF) and the rest moisture, protein, ash, carbohydrate and minerals (Ali and Blunden, 2003; Kandil 2002; Kanter *et al.*, 2006). Thymoquinone is the major bioactive component in VO extract of NS seed (Arslan *et al.*, 2005). NSF isolated from NS seed oil contains TQ rich VO and total sterols including phytosterols (Kandil, 2002). Phytosterols are of great interest due to their strong health beneficial antioxidant activity (Ramadan and Morsel, 2004). The GC-MS analysis of 18 VO samples led to the identification of 32-46 components (Burits and Bucar, 2000; Nickavar *et al.*, 2003; Benkaci-Ali *et al.*, 2006). The major components of each VO samples consisted of a mixture of compounds with low molecular weight monoterpenes, representing about 16-86 % of the total VO content (Burits and Bucar, 2000; Nickavar *et al.*, 2003; Arslan *et al.*, 2005; Benkaci-Ali *et al.*, 2006). The main compounds were TQ (8-57 %), limonene (LMN) (0.29-4.30 %), p-cymene (7-15 %), carvacrol (0.32-11.60 %), 4-terpineol (0.30-6.59 %), t-anethole (0.25-38.30 %), and sesquiterpene longifolene (0.70-8.00 %) (Burits and Bucar, 2000; Nickavar *et al.*, 2003; Benkaci-Ali *et al.*, 2006). Thymoquinone is the principal active ingredient in VO extract of NS seed (Arslan *et al.*, 2005). It has been reported to produce multiple health beneficial activities, which include antitumour (Thabrew *et al.*, 2005), anti-inflammatory (Houghton *et al.*, 1995), antibacterial (Morsi, 2000), antidiabetic (Al-Hader *et al.*, 1993) and antioxidative (El-Saleh *et al.*, 2004) effects. TQ has been also shown to attenuate eicosanoid generation (Houghton *et al.*, 1995), cisplatin nephrotoxicity (Badary *et al.*, 1997), tetrachloride hepatotoxicity (Al-Gharably *et al.*, 1997), rheumatoid arthritis (Budancamanak *et al.*, 2006) and gastric mucosal damage Kanter *et al.*, 2006). In addition, it has been shown that NS seed oil and its active compound TQ are capable of lowering plasma cholesterol levels in normal rats (Settaf *et al.*, 2000; Zaoui *et al.*, 2002).

n-hexane extract from NS seed that showed in vitro radical scavenging toward 2,2-diphenyl-1-picryl hydrazyl (DPPH) radicals (Ramadan *et al.*, 2003). Fixed oil of NS seed extracted with light petroleum (40-60 °C) also inhibited nonenzymatic lipid
peroxidation in ox brain phospholipid liposomes (Houghton et al., 1995). The methanol extract of the cold-pressed NS seed oil exhibited significant radical scavenging activities against ABTS•+, DPPH•, chelating activity and oxygen radical absorbing capacity (Yu et al., 2005). In addition, methanol extract also suppressed the lipid peroxidation in human LDL with reduction in the formation of thiobarbituric acid-reactive substances (TBARS) (Yu et al., 2005). Aqueous extract of NS seeds showed NO radical inhibition (Zaher et al., 2008). The VO from NS seed and TQ have been shown to exhibit antioxidant activities against DPPH radical by hydrogen atom or electron donating activity and also against OH radical in non-enzymatic lipid peroxidation in ox brain phospholipid liposomes and the deoxyribose degradation (Burits and Bucar, 2000). Limonene, an important constituent of NS seed oil and other components of it like carvone, anethole have also been shown to exhibit antioxidant property, measured by it’s ferric reducing ability (Lado et al., 2004). Administration of 25-200 ppm of LMN to cockerels elicited a significant suppression of HMG-CoA reductase activity and lowering of serum cholesterol. In addition, feeding of 200 ppm LMN to rats attenuated the effect of 1 % dietary cholesterol on serum cholesterol, while 1 % LMN given for 3 months, effectively decreased both hepatic HMG-CoA reductase activity and serum LDL-C, when compared to control rats (Qureshi et al. 1988).

Feeding of NS seed powder (30 mg/kg body weight) to normal rats for 20 weeks was associated with significant decrease in serum TC and LDL-C as well as significant increase in HDL-C levels when compared to control group of rats (Dahri et al., 2005). High levels of plasma TC and LDL-C, in rats fed a diet enriched with 1 % cholesterol and treated with thymoquinone rich fraction (TQRF) from NS seeds at 0.5, 1 and 1.5 g/kg or pure TQ at 20, 50 and 100 mg/kg body weight for 4 and 8 weeks, were significantly reduced when compared to control rats, with no significant changes in plasma TG and HDL-C levels (Al-Naqeep et al., 2009). This TQRF or TQ mediated reduction in plasma cholesterol concentrations involved two main mechanisms: first, significant suppression (up to 67 %) of liver HMG-CoA reductase mRNA expression, which caused inhibition of cholesterol synthesis and second, increased uptake of LDL-C via up-regulation of (up to 5-7 fold) of LDL receptor gene (Al-Naqeep et al., 2009). Recently, Ismail et al. (2010) have reported that treatment of 1 % cholesterol enriched diet fed rats for 8 weeks with TQRF (1.5
g/kg) or pure TQ (100 mg/kg body weight) increased the plasma hydroxyl radical scavenging activity by 3- and 1.5-fold, respectively. Treatment of 1 % cholesterol fed rats with TQRF (0.5-1.5 g/kg) or pure TQ (20-100 mg/kg body weight) for 8 weeks resulted in a dose dependent up-regulation of hepatic SOD1, CAT and Gpx genes as well as increase in SOD1 and Gpx activities (Ismail et al., 2010). Ismail et al. (2010) have reported that treatment of 1 % cholesterol enriched diet fed rats for 8 weeks with TQRF (0.5-1.5 g/kg) or pure TQ (20-100 mg/kg body weight) significantly decreased the plasma TC and LDL-C levels on the other hand, no significant changes were found in plasma HDL-C levels in the TQRF and TQ groups. Bamosa et al. (2002) reported strong hypocholesterolemic effect of TQ on the blood levels of cholesterol in doses ranging from 0.5 to 8 mg/kg/day for 14 days that reduced TG, LDL-C and HDL-C in normal albino rats.
1.7 **Objectives of the Present Study**

In foods, lipid peroxidation and enzymatic hydrolysis cause shelf life problems. The most limiting factor in determining the shelf life of dehydrated convenience foods is the autoxidation of fats and oils, causing “off-flavours” (Semwal *et al.*, 1999). Rancidity development is an oxidative process that can be blocked by antioxidants, by preventing the formation of free radicals, through the donation of electrons or hydrogen ions. Synthetic antioxidants are widely used to prevent the oxidation of oils and fats and extend the shelf life of lipid containing foods. In recent years, their use in foods has suffered severe criticism, as consumers are becoming increasingly conscious of the safety of synthetic chemical additives (Ozcan, 2003). The use of these synthetic antioxidants, however, is restricted because of their toxicity (Kahl and Kappus, 1993; Walton *et al.*, 1999; WHO, 1996). Therefore, the natural products that can act as antioxidants, either alone or synergistically with other additives, have gained importance (Baratta *et al.*, 1998; Pokorny *et al.*, 1997). On the other hand, reactive oxygen species (ROS) and other free radicals are characterized by their ability in causing oxidative damage to the body. They contribute to the etiology of a number of pathological conditions including cardiovascular diseases (CVD). Epidemiological studies suggest that dietary intake of antioxidants is associated with a reduced risk for CVD (Gey, 1993; Mayne, 1997). Minimizing oxidative damage may well be one of the most important approaches to the primary prevention of aging associated diseases and health problems, since antioxidants terminate direct ROS attacks and radical-mediated oxidative reactions, and appear to be of primary importance in the prevention of these diseases and health problems. Antioxidants have been detected in a number of food and agricultural products, including cereal grains, vegetables, fruits, and oil seeds (Burits and Bucar, 2000; Kalt, *et al.*, 1999; Yu *et al.*, 2002a; Yu *et al.*, 2002c). Recently, cold-pressed edible seed oils, including black caraway (*Nigella sativa*), carrot, hemp, and cranberry seed oils, have become commercially available. The antioxidant properties of NS seed/oil, rich in phenolic compounds are related to their abilities to donate electrons and act as free radical scavengers by the formation of stable phenoxy radicals (Croft, 1999). Yu *et al.* (2005) have reported a strong radical scavenging activities in methanol extract of cold-pressed NS seeds against DPPH•, ABTS**, Fe**2+-chelating, and oxygen radical absorbing capacities. In
addition, methanol extract also suppressed the Cu\(^{2+}\)-induced lipid peroxidation in human LDL with reduction in MDA/TBARS formation. The volatile oil (VO) extract from NS seeds and pure thymoquinone (TQ) have been shown to exhibit antioxidant activities against DPPH radical and also against OH radical in nonenzymatic lipid peroxidation in phospholipid liposomes, and the deoxyribose degradation (Burits and Bucar, 2000). Aqueous extract of NS seeds showed NO radical inhibition (Zaher et al., 2008).

In recent years, experiments have been performed on supplementation of diet with natural antioxidant compounds in order to attenuate oxidative stress-induced pathogenesis of diseases. These experiments have also dealt with the use of natural antioxidants to alleviate atherosclerosis induced by lipidemic oxidative stress. Dietary intake of antioxidants could also inhibit oxidation of LDL and thereby reduce the risk factors for CVD (Vinson et al., 2004; Meyer et al., 1997; Agarwal and Rao, 1998). Several studies have reported the antioxidant activity of NS seed oil and its principal active constituent, TQ against biologically hazardous ROS (Houghton et al., 1995; Nagi and Mansour, 2000; Mahgoub, 2003; Kruk et al., 2000). Ismail et al., 2010 have reported that feeding rats 1 % cholesterol rich diet for 8 weeks resulted in a significant decrease in plasma antioxidant capacity, as measured by the efficiency to scavenge OH radicals. As expected, rats treated with TQ rich fraction (TQRF) from NS seeds or pure TQ at various concentrations showed significant inhibitory activity toward the formation of \(^{\bullet}\)OH compared to untreated rats. In addition, in these treated rats, TQRF and TQ mediated a dose dependent up-regulation of hepatic antioxidant enzymes, SOD1, CAT and Gpx genes (p < 0.05), which apparently was responsible for the increase in enzymatic activities of SOD1 and Gpx, compared to cholesterol fed untreated rats (p < 0.05). There are only couple of reports indicating that daily administration of TQRF or pure TQ to rats fed 1 % cholesterol enriched diet for 8 weeks significantly reduced the plasma TC and LDL-C, with no effect on TG and HDL-C, compared to untreated cholesterol fed rats (Al-Naqeep et al., 2009; Ismail et al., 2010). This reduction in TC and LDL-C by TQRF and TQ is mediated by two mechanisms: (i) inhibition of cholesterol synthesis involving down regulation of HMG-CoA reductase gene and (ii) increased uptake of LDL-C via up-regulation of LDL receptor gene (Al-Naqeep et al., 2009).
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Limonene (LMN), an important constituent of VO fraction of NS seed oil, has been shown to exhibit antioxidant property, as measured by its ferric reducing ability (Lado et al., 2004). Qureshi et al. (1988) have reported that feeding of 200 ppm LMN to rats attenuated the effect of 1% dietary cholesterol on serum TC, while 1% LMN given for 3 months, effectively decreased both hepatic HMG-CoA reductase activity and serum LDL-C.

Based on the above discussion it appears that limited number of studies pertaining to antioxidant and cholesterol lowering property of TQRF of NS seeds and pure TQ have been reported in cholesterol fed rats. In the present study, in the first part, Nigella sativa (NS) seed oil and its methanol/nonsaponifiable (NSF) or volatile oil (VO) extracts, pure thymoquinone (TQ) and pure limonene (LMN), two constituents of VO fraction of NS seed oil, were evaluated for their scavenging activities against 2,2-diphenyl-1-picryl hydrazyl (DPPH*), 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS**), nitric oxide (NO*) and hydroxyl (*OH) radicals in both the assay for the deoxyribose test and nonenzymatic lipid peroxidation in phospholipid liposomes. The total phenolic compounds and chelating activities against Fe^{2+} in NS seed oil, and its NSF and VO extracts were also determined.

The putative preventive effects of NSF and VO extracts of NS seed oil, pure TQ and pure LMN on enzymatic and nonenzymatic antioxidant status and lipid peroxidation in plasma, erythrocytes and liver in the presence of lipemic-oxidative stress, induced in rats by feeding an atherogenic suspension containing 5 mg cholesterol, 30 mg coconut oil and 2.5 mg cholic acid per rat per day for 30 days, were investigated. Erythrocytes and LDL are important blood components that are constantly exposed to both extracellular and intracellular sources of ROS. Hypercholesterolemia leads to increased cholesterol accumulation in the erythrocyte and endothelial cells thereby activating and enhancing them to produce additional oxygen free radicals (Prasad and Kalra, 1989; Kay, 1991). Thus, exposure of erythrocytes to these prooxidant conditions can lead to a number of membrane changes including lipid peroxidation (Sato et al., 1995; Moore et al., 1990), protein crosslinking (Moore et al., 1990) and sulfhydryl group oxidation (Soszynski and Bartosz, 1997), resulting subsequently in membrane damage and hemolysis (Sato et al., 1995; Niki et al., 1988). Membrane enzymes such as ATPases are also targets of free radical attack (Moore et al., 1990; Dwight and Hendry, 1996). Decrease in
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erthrocyte ATPase activities has been found to coincide with pathological changes of other clinical parameters in CHD (Zhou et al., 1999). Thus, the protective effects of test fractions/compounds against oxidative damage in erythrocytes was investigated in atherogenic suspension fed rats simultaneously treated with 100 mg NSF, 20 mg VO 10, mg pure TQ or 400 mg LMN, by quantifying : (a) both ex vivo basal MDA/TBARS and in vitro H$_2$O$_2$-induced MDA formation in erythrocyte membranes, (b) both free and membrane protein-bound sulfhydryl groups of glutathione including enzymatic antioxidants, CAT, SOD, Gpx, Gred, reduced and oxidized glutathione, and (c) membrane bound total, Mg$^{++}$- and Na$^+$, K$^+$-dependent ATPases.

Several studies in animals (Boccio et al., 1990; Smith et al., 1985; Thiery and Seidel, 1987; Tsai and Kelley, 1978; Uysal, 1986; Uysal et al., 1988) and humans (Uzel et al., 1985; Yalcin et al., 1989) have shown that there is a close relationship between lipid peroxidation and hypercholesterolemia and/or hypercholesterolemic atherosclerosis. Hypercholesterolemia, especially elevated levels of LDL-C, is one major risk factor for CAD, and therapeutic reductions of total cholesterol (TC) and LDL-C levels result in a decrease in cardiovascular morbidity and mortality (Scandinavian simvastatin survival study, 1994; Shepherd et al., 1995; Sacks et al., 1996; Downs et al., 1998). The pooled results of many trials using different cholesterol lowering regimens indicate that for every 10 % reduction in cholesterol level, CHD deaths will be reduced by at least 15% (Gould et al., 1998). Hypertriglyceridemia and elevated levels of TG rich VLDL have reemerged as risk factors for atherosclerosis (Austin et al., 1998; Stampfer et al., 1996). On the other hand, plasma HDL-C levels are inversely correlated with the risk of atherosclerosis (Gordon et al., 1989). In agreement with these concepts, therapies that raise HDL and decrease triglyceride reduce the morbidity and mortality from CAD (Gordon et al., 1989; Frick et al., 1987). These intervention trials support the concept that LDL and possibly VLDL play a pivotal role in lipid uptake and that HDL is a key lipoprotein in lipid removal from macrophage foam cells in the arterial wall. Based on the above information, the hypolipidemic/antiatherogenic efficacy of feeding NSF, VO, pure TQ or LMN to atherogenic suspension fed rats was investigated.

Several studies have shown a 2- to 3-fold increase in CHD risk in patients with a predominance of sd-LDL particles than those mainly consist of lb-LDL (Austin et al., 1988; Austin et al., 1990; Stampfer et al., 1996). Furthermore, both the
prevalence and concentration of more proatherogenic sd-LDL subpopulation was significantly increased in subjects with various types of hyperlipidemia such as hyperLDLcholesterolemia, hypertriglyceridemia, combined hyperlipidemia and chylomicronemia, hyperlipidemia with CHD, and type 2 diabetic patients with CHD (Hirano et al., 2004). Similarly, Koba et al. (2006) have reported that the progression of CHD was not linked to the LDL particle size, but to the concentration of sd-LDL subfraction. Based on these and other published research highly atherogenic sd-LDL has been highlighted as a strong and more precise CHD risk marker (Austin et al., 1995; Hirano et al., 2004; Koba et al., 2006). Based on these reports, in the present study we have measured the cholesterol and apoB contents in LDL and its density subfractions, sd-LDL and lb-LDL, isolated from plasma of NLP-C, HLP-C and HLP rats treated with above test fractions/compounds. In order to establish the shift in buoyancy from less atherogenic lb-LDL to highly atherogenic sd-LDL, under lipidemic oxidative stress, the percent share of LDL in sd-LDL and lb-LDL subpopulations was examined. In addition, in treated groups, the role of NSF, VO, pure TQ and LMN in blocking this shift in buoyancy and in restoration of percent distribution of LDL cholesterol and apoB into sd-LDL and lb-LDL subfractions were analyzed.

Oxidative stress and oxidative modification of LDL have been postulated to play a pivotal role in initiation of the atherosclerotic process (Steinberg and Witztum, 2002). Relative to lb-LDL, sd-LDL subpopulation with a much greater tendency to become oxidized might thus be more likely to participate in proatherogenic events in the vessel wall of humans with hyperlipidemia, similar to atherogenic lipoprotein phenotype pattern B subjects (De Graaf et al., 1991; Tribble et al., 1992; Dejager et al., 1993; Chait et al., 1993; Tribble et al., 1994; Tribble et al., 1995; Tribble et al., 2001). This substantial difference in the oxidative susceptibility between sd-LDL and lb-LDL from normal and cholesterol-induced dyslipidemic rats is consistent with the previous reports indicating an inherently reduced content of free cholesterol and antioxidants, increased amount of more oxidizable polyunsaturated fatty acids and preformed hydroperoxide in sd-LDL particles from healthy, combined hyperlipidemic and atherogenic lipoprotein phenotype pattern B subjects (De Graaf et al., 1991; Thomas et al., 1994; Sevanian et al., 1996; Tribble et al., 2001). Therefore, the antioxidant protection of test
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fractions/compounds afforded to LDL and it’s density subfractions, sd-LDL and lb-LDL, from in vivo and Cu^{2+}-catalyzed in vitro oxidation was investigated. This objective was achieved by measuring ex vivo basal and in vitro maximal CD formation and lag times, as well as ex vivo basal and maximal production of MDA in LDL, sd-LDL and lb-LDL isolated from plasma of rats in NLP-C, HLP-C, HLP-NSF, HLP-VO, HLP-TQ and HLP-LMN groups.