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Full Length Research Paper

The potential influence of high cholesterol diet-induced oxidative stress on composition and properties of red blood cells in rabbits

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Atherosclerosis and heart diseases are major causes of morbidity and mortality in adults in industrialized nations. The aim of this study was to assess the potential influence of high-cholesterol diet-induced oxidative stress on composition and properties of red blood cells (RBCs) in rabbits. Thus, percentage of hematocrit, RBCs, white blood cells (WBCs) and platelets counts, total cholesterol (TC), low density lipoprotein (LDL), triglycerides (TG) and high density lipoprotein (HDL), Thiobarbituric acid reactive substances (TBARS) serum level, antioxidant enzymes activity (Superoxide dismutase: SOD; Glutathione peroxidase: GPx), hemoglobin (Hb) and Hb derivatives (oxyhemoglobin: HbO_2; carboxyhemoglobin: HbCO; sulfohemoglobin: SHb; met-hemoglobin: Met-Hb) were measured in control and high fat diet (HFD) rabbits. We found that the TC, LDL, TG and HDL (mg/dl) were significantly ($p < 0.001$) increased in HFD rabbits compared with control rabbits. A significant ($p < 0.05$) decrease in Hb (g/dl), percentage of hematocrit and RBCs count was observed in HFD rabbits compared with control rabbits while a significant increase in platelet and WBCs counts was observed. The TBARS was significantly ($p < 0.05$) increased in HFD rabbits compared with control rabbits while antioxidant enzymes SOD and GPx activity were significantly ($p < 0.05$) decreased. A significant increase in percentage of Met-Hb, HbCO and SHb was observed in HFD rabbits compared with control rabbits while a significant decrease in percentage of HbO_2 was observed. This study shows that hypercholesterolemia affects the level of Hb and Hb derivatives which causes anemia and may produce reactive oxygen species (ROSs) and other free radicals increasing TBARS and decreasing SOD and GPx enzymes activities. Hypercholesterolemia may promote the conversion of HbO_2 and the fraction of unstable Hb molecules to Met-Hb, SHb and HbCO. Furthermore, increased platelet and WBCs count in HFD rabbits may be of pathophysiological importance for the progression of atherosclerosis and thromboembolic complications. This study suggests that hypercholesterolemia may produce free radicals which promote oxidation of Hb and reduce its concentration and conversion of HbO_2 to Met-Hb and the fractions of unstable Hb molecules to Met-Hb, SHb and HbCO. Furthermore, an imbalance between free radical production and antioxidant enzymes activities may lead to oxidative stress.

Key words: High cholesterol diet, oxidative stress, antioxidant enzyme activates, red blood cells, atherosclerosis.

INTRODUCTION

Atherosclerosis is the primary cause of coronary and cardiovascular diseases. It can generally be viewed as a form of chronic inflammation that is induced and perturbed by lipid accumulation (Glass and Witztum, 2001; Ross, 1999) . Hyperlipidemia or a high level of serum triacylglycerol and cholesterol is a risk factor for premature atherosclerosis (Chobanian, 19913; Ghosh et al., 2001). Oxygen free radicals have been implicated in the pathogenesis of hypercholesterolemic atherosclerosis and antioxidants suppress the development of hyper-

Abbreviations: TC, Total cholesterol; **LDL,** low-density lipoprotein; **TG,** triglycerides; **HDL,** high density lipoprotein; **TBARS,** thiobarbituric acid reactive substances; **SOD,** superoxide dismutase; **GPx,** glutathione peroxidase; **RBCs,** red blood cells; **WBCs,** white blood cells; **Hb,** hemoglobin; **HbO_2,** oxyhemoglobin; **HbCO,** carboxyhemoglobin; **SHb,** sulfohemoglobin; **Met-Hb,** met-hemoglobin; **NOR,** normal; **HFD (CHO),** high fat diet.

cholesterolemic atherosclerosis (Prasad et al., 1994; Prasad et al., 1997; Kojda and Harrison, 1999; Chisolm and Steinberg 2000). Hypercholesterolemia can increase the cholesterol content of platelets, polymorphonuclear leukocytes and endothelial cells so that endothelial and smooth muscle cells, neutrophils and platelets may be sources of oxygen free radicals (Esterbauer et al., 1992).

The high polyunsaturated fatty acid content of RBC membrane and the continuous exposure to high concentrations of oxygen and iron in hemoglobin (Hb) are factors which make RBCs very sensitive to oxidative injury, making them an appropriate model to study oxidative stress (Kusmic et al., 2000). Oxidative stress produces profound alterations to cellular membrane lipids, proteins and nucleic acids, impairing cell metabolism and viability, and has been considered to be involved in several diseases (Son et al., 2004; Vaziri, 2004; Balkan et al., 2002; Stocker and Keane, 2001; Hitchon and Gabalawy, 2004; Bautista, 2001; Van et al., 2000).

A cardiovascular damage is produced by Met-Hb mediated oxidation of light density lipoproteins, accelerating arteriosclerosis. In addition, the release of heme from Met-Hb is an important factor in inflammation. Met-Hb becomes the mediator between hypoxia, RBC lysis and increased inflammation (Balkan et al., 2002). The degree of oxidation of hemoglobin senses the oxygen level in the blood and uses its ability to produce nitric oxide from nitrite to control vascular tone, increasing blood flood when the proportion of oxygenated hemoglobin falls (Edwards and Fuller 1996).

It has been reported that feeding rabbits a high cholesterol diet resulted in alterations in prooxidant-antioxidant status in several tissues as well as typical atherosclerotic changes in the aorta (Warnholtz et al., 2001; Aldons, 2000; Kohen and Nyska, 2002). One of the mechanisms responsible for the structural changes of RBCs in hypercholesterolemia has been reported to be oxidative stress (Balkan et al., 2002; Libby, 2002; Moussa et al., 2009; Abdelhalim and Moussa, 2010). It becomes evident that oxidative stress occurs when there is an excessive production of free radicals in the face of defective anti-oxidant defenses, but further evidences are required to support this hypothesis. It is thus well-motivating to investigate the potential influence of high cholesterol diet-induced oxidative stress on composition and properties of RBCs in control and HFD rabbits.

MATERIAL AND METHODS

Animal protocol and atherosclerosis samples

The rabbits used in this study were twenty five (12-weeks) New Zealand white male rabbits, weighed between 1.2 and 1.5 kg and obtained from the Laboratory Animal Centre (College of Pharmacy, King Saud University, Saudi Arabia). The rabbits were randomly assigned to control group in which rabbits consumed a standard diet and cholesterol group in which rabbits consumed an atherogenic diet. Animal food was stored at -20°C until use to prevent oxidation. The composition of the commercially basal diet is shown in Tables 1 and 2. The control group (number of animals: n = 10) was fed 100 g/day of normal diet (Purina Certified Rabbit Chow # 5321; Research Diet Inc., New Brunswick, NJ 08901, USA) for a period of 15 weeks. The HFD group (number of animals: n = 15) was fed a normal Purina Certified Rabbit Chow # 5321 with 1.0% added cholesterol plus 1.0% olive oil (100 g/day) for the same period of time. During the experiment, rabbits were housed in a room maintained at constant temperature and humidity and allowed free access to food and water. At the end of the protocol, the rabbits were anesthetized with pentobarbital sodium (40 mg/kg intravenously), blood samples were collected and aortas were removed for assessment of the atherosclerotic changes.

Collection of blood and preparation of serum

Blood samples were collected following an overnight fast and were collectively analyzed for both groups of animals. Blood samples of 2 ml were obtained from the rabbits via venepuncture of an antecubital vein. Blood was collected into two polypropylene tubes, one for serum and one for plasma. The blood for plasma was collected in heparin. Serum was prepared by allowing the blood to clot at 37°C and centrifuge at 3000 rpm for 10 min.

Serum lipids and blood cells count

TC, LDL, HDL, TG, Hb, hematocrit and RBCs, WBCs and platelet counts were measured in a Clinical Laboratory Center using an ADVIA 120 Hematology System (Bayer Medical, New York, USA).

Antioxidant enzyme activates SOD and GPx

Measurement of SOD plasma enzyme activity

The plasma enzyme activity of SOD (U/ml) was measured at wavelength of 500 nm with a commercially available kit (Randox Laboratories, kit Ransod superoxide dismutase) by testing the inhibition degree of a tetrazolium salt oxidation reaction. The coefficient of variability between assays was 4.2% (Sun et al., 1988).

Measurement of GPx plasma enzyme activity

The plasma enzyme activity of GPx (U/ml) was measured with a commercially available kit (Ransel glutathione peroxidase, Randox Laboratories) at wavelength of 340 nm by measuring the decrease of NADPH absorbance. The coefficient of variability between assays was 4% (Paglia and Valentine 1967).

Measurement of TBARS serum level

TBARS serum levels (µmol/ml) were measured by the previously described methods (Prasad et al., 1996; Ohkawa et al., 1979). TBARS were extracted in a mixture of butanol/pyridine (15:1). The butanol/pyridine layer was separated by centrifugation at 3000 rpm for 15 min. The fluorescence intensity of the butanol/pyridine solution was measured at wavelength of 553 nm with excitation at wavelength of 513 nm using a spectrophotometer. The TBARS content of serum was expressed as mol/ml.

Measurement of Hb derivatives

HbO_2, HbCO, SHb and Met-Hb were measured using the

Table 1. Chemical composition of laboratory normal (NOR) diet (Nutrients: Purina certified rabbit Chow # 5321).

Nutrients (%)			
Protein	16.20	Cholesterol, ppm	0.00
Arginine	0.84	Fat (acid hydrolysis)	4.00
Cystine	0.25	Linoleic acid	1.31
Glycine	0.77	Linolenic acid	0.08
Histidine	0.38	Arachidonic acid	0.00
Isoleucine	0.88	Omega-3 fatty acids	0.08
Leucine	1.30	Total saturated fatty acids	0.43
Lysine	0.78	Total monounsaturated fatty acids	0.70
Methionine	0.35	Fiber (Crude)	14.00
Phenylalanine	0.80	Neutral detergent fiber	27.40
Tyrosine	0.50	Acid detergent fiber	17.10
Threonine	0.64	Nitrogen-free extract (by difference)	50.00
Tryptophan	0.14	Starch	21.50
Valine	0.84	Glucose	0.34
Serine	0.85	Fructose	0.90
Aspartic Acid	.87	Sucrose	2.44
Glutamic Acid	3.33	Lactose	0.00
Alanine	0.85	Total digestible nutrients	66.00
Proline	1.31	Gross Energy, kcal/gm	3.81
Taurine	<0.01	Physiological fuel value, kcal/gm	2.88
Fat (ether extract)	2.50	Metabolizable energy, kcal/gm	2.49

Purina certified rabbit Chow # 5321; Research Diet Inc., New Jersey, USA.

Table 2. Chemical composition of laboratory NOR diet (Minerals and Vitamins; Purina certified rabbit chow # 5321).

Minerals (%)		Vitamins (%)	
Ash	7.30	Carotene, ppm	28.00
Calcium	1.10	Vitaimn K, ppm	2.90
Phosphorus	0.50	Thiamin Hydrochloride, ppm	4.80
Phosphorus (non-phytate)	0.27	Riboflavin, ppm	5.00
Potassium	1.20	Niacin, ppm	54.00
Magnesium	0.25	Pantothenic Acid, ppm	19.00
Sulfur	0.24	Choline Chloride, ppm	1600.00
Sodium	0.30	Folic Acid, ppm	8.40
Chlorine	0.66	Pyridoxine, ppm	4.50
Fluorine, ppm	11.00	Biotin, ppm	0.20
Iron, ppm	340.00	B_{12} mcg/kg	6.60
Zinc, ppm	120.00	Vitamin A, IU/gm	20.00
Manganese, ppm	121.00	Vitamin D, IU/gm	1.10
Copper, ppm	17.00	Vitamin E, IU/gm	44.00
Cobalt, ppm	0.50	Ascorbic Acid, mg/gm	-
Iodine, ppm	1.10	-	-
Chromium, ppm	0.70	-	
Selenium, ppm	0.25	-	-

Purina certified rabbit Chow # 5321; Research Diet Inc., New Jersey, USA.

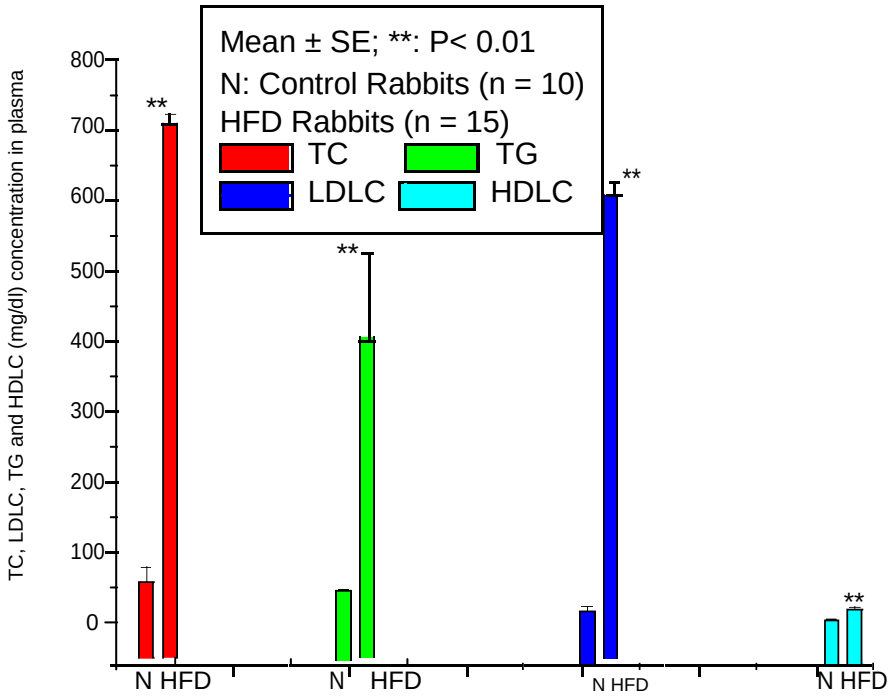

Figure 1. TC, LDLC and TG concentration in the serum of control and HFD rabbits.

multicomponent spectrophotometric method for the simultaneous determination of Hb derivatives described by Atef et al. (1995).

Statistical analysis

Statistical analysis was performed by means of InStat® package for personal computers (GraphPad™ Software, Inc., San Diego, USA). Values were considered significant if $P < 0.05$. Each sample was run in duplicate. All data were expressed as mean ± standard error (SE), for control rabbits (n = 10 animals) and for HFD rabbits (n = 15 animals). The data of control and HFD rabbits were compared using one-way ANOVA analysis followed by Turkey's test for multiple comparisons.

RESULTS

Figure 1 shows TC, LDL and TG concentrations (mg/dl) in serum of control and HFD rabbits. The TC (mg/dl) significantly ($p < 0.001$) increased in HFD rabbits compared with control rabbits with percentage normalized change of 1198%. The LDL (mg/dl) significantly ($p < 0.001$) increased in HFD rabbits compared with control rabbits with percentage normalized change of 1591%. The TG (mg/dl) significantly increased in HFD rabbits compared with control rabbits with percentage normalized change of 710%. The HDL (mg/dl) significantly increased in HFD rabbits compared with control rabbits with percentage normalized change of 136.36%.

Figures 2 and 3 show that in HFD rabbits compared with control rabbits, a significant ($p < 0.05$) decrease in Hb (g/dl), percentage of hematocrit and RBCs count was observed with percentage normalized changes of 18.94, 18.99 and 31.08%, respectively, while a significant increase in platelet and WBCs counts was observed in HFD rabbits compared with control rabbits with percentage normalized changes of 16.97 and 51.89%, respectively.

Figures 4 and 5 show the antioxidant enzymes (SOD and GPx) and oxidative stress (Malondialdehyde: TBARS) parameters in control and HFD rabbits. The SOD and GPx were significantly ($p < 0.05$) decreased in HFD rabbits compared with control rabbits with percentage normalized changes of 11.36 and 36.84%, respectively, while the TBARS serum level was significantly ($p < 0.05$) increased in HFD rabbits compared with control rabbits with percentage normalized change of 55.71%.

Figures 6 and 7 show percentage of HbO_2, HbCO, SHb and Met-Hb in HFD rabbits compared with control rabbits. A significant increase in percentage of Met-Hb, HbCO and SHb was observed in HFD rabbits compared with control rabbits with percentage normalized change of 300% for each; while a significant decrease in percentage of HbO_2 was observed in HFD rabbits compared with control rabbits with percentage normalized change of 14.14%.

DISCUSSION

In the present study, the elevations in serum TC, TG, LDL and HDL levels observed in this study were in

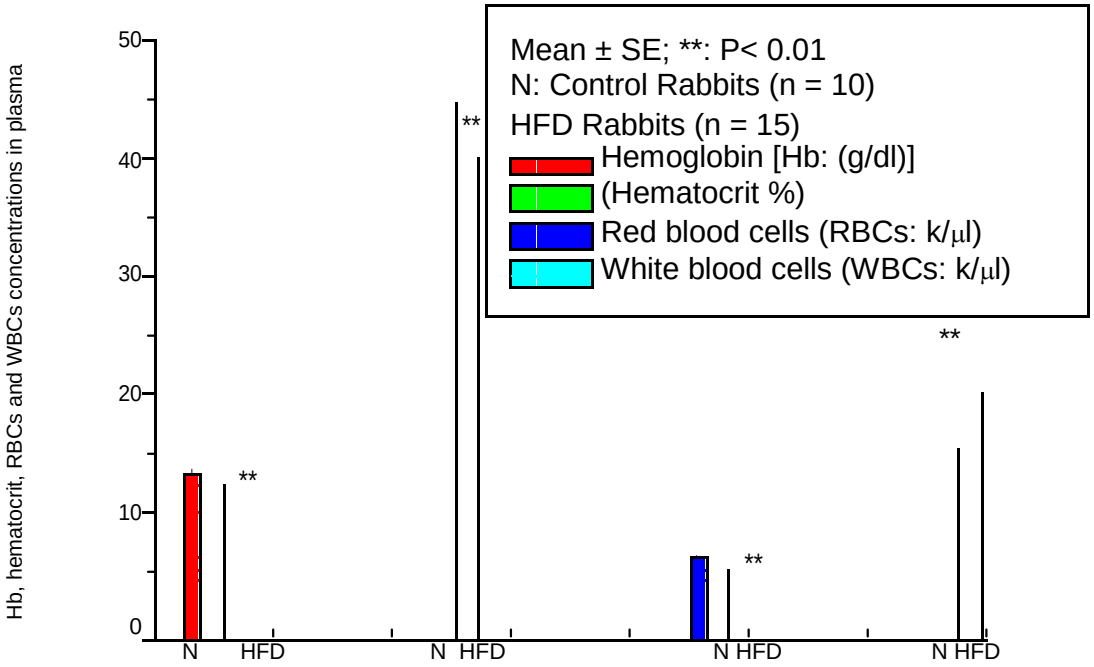

Figure 2. Hb, percentage of hematocrit and RBCs and WBCs count in control and HFD rabbits.

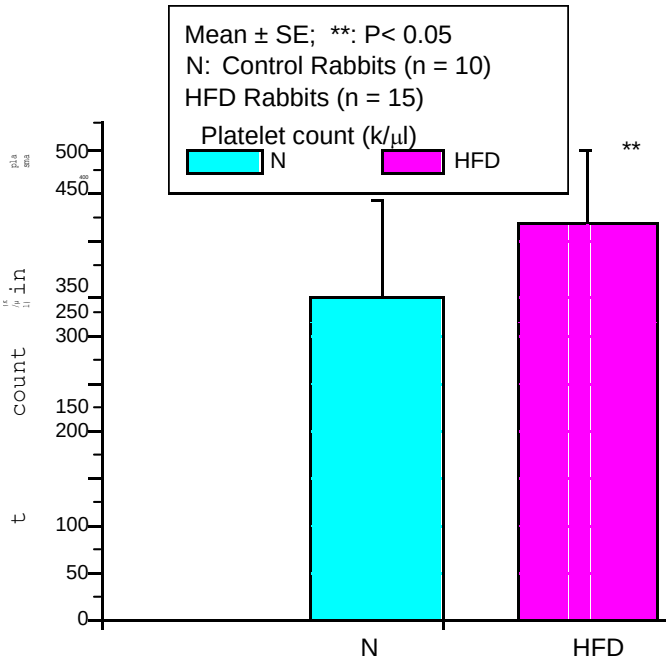

Figure 3. Platelet count in control and HFD rabbits.

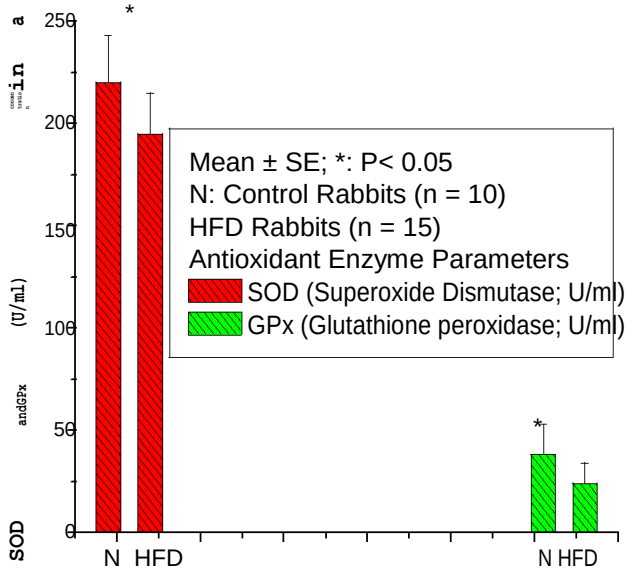

Figure 4. Plasma antioxidant enzymes SOD and GPx in control and HFD rabbits.

agreement with those reported in several studies (Abdelhalim and Alhadlaq, 2008; Tanaka et al., 2001) . It has been reported that high serum abnormally levels of TC and LDL are associated with an increased risk for atherosclerosis (Abdelhalim and Alhadlaq, 2008; Korhonen et al., 1996; Duverger et al., 1996). Hypercholesterolemia and hypertriglyceridemia are independent risk factors that alone or together can accelerate the development of coronary artery disease and the progression of atherosclerotic lesions (McKenney, 2001).

In this study, a significant increase in HDL concentration indicates that HDL may play a protective role through reversing cholesterol transport, inhibiting the oxi-

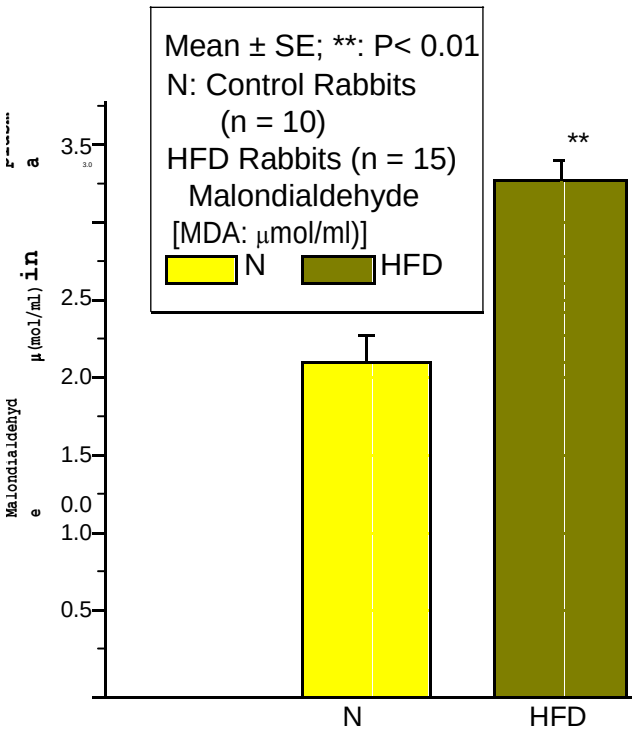

Figure 5. Plasma MDA (?mol/ml) concentration in control and HFD rabbits.

dation of LDL and neutralizing the atherogenesis effects of oxidized LDL. This study shows that the Hb concentration, percentage of hematocrit and RBCs count were significantly decreased in HFD rabbits compared with control rabbits while a significant increase in platelet and WBCs counts was observed. Hb is the most abundant and functionally important protein in RBCs, once it is released from RBCs, it becomes highly toxic because of the oxidative properties of heme which participates in Fenton reaction to produce ROSs causing cell injury (Moussa et al., 2009; Abdelhalim and Moussa, 2010; Puppo and Halliwell, 1988; Fabiana et al., 2009; Baynes, 1991; Tribe and Poston. 1996). This study suggests that hypercholesterolemia, lipid peroxidation and production of free radicals promote oxidation of Hb and reduce its concentration. The decrease in Hb concentration and RBCs count in HFD rabbits reflects the presence of anemia in these rabbits. An increase in oxidative stress has also been suggested as a cause of the haemolytic anemia, which was seen following high cholesterol diet (Balkan et al., 2002).

A higher WBCs count may reflect the existence of clinical or subclinical *in vivo* harmful inflammatory activity. Many of the non-infectious health problems, such as atherosclerosis and hypertension, associated with a higher WBCs count are considered risk factors for cardiovascular diseases (Facchini et al., 1992; Huang et al., 2001). An increased pro-inflammatory state enhances activation of WBCs and endothelial cells, thereby promoting platelet aggregation and thrombus formation (Ross, 1999; Harrison, 2005; Lohsoonthorn et al., 2007). It has been reported that severe hypercholesterolemia leads to alterations in the RBC lipid composition, conti-nuous exchange of lipids between plasma lipoprotein and RBC membrane, an increase in RBC lipid peroxidation and a decrease in the anti-oxidant system (Chisolm and Steinberg, 2000; Kusmic et al., 2000; Cimen, 2008). In this study, the diminished antioxidant defense system in HFD rabbits leads to lipid peroxidation and plasma oxidative damage as characterized by significant increase in TBARS serum level and significant decrease in plasma enzymes activity of SOD and GPx. The attack of ROSs on membrane lipoproteins and polyunsaturated fatty acids results in formation of numerous oxygenated compounds, particularly malondialdehyde (Esterbauer et al., 1991).

This study suggests that an imbalance between free radical production and antioxidant level leads to oxidative stress, which is obvious from the depressed antioxidant enzyme activities in HFD rabbits. The lipid peroxidation marker malondialdehyde has been reported to be increased in hyperlipidemic rabbits (Esterbauer et al., 1991). The increase in serum and aortic malondialdehyde indicates an increase in the levels of oxygen radicals, which may induce atherosclerosis by inducing endothelial cell injury and modulation of cell adhesion molecules (Maxfield and Tabas 2005).

It became evident from this study that hyperlipidemic rabbits had a significantly higher percentage level of Met-Hb which is the non-functional part of Hb, and a significantly lower percentage level of HbO_2 which is the most important part of Hb that carries oxygen to all parts of the body. As a consequence of their physiological role, RBCs are exposed to continuous oxidative stress. This study also suggests that hypercholesterolemia may promote the conversion of HbO_2 to Met-Hb, and consequently, the fractions of unstable Hb molecules that undergo abnormal dissociation to Met-Hb, SHb and HbCO were increased in HFD rabbits compared with control rabbits.

Conclusion

This study shows that hypercholesterolemia affect the level of Hb and Hb derivatives which may cause anemia and produce reactive oxygen species (ROSs) and other free radicals increasing TBARS serum level and decreasing plasma enzymes SOD and GPx activity. Hypercholesterolemia may promote the conversion of HbO_2 and the fraction of unstable Hb molecules to Met-Hb, SHb and HbCO. Furthermore, increased platelet and WBCs count in HFD rabbits may be of pathophysiological importance for the progression of atherosclerosis and thromboembolic complications. This study suggests that hypercholesterolemia may produce free radicals which promote oxidation of Hb and reduce its concentration and

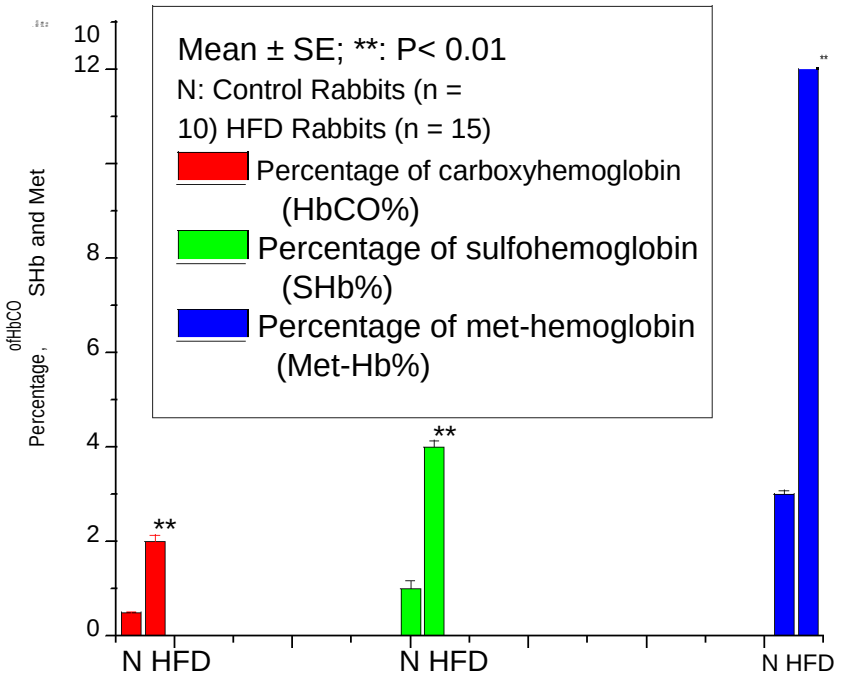

Figure 6. Percentage of HbCO, SHb and Met-Hb in control and HFD rabbits.

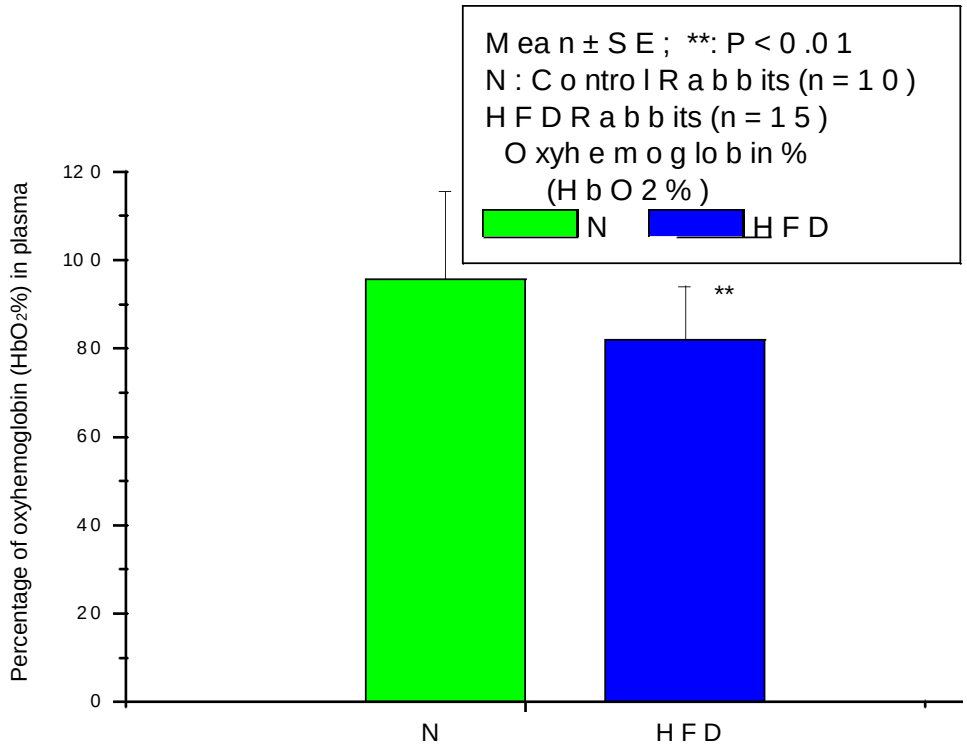

Figure 7. Percentage of HbO_2 in control and HFD rabbits.

convert HbO_2 to Met-Hb and the fractions of unstable Hb molecules to Met-Hb, SHb and HbCO. Furthermore, an imbalance between free radical production and antioxidant enzymes activities may lead to oxidative stress.

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