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Biochemical Changes of Hemoglobin and Osmotic Fragility of Red Blood Cells in High Fat Diet Rabbits

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Abstract: The aim of this study was to assess the effects of hyperlipidemia on auto-oxidation rate of hemoglobin (Hb; absorbance at 630 nm versus time), Hb derivatives and osmotic fragility of Red Blood Cells (RBCs). These parameters were measured in twenty five 12-week-old male New Zealand white rabbits fed on a High Fat Diet (HFD) for a feeding period of 10 weeks. We found that Hb concentration and RBC count were significantly decreased while white blood cell and platelet counts were significantly increased in HFD rabbits compared with control rabbits. The Total Cholesterol (TC) was significantly increased ($p < 0.01$) in HFD rabbits compared with control rabbits with percentage normalized change of 1198% and Low Density Lipoprotein Cholesterol (LDLC) significantly increased ($p < 0.01$) in HFD rabbits compared with control rabbits with percentage normalized change of 1591%. In HFD rabbits, oxyhemoglobin (HbO₂) percentage was significantly decreased while met- hemoglobin (Met-Hb) percentage was significantly increased compared with control rabbits. The auto-oxidation rate was significantly higher in HFD rabbits compared with controls. Hyperlipidemia induced an increase in the osmotic fragility of RBCs and a decrease in their membrane elasticity compared with controls. This study suggests that hyperlipidemia may produce reactive oxygen species and other free radicals which increase the auto-oxidation rate of Hb and promote the conversion of HbO₂ and the fractions of unstable Hb molecules to Met-Hb and carboxyhemoglobin. Increased platelet activation in hyperlipidemic rabbits may be of pathophysiological importance for the progression of atherosclerosis and thromboembolic complications. The increase in osmotic fragility of RBCs may be attributed to the disturbance of ionic motion through the membrane and the change in molecular properties of the membrane macromolecules.

Key words: Atherosclerosis, auto-oxidation, hemoglobin derivatives, lipid peroxidation, osmotic fragility of red blood cells

INTRODUCTION

The high polyunsaturated fatty acid content of the Red Blood Cell (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). The attack of Reactive Oxygen Species (ROS) on cell membranes results in formation of lipid peroxidation products such as malondialdehyde (MDA).

Atherosclerosis is the underlying cause of heart attack, stroke and peripheral vascular disease. It is a major cause of morbidity and mortality worldwide. The disease can generally be viewed as a form of chronic inflammation

that is induced and perturbed by lipid accumulation (Ross, 1999; Glass and Witztum, 2001). One of the initial events during the development of atherosclerosis is the accumulation of cells containing excess lipids within the arterial wall. Hyperlipidemia or a high level of serum triacylglycerol and cholesterol is a risk factor for premature atherosclerosis (Chobanian, 1991). In addition, it has been demonstrated that an increased intracellular generation of ROS plays an important role in chronic inflammatory responses to atherosclerosis (Berliner *et al.*, 1995; 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). Numerous oxygenated compounds, particularly aldehydes such as MDA and conjugated dienes, are produced during the attack of free radicals on membrane lipoproteins and polyunsaturated fatty acids (Esterbauer *et al.*, 1991).

It is well known that ROS can bring about damage to proteins by site-specific damage at metal binding sites and disulfide bonds in addition to fragmentation, cross-linking and aggregation, which may damage or change the functions of proteins. Moreover, it has been recognized that ROS are widely used as secondary messengers to influence gene expression (Allen and Tresini, 2000; Hensely *et al.*, 2000). The aim of this study was to assess the effects of hyperlipidemia on auto-oxidation rate of Hb, Hb derivatives and osmotic fragility of RBCs in rabbits fed on a HFD for a feeding period of 10 weeks.

MATERIALS AND METHODS

High fat diet rabbits: This project was conducted at 2009 in college of Science, King Saud University, Saudi Arabia. The HFD rabbits used in this study were twenty five (12-weeks) New Zealand white male rabbits obtained from the Laboratory Animal Centre (College of Pharmacy, King Saud University, Saudi Arabia). The animals were sacrificed by intravenous injection of Hypnorm (0.3 mL kg⁻¹) in accordance with the guidelines approved by King Saud University Local Animal Care and Use Committee. The rabbits were individually caged and divided into control group and HFD group. The control group (n = 10) was fed 100 g day⁻¹ of normal diet (Purina Certified Rabbit Chow # 5321; Research Diet Inc., New Brunswick, NJ 08901, USA) for a period of 10 weeks. The HFD group (n = 15) was fed a normal Purina Certified Rabbit Chow # 5321 with 1.0% added cholesterol plus 1.0% olive oil (100 g day⁻¹) for the same period of time. The rabbits were sacrificed following intravenous injection of heparin (400 U kg⁻¹ b.wt.).

Complete blood count: Blood samples of 2 mL were obtained from the rabbits via puncture of an antecubital vein. Blood was collected into two test 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. TC, LDLC, Hb, RBC, White Blood Cell (WBC), platelet, lymphocyte and neutrophil levels were measured at a Clinical Laboratory Center using an ADVIA 120 Hematology System (Bayer Medical, New York, USA).

Hemoglobin derivatives: Oxyhemoglobin (HbO₂), carboxyhemoglobin (HbCO), sulfohemoglobin (SHb) and met-hemoglobin (Met-Hb) were measured using the multicomponent spectrophotometric method for the simultaneous determination of Hb derivatives described by Atef *et al.* (1995).

Hemoglobin auto-oxidation rate: Measurement of the auto-oxidation rate, i.e. the absorbance of Hb at 630 nm versus time, was carried out spectrophotometrically as described by Guillochon *et al.* (1986).

Osmotic fragility of red blood cells: The osmotic fragility of RBCs was measured according to the method of Turgeon (1993). The test was performed within 1 h of blood collection. Whole blood was added to various concentrations of buffered NaCl (pH = 7.4 and temperature maintained at 25°C). The hemolysis percentage was calculated from the following equation:

$$\text{RBC hemolysis at a certain NaCl concentration (\%)} = \frac{A_1}{A_0} \times 100$$

where, A₁ is the absorbance of solution at a certain NaCl concentration and A₀ is the absorbance of solution at 0% NaCl concentration. Absorbance was measured using a Shimadzu UV-model 1601 spectrophotometer at wavelength of 540 nm.

Statistical analysis: The results were expressed as Mean±SE. To assess the significance of the differences between the control group and HFD group of rabbits, statistical analysis was performed using statistical software (SPSS/10 Windows) for repeated measurements, with significance assessed at 5% confidence level.

RESULTS

Table 1 shows the effect of HFD on TC and LDLC concentration (mg dL⁻¹) in serum of control and HFD rabbits. The TC concentration significantly increased (p<0.001) with percentage normalized change of 1198% in HFD rabbits compared with control rabbits. The LDLC concentration significantly (p<0.001) increased with percentage normalized change of 1591% in HFD rabbits compared with control rabbits.

Table 2 shows the percentage levels of HbO₂, HbCO, SHb and Met-Hb of HFD rabbits compared with control rabbits. A significant increase of the percentage level of

Table 1: TC and LDLC concentration in serum of control and HFD rabbits

Concentration (mg dL ⁻¹)	Control group (n = 10)	HFD group (n = 15)
Total cholesterol (TC)	54.6±16.4	708.7±14.8**
Low density lipoprotein (LDLC)	36.0±10.1	608.7±24.1**

Data expressed as Mean±SD; n: No. of animals; **p<0.01

Table 2: Hb derivatives of HFD rabbits compared with control rabbits

Parameters (%)	Control rabbits (n = 10)	HFD rabbits (n = 15)
HbO ₂	95.5±0.29	82±0.27**
HbCO	0.5±0.02	2±0.15
SHb	1.0±0.17	4±0.14
Met-Hb	3.0±0.09	12±0.18**

Data expressed as Mean±SE; n: number of animals; **p<0.01

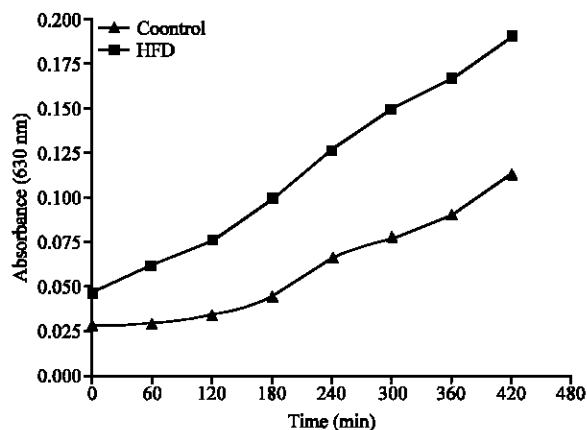


Fig. 1: Auto-oxidation rate of Hb of HFD rabbits compared with control rabbits

Met-Hb while a significant decrease of the percentage level of HbO₂ of the HFD rabbits compared with the control rabbits was observed. Non-significant increase of the percentage levels of HbCO and SHb of the hyperlipidemic rabbits compared with the control rabbits was observed.

Figure 1 shows the auto-oxidation rate of Hb of HFD rabbits compared with control rabbits. A significant (p<0.01) increase of the auto-oxidation rate of Hb of the HFD rabbits compared with the control rabbits was observed.

Table 3 shows the complete blood count of HFD rabbits compared with control rabbits. Significant decrease of the Hb concentration, percentage of hematocrit, RBC count and percentage of neutrophils while significant increase of the platelet count, WBC count and percentage of lymphocytes were observed in the HFD rabbits compared with control rabbits.

Figure 2 shows the results of osmotic fragility measurement for RBCs collected from HFD rabbits and control rabbits, where the percentage of hemolyzed cells has been plotted as a function of the percentage

Table 3: Complete blood count of HFD rabbits and control rabbits

Parameters	Control rabbits (n=10)	HFD rabbits (n = 15)
Hb (g dL ⁻¹)	13.36±0.30	10.83±1.50*
Hematocrit (%)	43.82±1.04	35.50±5.10*
MCV (fl)	71.56±1.58	82.34±2.59
MCH (pg)	19.73±1.50	23.11±1.66
MCHC (g dL ⁻¹)	27.67±2.48	28.06±2.02
RBC count (k μL ⁻¹)	6.21±0.12	4.28±0.93*
Platelet count (k μL ⁻¹)	358.79±85.75	419.66±78.80*
WBC count (k μL ⁻¹)	13.78±1.76	20.93±4.51*
Neutrophils (%)	57.80±9.94	36.33±18.83*
Lymphocytes (%)	39.40±8.96	60.00±2.00**

p<0.05; n = No. of animals; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration

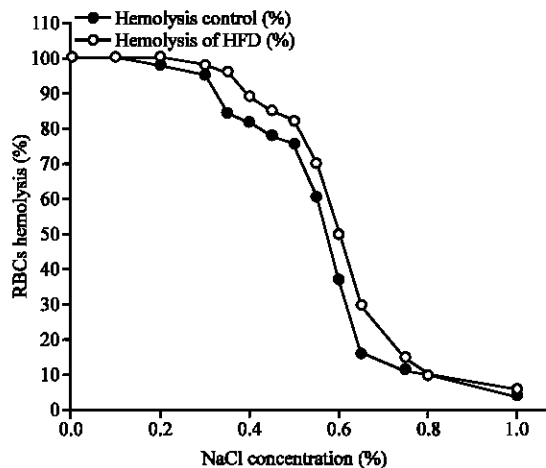


Fig. 2: Osmotic fragility of RBCs of HFD rabbits compared with control rabbits

concentration of NaCl. It is clear from the Fig. 2 that there was an increase of the hemolysis of RBCs of HFD rabbits compared with control rabbits.

DISCUSSION

It is known that the most important factor for the increase of free radical production is the condition of hypercholesterolemia, which can induce damage through overproduction of superoxide radicals in mitochondria (Baynes, 1991). Superoxide is converted to hydroperoxyls, which can diffuse through membranes and initiate lipid peroxidation. The oxidation of unsaturated lipids, specifically unsaturated fatty acids, has implications not only for atherosclerosis, but also for the stability and integrity of red cell membranes (Tribe and Poston, 1996). The lipid peroxidation marker MDA has been reported to be increased in hyperlipidemic rabbits (Esterbauer *et al.*, 1991; Gallow *et al.*, 1994). Hypercholesterolemia and abnormalities in lipoprotein metabolism, including their oxidation, are the most serious risk factors and important

early events in the pathogenesis of atherosclerosis (Maxfield and Tabas, 2005; Mallika *et al.*, 2007).

Hb (the most abundant and functionally important protein in RBCs), once released from RBCs, becomes highly toxic because of the oxidative properties of heme, which participates in the Fenton reaction to produce ROS causing cell injury (Puppo and Halliwell, 1988). The toxicity of heme is increased by heme hydrophobicity, which enables it to intercalate into lipid membranes and other lipophilic compartments when not associated with proteins (Balla *et al.*, 1993).

From our data, it is evident that the 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 HbO₂ which is the most important part of Hb, carrying oxygen to all parts of the body. Hyperlipidemia promotes the conversion of HbO₂ to Met-Hb and consequently, the fractions of unstable Hb molecules that undergo abnormal dissociation (auto-oxidation) to Met-Hb, SHb and HbCO were found to be increased in the hyperlipidemic rabbits compared to the control rabbits. As shown in Fig. 1, the auto-oxidation rate of Hb in the hyperlipidemic rabbits was higher than that observed in the control rabbits. Hyperlipidemia produces more ROS and other free radicals, which increase the auto-oxidation rate of HbO₂ to Met-Hb.

Atherosclerosis is classified as an inflammatory disease, since the presence of humoral and cellular components of the immune response has been detected within the atherosclerotic lesion. Increased production and release of activated leukocyte-derived products, such as ROS, enzymes and pro-inflammatory mediators, contribute to the development of the atherosclerotic process (Ross, 1999; Libby, 2002; Smith *et al.*, 2004; Fabiana *et al.*, 2009). The results of present study showed a significant decrease of Hb concentration in HFD rabbits, while the WBC count and platelet count were significantly increased. Therefore, in hyperlipidemia, lipid peroxidation and free radicals promote oxidation of Hb and reduce its concentration. The decreases in Hb concentration and RBC counts in the hyperlipidemic rabbits reflect the presence of anemia in these rabbits. A higher WBC 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 total leukocyte count are considered risk factors for cardiovascular diseases (Facchini *et al.*, 1992). A higher total leukocyte count may be part of the causal chain that leads to atherosclerosis and ischemic arterial diseases (Huang *et al.*, 2001).

It has been noted that vascular endothelial cells are activated by the presence of atherosclerotic risk factors, such as hypertension, hypercholesterolemia and hyperglycemia, therefore, promoting the increased synthesis and release of cytokines and chemokines into the circulation. An increased pro-inflammatory state enhances activation of WBCs and endothelial cells, thereby promoting platelet aggregation and thrombus formation (Ross, 1999). Alternatively, it has been noted that increased cell mass, increased platelet number and activation of platelets and leukocytes promote the formation of platelet-leukocyte aggregates, which contribute to the pathophysiological processes leading to increased risk for atherosclerotic disorders (Harrison, 2005; Lohsoonthorn *et al.*, 2007). It has become evident that atherosclerosis is an inflammatory disease, which involves hypercholesterolemia, low-density lipoprotein oxidation, increased oxidative stress and leukocyte recruitment and infiltration within the atheroma (Ross, 1999; Libby, 2002). The results of RBC membrane fragility presented here indicate a decrease in the membrane flexibility (increased fragility) of hyperlipidemic rabbits, which may be due to the disturbance of ionic motion through the membrane and/or the change in the molecular properties of the macromolecules forming the membrane. This change in the ionic mobility through the cellular membrane leads to changes in metabolic functions.

In conclusion, hyperlipidemia affects the level of Hb, which causes anemia, as well as affecting the auto-oxidation rate of Hb. Increased platelet activation in hyperlipidemic rabbits may be of pathophysiological importance for the progression of atherosclerosis and thromboembolic complications.

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