

HYPERLIPIDEMIA INDUCED BY ATHEROGENIC DIET ENHANCED OXIDATIVE STRESS IN THE KIDNEY AND INFLAMMATORY RESPONSES: AN IN-VIVO STUDY

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ABSTRACT

This study evaluated inflammatory processes and oxidative stress in hyperlipidemic condition induced by high lipid diet. Atherogenic diet prepared by enriching standard diet with 0.2% cholesterol and 0.6% groundnut oil was given to a set of rats while standard diet was given to control rats. Markers of inflammatory processes (interleukin 2 and tumour necrosis factor alpha), oxidative stress index (malondialdehyde), markers of renal damage (urea and creatinine), pro- and anti-oxidant elements (copper, zinc, iron, manganese, chromium, and calcium) were investigated and compared in the two groups. Results showed increased concentrations of serum total cholesterol, serum triglyceride, serum very low density lipoproteins; tissue total cholesterol and triglycerides in rats given atherogenic diet. In group where atherogenic diet induced high lipids concentrations, enhanced inflammatory processes, oxidative stress in the kidney, as well as higher serum concentrations of pro- and anti-oxidant elements were observed when compared with control rats. Conclusively, hyperlipidemia enhanced oxidative stress in renal tissue and inflammatory reactions.

Keywords: Hyperlipidemia, Lipids, Inflammation, Atherogenic, Elements, Cardiovascular

INTRODUCTION

One of the predisposing factors for atherosclerosis includes hyperlipidemia. Hyperlipidaemia is one of the major risk factors of CVD, which can be modified either by proper lifestyle changes, medical management or by the combination of both. It has emerged as the most important preventable and modifiable risk factors for coronary heart disease (CHD). Clinical signs of this condition are an increase in the fasting serum cholesterol level (hypercholesterolemia) or the fasting serum triglyceride level (hypertriglyceridemia) or both. This makes study of lipid profile in the general population very important in society (limbu *et al.*, 2008). In all the Asian countries, there is a concomitant rise in the level of serum total cholesterol (TC), and with it a rise in cardiovascular diseases. Serum TC levels had been reported to be higher in the urban compared with the rural population (khoo *et al* 2003). Hyperlipidemia is called primary if it is inherited and secondary if it is caused by illness or other health problem. Observations have shown that hyperlipidemia, if not managed on time, results in cardiovascular diseases such as, atherosclerosis, stroke, hypertension etc. Plasma hyperlipidemia generates increased transcytosis of atherogenic lipoproteins, leading to their accumulation within and outside the endothelial cell hyperplastic basement membrane, against the fragmented internal elastic lamina. (Simionescu *et al* 2009). Modification of the low density lipoproteins (LDL), mostly by oxidation, is believed to play an important role in human atheroma formation. (Holvoet *et al* 2000). Series of hypotheses have been postulated to be the cause of atherosclerosis and other cardiovascular disorders. For instance, Williams

and Tabas, (1995) attributed atherosclerosis to inflammatory processes in the vessel wall in response to retained low density lipoprotein (LDL) molecule. Also, Fabricant and Fabricant, (1999) believe that atherosclerosis may be due to an infection of the vascular smooth muscle cells. However, growing evidence suggests that atherosclerosis is an immune-mediated inflammatory process and that cytokines participate as mediators in this process. Interleukin 2 (IL-2), a cytokine produced by activated T-lymphocytes, has been found to further activate the T-cells and may potentially enhance atherogenesis. Study had shown that IL-2 is involved in the pathogenesis of atherosclerosis and vascular disease by modulating the responsiveness to angiotensin II in vascular smooth muscle cells (Carlos, 2006). Serum levels of IL-2, a pro-inflammatory cytokine, are associated with carotid artery IMT, a predictor of stroke and vascular disease.

Conflicting reports have shown involvement of elevated triglyceride and cholesterol in the enhancement of oxidative stress and inflammatory processes. This study evaluated in-vivo, the possible modulatory role of hyperlipidemia induced by atherogenic diet on inflammatory processes and oxidant/antioxidant status using an animal model.

MATERIALS AND METHODS

Preparation Of Atherogenic Diet

Atherogenic diet is a type of diet capable of starting or speeding up the development of atherosclerosis. Atherogenic diet consist of high fats, high cholesterol foods and can lead to serious health problems overtime (Allison, 2011). Atherogenic diet was prepared according to method of Saso et al., 1992.

Table 1. Compositions of both standard and atherogenic diets

<i>Compositions</i>	<i>Weight in Kg</i>	<i>Standard diet (%)</i>	<i>Atherogenic diet (%)</i>
Maize	8.97	18.00	17.678
Soya bean meal	3.74	8.00	7.37
Brewery dry grain (BDG)	17.50	35.00	34.49
Wheat bran (WB)	5.00	10.00	9.85
Rice bran (RB)	12.50	25.00	24.635
oyster shell (OS)	1.00	2.00	1.97
Bone meal (BM)	0.50	1.00	0.985
Common salt (CS)	0.13	0.50	0.256
methionine	0.50	0.10	0.985
Fish meal	0.50	0.10	0.985
Groundnut oil	0.30	-	0.59
Cholesterol	0.10	-	0.197

Animals and Treatments

Twenty rats with average weight of 200g were grouped into 2. They were kept in a well-ventilated animal house of the Department of Anatomy, Ladoke Akintola University of Technology, Nigeria. The animals had unrestricted access to clean water. Animals in group 1 was given a standard diet (table 1) while those in group 2 were given atherogenic diet containing 0.2% cholesterol and 0.6% groundnut oil in standard diet (table 1). The feeding was done for 8 consecutive weeks. During this period, the weight of each of the rats was measured and daily consumptions were monitored. At the 8th week, the animals were sacrificed for collection of samples which included blood and organs for further analyses.

Collection of Blood Sample

Blood was collected directly from the heart into plain and well-labelled sample bottles and were centrifuged to obtain serum for analysis of biochemical parameters which included the lipids and lipoproteins.

Histological Study

The liver was quickly excised and immediately placed on blotting paper to remove blood. The tissues were then placed in 10% formalin solution in appropriately labeled sample bottles for histological studies. The tissues of the organ was removed and fixed in Bouin's fluid for 24 h. After fixation, the tissues were dehydrated through ascending grades of ethanol. Thereafter, it was cleared in xylene and finally embedded in paraffin wax. Using a rotary microtome, specimens were sectioned at 5 mm and sections were mounted on clean slides and stained with sudan blue.

Preparation of Tissue Homogenates

The liver was quickly excised and immediately placed on a blotting paper to remove the blood. Samples of organs were immediately rinsed in 1.15% of potassium chloride solution to remove the hemoglobin. The organ samples were homogenized in aqueous potassium phosphate buffer (0.1M, pH 7.4) in volumes of four times the weight of samples using a Teflon homogenizer. The resultant homogenates were centrifuged at 10,000g for 20 minutes to obtain the post-mitochondrial supernatant fraction (PMF). The PMF was decanted into sample bottles and stored at - 80°C prior to use. The tissue homogenates of the organs were used to assay for the lipids and lipoproteins.

Analyses of Lipids, Lipoproteins, Total Proteins and Albumin

Total cholesterol, HDL-C, LDL-C, triglyceride, total proteins and albumin were analyzed using spectrophotometric methods. Globulin concentration was calculated from the difference between total protein and albumin concentrations. Total cholesterol concentration in the serum was determined spectrophotometrically using the cholesterol oxidase method at 546 nm, 37°C (Allain, 1974). HDL-C was determined by spectrophotometric method of (Assmann, 1983) at 500 nm, 37°C. Triglycerides were determined using spectrophotometric method (Buccolo, 1973). Phospholipids were determined by method described by Takayama, 1977. Total protein concentration was determined using the Biuret method (Weischselbaum 1946).

Estimation of Serum Trace Elements

The sample i.e. serum was digested using 0.2N hydrochloric acid. Briefly, 0.6ml of serum was pipette into test tube and 10ml of 0.2N hydrochloric acid was added. The mixture was kept overnight. The following morning, the mixture was placed in water bath with temperature of 37°C till colour changed to pale yellow. The tube was removed from the water

bath and volume of mixture was made up to 15ml with 0.2N hydrochloric acid. Direct measurements of elements were carried out using atomic absorption spectroscopy (AAS) Bulk Scientific, Model 200A.

Statistics

All data are presented as means \pm SEM; where N is the number of experiments. Statistical significance was determined by Student's *t* test for independent samples; $P < 0.05$ was considered statistically significant.

RESULTS

Table 2. Serum concentrations of cytokines, albumin, creatinine and urea in animals given standard diet and atherogenic diet

Biochemical parameters	Group given standard diet	Group given atherogenic diet	p-value
IL-2	4.48 \pm 0.57	7.65 \pm 3.83	0.14
TNF- α	370 \pm 279	1383 \pm 1040	0.10
Creatinine	1.22 \pm 0.26	1.16 \pm 0.06	0.68
Urea	43.80 \pm 3.85	43.35 \pm 2.32	0.32
Albumin	1.382 \pm 0.155	1.342 \pm 0.155	0.73
Total Protein	4.27 \pm 0.51	3.69 \pm 0.45	0.16

Table 3. Serum concentrations of lipids, lipoproteins in both control animals and those given atherogenic diet

Biochemical Characteristics	Group given standard diet	Group given atherogenic diet	p-value
Total cholesterol (mg/dl)	30.78 \pm 3.52	36.60 \pm 12.60	0.44
Triglyceride (mg/dl)	50.63 \pm 6.16	64.30 \pm 12.70	0.24
Phospholipid mg/dl	303.00 \pm 110	306.30 \pm 82.5	0.97
VLDL-C (mg/dl)	18.71 \pm 5.98	35.80 \pm 2.71	0.007

Table 4. Tissue concentrations of lipids, lipoproteins and proteins in both control animals and those given atherogenic diet

Biochemical Parameters	Kidney		Liver		Heart	
	Group given standard diet	Group given atherogenic diet	Group given standard diet	Group given atherogenic diet	Group given standard diet	Group given atherogenic diet
T. Cholesterol	29.81 \pm 7.84	174.14 \pm 8.18	36.1 \pm 24.9	182.14 \pm 9.47	4.19 \pm 1.24	174.90 \pm 14.7
Triglyceride	18.47 \pm 1.93	63.44 \pm 6.38	17.19 \pm 3.81	11.91 \pm 4.33	8.33 \pm 2.12	37.26 \pm 4.54
Albumin	2.57 \pm 0.01	2.79 \pm 0.20	2.64 \pm 0.12	2.81 \pm 0.25	2.66 \pm 0.02	2.54 \pm 0.06
Malondialdehyde	615 \pm 235	789 \pm 105	747 \pm 142	587 \pm 111	626.1 \pm 72.0	393 \pm 245

Table 5. Serum concentrations of elements in both control animals and those given atherogenic diet

<i>Biochemical Characteristics</i>	<i>Group given standard diet</i>	<i>Group given atherogenic diet</i>	<i>p-value</i>
Magnesium	28.83±4.06	27.72±4.12	0.72
Copper	2.73±0.31	2.94±0.24	0.32
chromium	0.78±0.15	1.14±0.10	0.01
Calcium	52.40±17.7	97.4±31.6	0.06
Iron	11.06±0.64	11.74±1.25	0.39
Zinc	1.32±0.39	1.40±0.21	0.75
Manganese	0.10±0.06	0.12±0.05	0.69

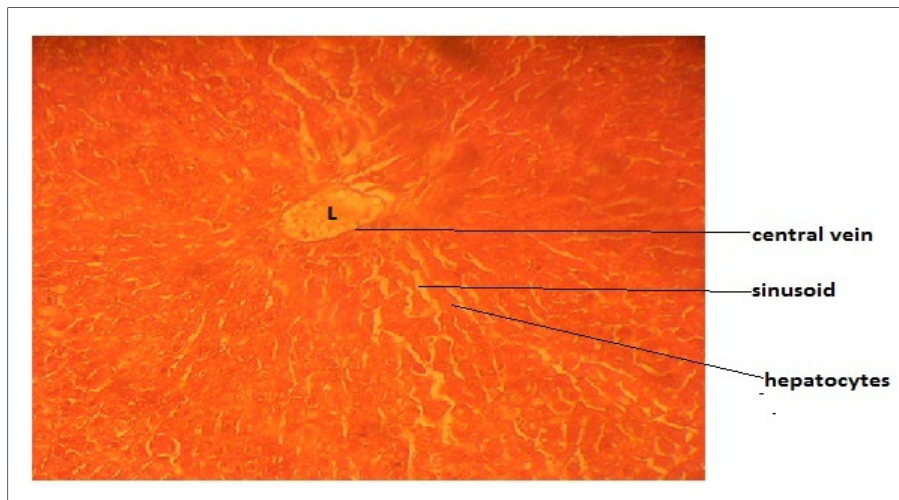


Figure 1. Cross section of liver of rat given normal diet, Normal central vein and hepatocyte sinusoid. Stain: Sudan blue, Magnification=400

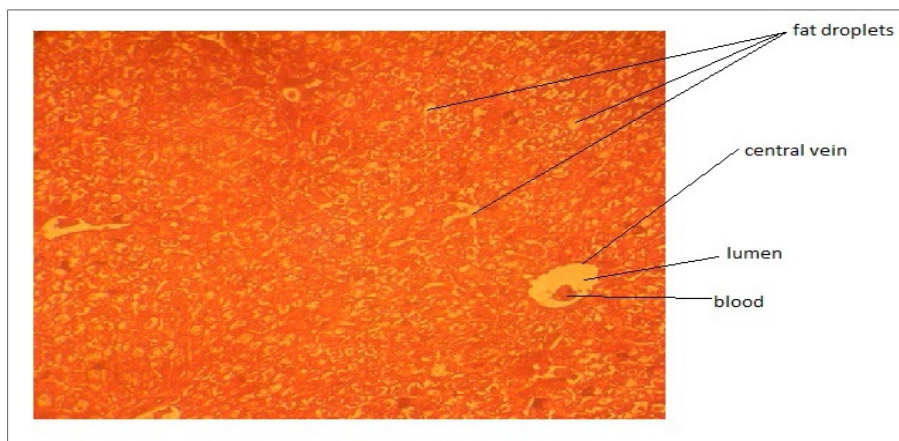


Figure 2. Cross section of liver of rat given atherogenic diet, Degeneration of hepatocytes with evidence of multifocal cellular fatty inclusion. Stain: Sudan blue, Magnification= 400

DISCUSSION

Cardiovascular diseases, as a group, is a leading cause of the death worldwide, causing over 16.7 million deaths globally in 2002 (Mackay and Mensah, 2003). **In fact**, since 1990, greater than 85,000,000 disability-adjusted life-years were lost worldwide due to coronary heart diseases (CHD) and stroke; this CHD disease burden is projected to rise to 143,000,000 disability-adjusted life-years by 2020 (Mackay and Mensah, 2003; Fon Tacer *et al.*, 2007). The increases recorded in the serum concentrations of total cholesterol, triglycerides, phospholipids, low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) in rats fed atherogenic diet (Table 3), may be adduced to excess lipids supplied in their diet. Various reports have it that the consumption of lipid rich diets correlate with increases in serum lipid levels (Hegsted *et al.*, 1965; Hu *et al.*, 2001). Therefore, the increased serum levels in the lipid rich lipoproteins (LDL-C and VLDL-C) indicate that more cholesterol and triglyceride are been transported from the liver to the extra-hepatic tissues to be taken up by those tissues. The increase in the serum HDL concentration (Table 3) may be due to the boost of HDL- C biosynthesis majorly in the liver and partly in the small intestine. HDL- C particles are formed basically from ApoAI and apoAII Apo lipoproteins, whose expression were shown to be influenced by nutritional interventions, such as a switch from high-carbohydrate to a high-fat diet to lipid rich diet that was reported to increase the production rate of apoA-I rather than its clearance (Lewis and Rader, 2005; **Jiang *et al.*, 2006**). In addition, HDL-C plays a key role in the reverse cholesterol transport pathway, and a balanced homeostasis of lipid and lipoprotein metabolisms are ensured under normal physiological conditions Genest *et al.*, 1990; Jiang *et al.*, 2006). Thus, an increased intake in dietary lipids, under normal physiological conditions proportionately increases the need for lipoproteins for effective distribution and metabolism of the exogenous lipids.

The pattern obtained in the serum cytokine concentrations [interleukin 2 (IL-2) and tumor necrosis factor alpha (TNF- α)] in rats fed with atherogenic diet (Table 2) is an indicator of chronic inflammation that might be associated with hyperlipidemia, as recorded in this study. Cytokines have been established to regulate the metabolism of lipids and lipoproteins. For instance, high serum levels of TNF- α , IL-6 and IL-1 are reported to inhibit cholesterol and triglyceride biosynthesis *in vivo*, while IL-2 could induce severe chronic hypocholesterolemia that is mediated by the inhibition of lecithin: cholesteryl acyltransferase (LCAT) activity (Michiel and Oppenheim, 1992; Kwong *et al.*, 1997).

The interplay between inflammation and lipid metabolism has recently been the focus of research aimed at understanding the development of metabolic syndrome and mechanisms of atherogenesis (Getz, 2005; Steinberg, 2005). In fact, over fifteen years, the prominent role for inflammation in the pathogenesis of atherosclerosis has been established (Libby *et al.*, 2002; Langley-Evans and Carrington, 2006; Oyewo *et al.*, 2012). There is increasing evidence that cytokines in general, especially tumour necrosis factor (TNF- α) plays an important role in cardiovascular disease. This is not surprising since TNF- α modulates both cardiac contractility and peripheral resistance, which are the two most important haemodynamic determinants of cardiac function. Thus, increased levels of TNF- α or of its soluble receptors have been implicated in the pathophysiology of ischaemia-reperfusion injury, myocarditis, cardiac allograft and, more recently, also in the progression of congestive heart failure (Ferrari, 1999). High serum levels of IL-2 was reported also to result in adverse cardiovascular and hemodynamic effects similar to septic shock (White *et al.*, 1994; Vial and Descotes, 1995) that can lead to hypotension, vascular leak syndrome, and myocardial infarction, cardiomyopathy, and thrombotic events such as deep vein thrombosis, pulmonary embolism, and arterial thrombosis (Olsen *et al.*, 2001).

In addition, previous studies reported that cholesterol elimination through bile acid synthesis and export is strongly inhibited by increased serum levels of TNF- α , which also favours fatty acid synthesis rather than fatty acid oxidation (Khovidhunkit *et al.*, 2004; Tacer *et al.*, 2007). Therefore, cholesterol and its precursors can be subjected to harmful oxidation that can intensify atherogenesis, because increased blood levels of TNF- α is implicated in the down regulation of many genes involved in defense mechanisms against oxidative stress (Libby *et al.*, 2000; Li *et al.*, 2003). For example, increased serum TNF- α level inhibits the expression of paraoxonase, the major apo-lipoprotein that protects LDL-C from oxidative stress, which is a valuable marker of atherogenic changes (Feingold *et al.*, 1998; Li *et al.*, 2003).

The result obtained in tissue total cholesterol and triglycerides levels of rats fed with atherogenic diet (Table 4) supported the results of the serum lipids concentrations (Table 3). Since there were increases in the serum levels of LDL-C and VLDL-C, then more cholesterol and triglyceride are deposited and taken up (endocytosed) at the extrahepatic tissues, which is reflected in (Table 4). That is, the increase in dietary lipids in rats fed the atherogenic diet invariably increased the need for lipoproteins for the effective distribution and metabolism of the exogenous lipids.

Serum albumin levels are determined by rates of hepatic synthesis and secretion, exchanges between the intra- and extravascular compartments, lymphatic uptake, alterations in volume of distribution (including hemodilution), protein degradation, and body losses (Friedman and Fadem, 2010). The decreased serum total protein and albumin concentrations in the rats fed with atherogenic diet (Table 2) might be an indicator of nutrition imbalance, liver disease, increased protein utilization or degradation, increased glucocorticoids and the increased serum TNF- α level recorded in the study (Table 2). It is well established that albumin levels fall in patients with inflammatory disorders and other illnesses (Moshage *et al.*, 1987; Friedman and Fadem, 2010). Possible contributory mechanisms include downregulated production of albumin mRNA by the liver. The mRNA concentration available for action on ribosomes is an important factor controlling the rate of albumin synthesis. Trauma (oxidative stress) and disease processes will affect the albumin mRNA content by a decrease in gene transcription, as seen in the acute-phase reaction mediated by cytokines, mainly interleukin-6 (IL-6) and tumour necrosis factor α (TNF- α) (Peters, 1996^{a,b}). Therefore, the reduced levels of serum albumin might cause fluid to escape into extravascular tissues spaces, leading to increased vascular permeability, evident in localized oedema and reduce delivery of nutrients to the tissues.

In addition, the reduction in serum albumin and heart albumin concentrations might indicate a reduction in the antioxidant pool of the rats fed the atherogenic diet. Under physiological conditions, albumin may have significant antioxidant potential (Hu *et al.*, 1993). It is involved in the scavenging of oxygen free radicals, which have been implicated in the pathogenesis of inflammatory diseases. Physiological solutions of human serum albumin have been shown to inhibit the production of oxygen free radicals by polymorphonuclear leukocytes (Hu *et al.*, 1993). This may be related to the abundance of sulfhydryl (-SH) groups on the albumin molecule. These are important scavengers of oxidizing agents, such as hypochlorous acid (HOCl) formed from the enzyme myeloperoxidase, which is released by activated neutrophils (Phillips *et al.*, 1989; Hu *et al.*, 1993; Kaufmann *et al.*, 1995). *Although, the liver is the major organ involved in the synthesis of albumin, while the kidney is involved in the reabsorption and excretion of serum albumin, the result obtained the kidney and liver albumin concentrations did not support the reduction in the antioxidant activities.*

Serum urea and creatinine concentrations are important prognostics in kidney problems. They are molecules, whose blood levels are tightly regulated by the excretory activities of the

kidney. The pattern obtained the serum urea and creatinine concentration in the atherogenic rats (Table 2) did not indicate a gradual loss of kidney function. The concentrations of serum electrolytes are good markers for assessing kidney function. In this study, of the entire serum electrolytes quantified, the increase in serum Ca^{2+} concentration in the rats given atherogenic diet (Table 5) might not suggest a gradual loss of kidney function. This is because other markers of kidney functions indicated the kidney functions were not possibly affected. Nonetheless, the increase in the serum Ca^{2+} concentration might be caused by the concentration of TNF- α . Tracey and Cerami (1994) reported that increased serum levels of TNF- α caused calcium to be released from bone, promoting osteoporosis.

The fat droplets revealed in the photomicrograph of the liver of rats fed the atherogenic diet indicated that the hepatocytes were fat laden. This observation is in agreement with the result of the liver total cholesterol and TNF- α concentration (Table 4) and (Table 2) respectively. Fon Tacer *et al.* (2007) reported that increased serum TNF- α concentration induces proatherogenic alterations in the liver by blocking the elimination of cholesterol through bile acid synthesis and the inhibition of fatty acid oxidation.

Consumption of high lipid diet in this study caused increased serum concentration of copper. Also, an increased serum and tissue concentrations of lipids was accompanied by elevated serum copper concentration. Cells have highly specialized and complex systems for maintaining intracellular copper concentrations [Dijkstra, 1996]. If this balance is disturbed, excess copper can induce oxidative stress that could lead to chronic inflammation [Sternlieb, 1980; Thornburg, 2000]. Copper induced hepatitis has been described both in humans (Wilson's disease) as well as in dogs. It is most likely that the disturbed serum copper concentration induced by high lipid diet led to increased oxidative stress in kidney and inflammatory processes. On the other hand, copper ions participate in radical reactions such as the conversion of superoxide to hydrogen peroxide and hydroxyl radicals, and catalyze the oxidative modification of LDL in vitro and in the arterial wall; copper excess can induce oxidative damage to DNA (2,3 Bremner, 1996; Ferns *et al.*, 1997). In this study, increased concentration of lipid peroxides (malondialdehyde) which is an index of oxidative stress was found in rats given atherogenic diet.

There is evidence that copper and zinc have pro-oxidant and antioxidant properties, respectively, so that their imbalance may be expected to condition oxidative stress status (Guo and Wang, 2013). Oxidative stress is relevant in aging and in age-related degenerative diseases. In this study, blood content of copper, zinc, markers of inflammation (interleukine-2 and tumour necrosis factor- α) as well as of lipid peroxides (malondialdehyde) were investigated in rats given standard diet and atherogenic diet. Serum copper/zinc ratio, cytokines and malondialdehyde (in kidney) were significantly higher in the rats given atherogenic diet than control. Notably, the increased copper/zinc ratio found in atherogenic group was due to high copper values. The higher the serum copper/zinc ratio the higher the lipid peroxides kidney tissue content. Mezzetti *et al.*, 1998 concluded that there is a strict relationship between copper/zinc ratio and systemic oxidant burden. Moreover, advanced age and, particularly, advanced age-related chronic degenerative diseases are associated with a significant increase in the copper/zinc ratio and systemic oxidative stress.

Increased serum concentrations of manganese (20%), iron (6.15%), and chromium (46.15%) were seen in rats that consumed high lipid diet compared with control rats. Studies have shown beneficial effects of manganese, iron and chromium in protecting body against oxidative stress and related metabolic disorders. For instance, oxidative damage due to free radicals is associated with vascular disease in people with type 1 and those with type 2 diabetes mellitus (DM) [Oberley, 1988]. However, studies have reported the beneficial

effects of supplemental chromium on plasma glucose and related variables of people with type 2 DM and steroid-induced diabetes [Anderson et al., 1997; Ravina et al., 1999; Ravina et al., 1999]. There are also suggestive studies to show that chromium also improves cellular antioxidant capacity in rats [Tezuka et al., 1991; Preus et al., 1998; Ueno et al., 1998]. The increased concentrations of manganese, iron and chromium may be in response to elevated serum and tissue lipid concentrations caused by atherogenic diet.

Serum malondialdehyde was significantly elevated in kidney tissue of rats given high lipid diet compared with control (615 ± 235 vs. 789 ± 105), thus confirming that lipid peroxidation increases in hyperlipidemic rats. The source of the lipid peroxides is postulated to be the end products from membrane damage which are elevated in rats with atherogenic diet. These elevated levels of peroxides could result from the hyperlipidemic state in relation with auto oxidation of plasma glucose and other small autooxidizable molecules [Hunt and Wolf, et al., 1991] and are associated with poor metabolic control of plasma glucose [Nourooz et al., 1997].

CONCLUSION

The consumption of atherogenic diets (dietary cholesterol) triggered oxidative stress in rats with concomitant induction of inflammatory markers that could lead to cardiovascular diseases or the formation of arterial lesions.

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