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A Diet-induced Atherosclerosis in Rabbit Model Provides an Insight into Essential Elements Concentrations in Cardiovascular Disease

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Authors' contributions

This work was carried out in collaboration between all authors. Author ASA designed the work, wrote the protocol, wrote the first draft, actively involved in the laboratory works. Author TIA did the statistical analysis, actively involved in the laboratory works. Author JOF was actively involved in the laboratory works. Author BO was actively involved in the laboratory works, involved in literature searches. All authors read and approved the final manuscript

Research Article

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ABSTRACT

Aim: To assess the effect of hyperlipidemia and atherosclerosis on essential minerals.
Study Design: Animal model was used for this study.
Place and Duration of Study: Department of biochemistry and department of Anatomy, Ladoke Akintola University of Technology, Ogbomoso. January, 2012 to October, 2012.
Methodology: We included 8 white rabbits which were divided into 2 groups, 1 (control i.e. rabbits given normal diet) and 2 (rabbits given standard diet plus 0.2% cholesterol and 0.6% groundnut oil i.e. atherogenic diet). Feeding was for 8 weeks. The minerals were determined using atomic absorption spectrophotometer; lipids and lipoproteins were determined spectrophotometrically while the effects of atherosclerosis on heart and kidneys were determined chemically and histologically.
Results: The results showed elevated serum concentrations of total cholesterol (4.05%), triglyceride (28%), high density lipoproteins (12.17%), very low density

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lipoprotein (70%) in atherosclerotic group when compared with control. Atherosclerosis caused decreased serum concentrations of copper (13.88%), manganese (23%), iron (26.46%) in atherosclerotic group when compared with control. Atherosclerosis induced elevated serum concentrations of zinc (47.34%), chromium (37.21%), calcium (72.04%) and magnesium (125.13%). Except for chromium, significant positive correlations were observed between serum cholesterol and trace elements. Induction of atherosclerosis resulted in impaired renal function shown by elevated concentrations of urea (34.80%) and creatinine (147.54%). Renal histology showed cellular necrosis of the epithelial squamous cells. In the aorta and brachiocephalis of the atherogenic diet fed rabbits, there were large number of smooth muscle like cells and focal aggregation of foam cells resulting in intima thickness.

Conclusion: This study further emphasized the monitoring of systemic concentrations of essential minerals in cardiovascular disorder as this may prevent occurrence of another pathologic condition.

Keywords: Atherosclerosis; hyperlipidemia; minerals; rabbits; cardiovascular disease.

1. INTRODUCTION

Atherosclerosis is a complex multifactorial disease, which develops in the arterial wall in response to various stimuli and results in excessive inflammatory and fibro proliferative reactions. Cardiovascular disease (CVD) due to atherosclerosis is the leading cause of morbidity and mortality in westernised countries. Atherosclerosis is a complex disease, involving many cell types and circulating mediators and resulting in an inflammatory state. Atherosclerotic lesions form *de novo* from focal accumulation of lipoproteins, monocyte-derived macrophages, and lymphocytes within the arterial wall. These lesions can develop as early as the second decade of life and progress into clinical disease over time. The formation of plaque in the arterial intima may be due to hyperlipidemia which may include increased serum concentrations of total cholesterol and low density lipoprotein concentration. However, despite recent advances in cardiology, atherosclerosis remains an important medical problem.

In United States of America's data for the year 2004, the first symptom in 62% of man and 47% of women suffering from atherosclerotic cardiovascular disease, is heart attack or sudden death [1]. In Finland, cardiovascular heart disease is also the cause of every fourth death in the working-range population i.e. 15-64 years, and is the most single death in the whole population. The percentage of premature death from cardiovascular disease ranges from 4% in high-income countries to 42% in low-income countries. This high incidence of death in low-income countries therefore made the study of lipid profile in the general population important in this society. Study had equally shown rise in the level of serum total cholesterol (TC) particularly in urban setting in Asian countries [2].

Atherosclerosis affects many important biochemical and/or physiological processes in the body. These include loss of ability of intima endothelium to secrete nitric oxide (NO) which is a vasoprotective gas against vascular inflammation. Minerals and trace elements are very important requirements for proper functionality and susteinance of life. For trace elements, they are inorganic compounds that, like vitamins, are essential for health and needed only in small amounts, known as reference nutrient intake. They are needed for normal health and make up less than 0.01 percent of the body's dry weight [3]. Some of them function as cofactors for enzymes, hormones etc. The role of these elements cannot be over-

emphasized as their deficiencies can lead to unimagined debilitating diseases. Also, the disordered homeostasis of these all important elements have been reported to cause series of human diseases which include bone disease, infertility, hypochromic anemia, macrocytosis etc.

It is well established that several trace elements are of great importance in a number of biological processes, mostly through their action as activators or inhibitors of enzymatic reactions, by competing with other elements and proteins for binding sites, by influencing the permeability of cell membranes, or through other mechanisms. It is therefore reasonable to assume that these minerals would also exert an action, either directly, or indirectly, on the cardiac cell, on the blood vessel walls, on the blood-pressure-regulating centres, or on other systems related to cardiovascular function such as, e.g. the lipid and carbohydrate metabolism. Man-made alterations of the environment, such as the use of fertilizers, food additives, food processing and canning, treatment and softening of drinking water, and the industrial pollution of air and water, may bring about changes in the mineral balance and, as a consequence, in some biological functions, including the cardiocirculatory function. Other pathologies associated with deficiency and/or toxicities of the minerals/elements include infertility, osteomalacia, Wilson's disease, vitamin K deficiency, hypochromic anemia, lung cancer etc. In recent years, epidemiological, clinical, pathological and experimental evidence has accumulated which justified undertaking deeper studies of trace elements in cardiovascular diseases. The present study assessed the levels of selected elements in diet-induced atherosclerotic condition.

2. METHODOLOGY

2.1 Preparation of Atherogenic Diet

2.1.1 Preparation of diets

Both standard and atherogenic diets are prepared according to the table below.

Table 1. Compositions of both standard and atherogenic diets

Compositions	Weight in Kg	Standard diet (%)	Atherogenic diet (%)
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

2.2 Animals and Treatments

Eight rabbits with average weight of 800g 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 2). The feeding was done for 8 consecutive weeks. During this period, the weight of each of the rabbits was measured and daily consumptions were monitored. At the 8^{th} week, the animals were sacrificed for collection of samples which included blood and organs for further analyses.

2.3 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 analyses of biochemical parameters.

2.4 Histological Study

The kidney 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 haematoxylin and eosin.

2.5 Preparation of Tissue Homogenate

The kidney 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 - 80oC prior to use. The tissue homogenates of the organs were used to assay for the lipids and lipoproteins.

2.6 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 37ºC 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.

2.7 Analyses of Lipids, Lipoproteins

Total cholesterol was determined using enzymatic methods of Allain et al. [4], high density lipoprotein cholesterol was determined using method of Assmann et al. [5], triglyceride was determined using method of Buccolo and David [6], low density lipoprotein cholesterol was calculated using Friedwald equation [7], i.e. LDL-C=total cholesterol–(high density lipoprotein cholesterol+0.2(triglyceride)), very low density was calculated as 1/5(triglyceride). Phospholipids were determined using method of Takeyama [8].

2.8 Statistics

All data are presented as means ± standard mean of error; 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.

3. RESULTS

3.1 Pathological Findings of Aortic and Brachiocephalis.

3.1.1 Macroscopic findings

Luminal surface of the aorta and brachiocephalis of rabbits fed atherogenic diet were strongly stained compared with those given standard diet. Aorta and brachiocephalis walls of the atherogenic diet fed rabbits were thicker and harder than those fed standard diet (Figs. 1-4).

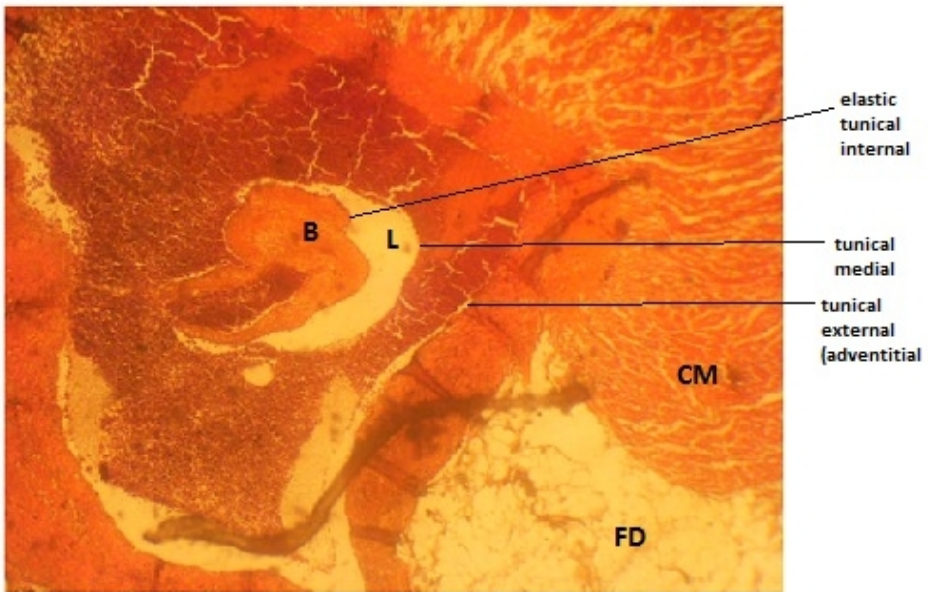

Fig. 1. Cross section of aorta of rabbits given normal diet. The features appears normal. The cross section exemplifies a typical elastic artery.
Stains : hematoxylin and eosin. Magnification=400. B=blood; L=lumen; CM=cardiac muscle.

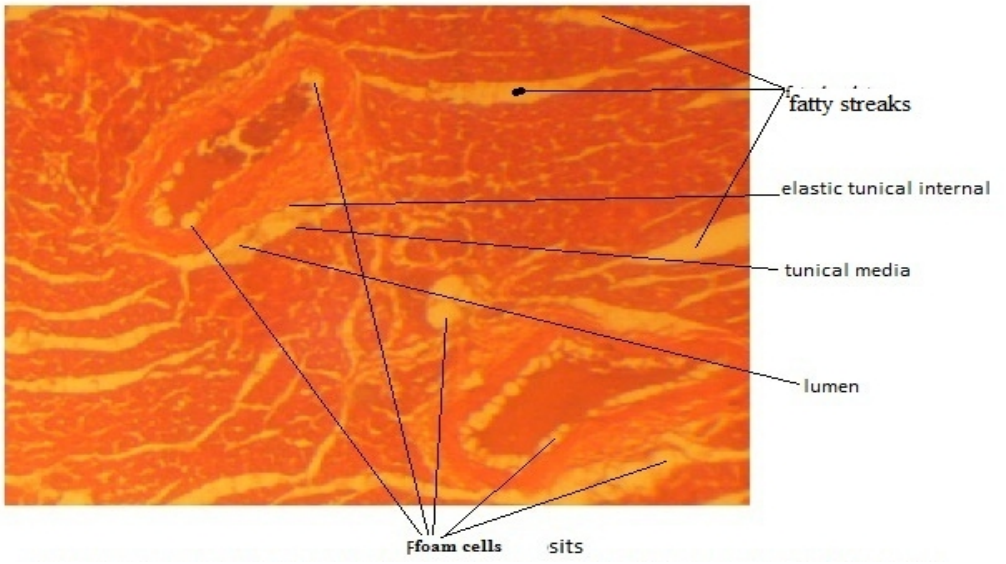

Fig. 2. Cross section of aorta of rabbits given atherogenic diet. Degeneration of the blood vessels as a result of fat deposition at the luminal side of the circular musculature

Stain : *Sudan blue. Magnification=400. B=blood; L=lumen; CM=cardiac muscle.*
There is increased thickness of the intima, aggregation of foam cells on the intima, formation of lesions as depicted by formation of fatty streaks.

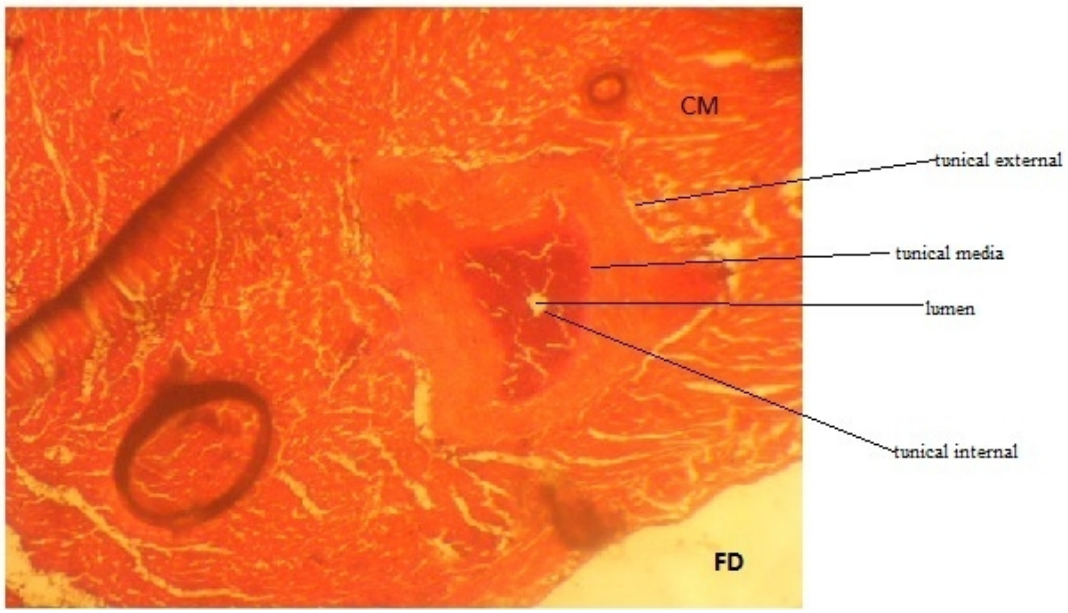

Fig. 3. Cross section of brachiocephalis artery of rabbits given normal diet. The features appear normal.

Stain : *Hematoxylin and eosin. Magnification=400. B=blood; L=lumen; CM=cardiac muscle.*

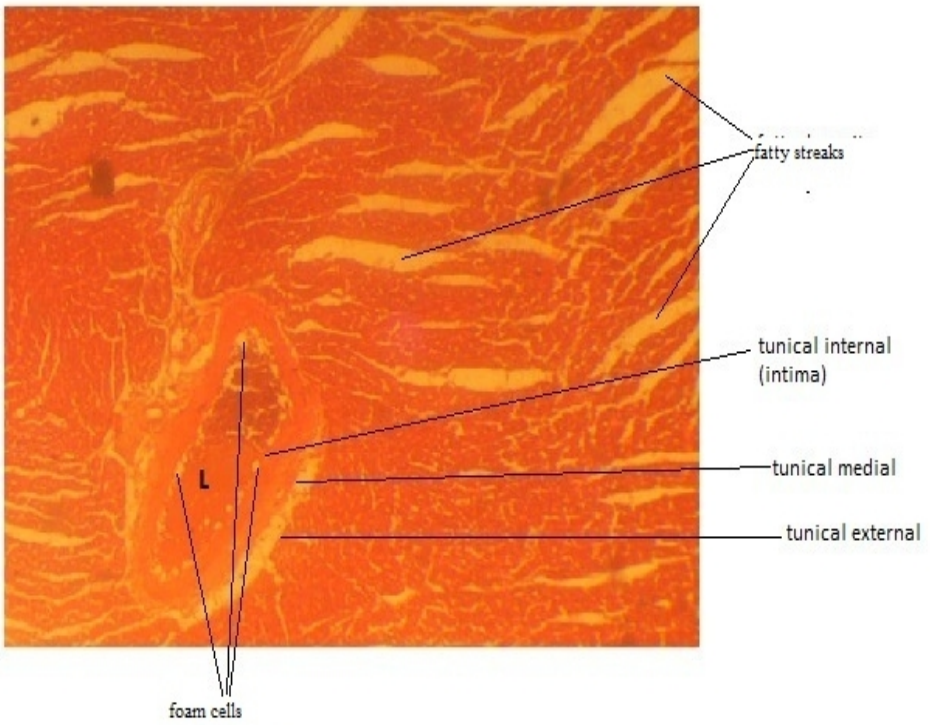

Fig. 4. Cross section of brachiocephalis of rabbits given atherogenic diet. There is severe fat droplet at the luminal side of circular musculature.
Stain: *Sudan blue. Magnification=400. B=blood; L=lumen; CM=cardiac muscle.*
There is increased thickness of the intima, aggregation of foam cells on the intima, presence of lesions as represented by occurrence of fatty streaks.

3.1.2 Light microscopic findings

In the aorta and brachiocephalis of the atherogenic diet fed rabbits, there were large number of smooth muscle like cells and focal aggregation of foam cells resulting in intima thickness. Lipid droplets and fatty streak were found in the smooth mucle cells and foam cells in the thickened intima (Figs. 2 & 4). Both foam cells and fatty streaks belong to TYPE II LESIONS [9]. These are based on classifications of atherosclerotic lesions by Fuster et al. [10] and Stary [11], which included the following foam cells, fatty streaks, preatheroma, atheroma, fibroatheroma and complicated lesions.

There were moderate to severe fat deposition in aorta and brachiocephalic tissues of the artery of the rabbits fed atherogenic diet when compared with normal.

Results also showed increased but non-significant ($p \geq 0.05$) concentrations of total cholesterol, triglyceride, high density and very low density lipoprotein cholesterol in group given atherogenic diet. Mean serum concentration of low density lipoprotein cholesterol was reduced in group given atherogenic, however, this difference was not statistically significant (Table 2).

For the essential minerals, serum concentrations of zin, chromium, calcium and magnesium were non-significantly ($P > .05$) increased in group given atherogenic diet when compared

with control. Cupper, iron and manganese, however, were reduced in group given atherogenic diet. The difference was only significant for iron (*P*=.05) (Table 2).

For the markers of nephritic effects, i.e. urea and creatinine, there were increased serum concentrations in group given atherogenic diet when compared with control group. However, the differences were not statistically significant (Table 2).

In the homogenates of heart and liver, total cholesterol and triglyceride showed divergent results. There were significant increases in tissue concentrations of total cholesterol in heart and liver of rabbits given atherogenic diet (*P*=.05). However, the concentrations of triglyceride in tissue homogenates of heart and liver were non-significantly reduced in group given atherogenic diet (*P*>.05) (Table 3).

Table 2. Concentrations of lipids, lipoproteins and elements in both control animals and those given atherogenic diet

Biochemical Characteristics	Control (N=5)	Atherosclerotic animals (N=5)	*P*-value
Total cholesterol (mg/dl)	173.00±10.30	180.10±13.50	0.44
Triglyceride (mg/dl)	50.63±6.16	64.30±12.70	0.24
HDL-C (mg/dl)	115.00±32.2	129.80±12.7	0.54
VLDL-C (mg/dl)	10.13±1.23	17.64±6.70	0.20
Zinc (mg/l)	1.69±0.21	2.46±0.60	0.17
Copper(mg/l)	2.09±0.33	1.80±0.25	0.32
Manganese(mg/l)	0.13±0.06	0.10±0.05	0.51
Iron(mg/l)	10.47±0.79	7.70±0.51	0.01
Chromium(mg/l)	0.43±0.15	0.59±0.09	0.22
Calcium(mg/l)	96.20±25.80	165.50±92.82	0.34
Magnesium(mg/l)	28.65±3.51	64.50±35.4	0.22
Creatinine(mmol/l)	3.02±1.53	1.22±0.16	0.10
Urea(mmol/l)	58.02±19.07	43.04±1.55	0.22

Table 3. Concentrations of lipids and lipoproteins in organs of both control animals and those given atherogenic diet

Biochemical parameters	Organs	Control (N=5)	Atherosclerotic animals (N=5)	p-value
Total Cholesterol (mg/dl)	Heart	27.09±2.24	144.34±1.06	0.00
	Liver	7.55±1.24	151.45±6.30	0.00
Triglyceride (mg/dl)	Heart	59.23±8.77	52.00±17.30	0.58
	Liver	81.10±15.5	63.79±5.29	0.21

For the histology, the cross section of kidney of rabbits given atherogenic diet showed mild necrosis of the epithelial squamous cells while that of the control group appeared normal (Figs. 5 and 6).

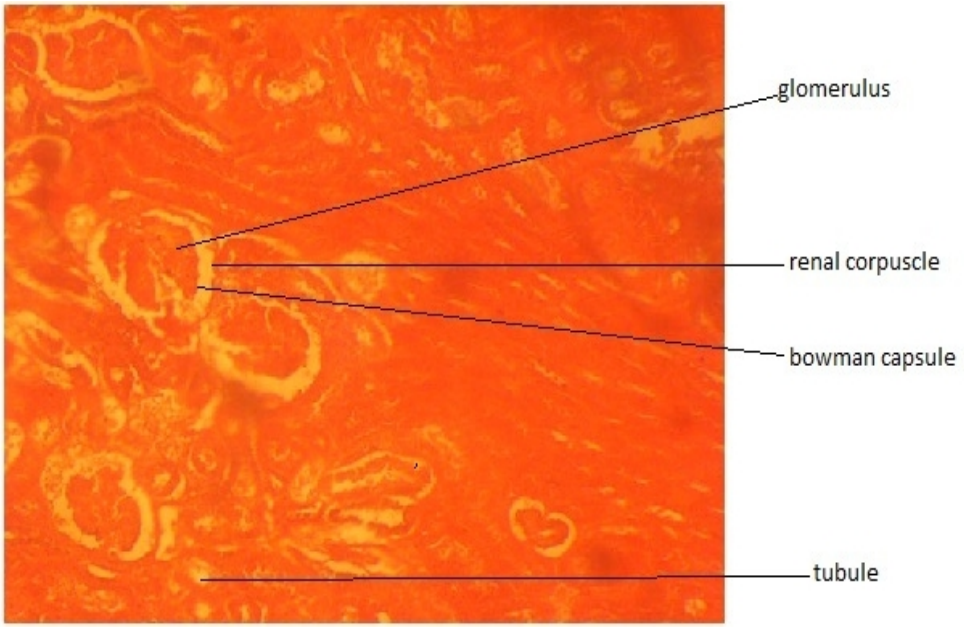

Fig. 5. Cross section of kidney of rabbits given normal diet. Normal renal corpuscle and ducts.
Stain: Sudan; Magnification: 400xs.

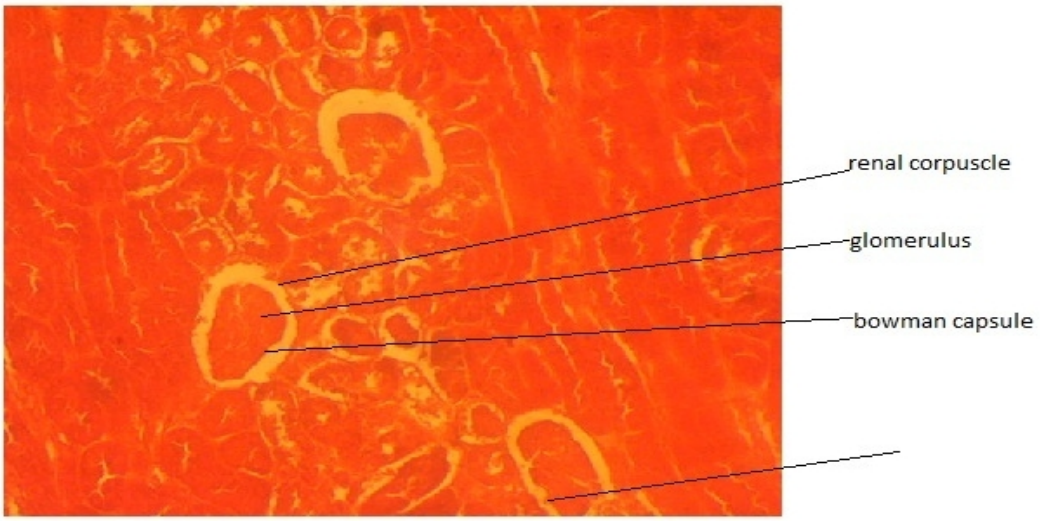

Fig. 6. Cross section of kidney of rabbits given atherogenic diet. Mild cellular necrosis of the epithelial lining (epithelial squamous cells)
Stain: Sudan; Magnification: 400xs.

4. DISCUSSION

The increase of serum cholesterol and triglyceride concentrations in this study was accompanied by structural changes in the arteries, the formation of an enlarged intima, the

appearance of fatty streaks, and later, of the atheroma (Fig.1). The sequence of cellular events occurring in lesion prone areas was as previously reported for the HL hamster by Sima et al. [12].

The reduced serum concentrations of copper and iron observed in the group given atherogenic diet may be due to their increased utilization due to their increased requirement in the oxidation of low density lipoprotein cholesterol (LDL-C). Free copper and iron are two of the most potent pro-oxidants. Extensive LDL oxidation in the atherosclerotic lesions may require a source of copper or iron as catalyst for the oxidation. Thus there is possibility for reactive oxygen species to distrupt copper bound to ceruloplasmin and iron sequestered inside ferritin thus impairing the protective function (antioxidant) of these carriers. This functional impairment may liberate copper and iron which in turn may be used up in promotion of oxidative reactions. With this, one would have expected elevated serum concentrations of these two elements; however, increase in their urinary excretion might have been induced by the hyperlipidemic condition. Unfortunately in this study, we do not have the urine data of these animals. This may be due to negative effect of the condition on the kidney as evident by increased serum concentrations of urea and creatinine. Elevated serum concentrations of urea and creatinine are a strong marker of nephrotic disorder. During hyperlipidemia, chronic damage had been reported to increase in renal [13]. Enhanced generation of reactive oxygen species (ROS), rise in oxidant enzyme activity, and generation of hypochlorite-modified proteins in renal tissue and urine were noticed. Another possibility for the reduced serum concentrations of the trace elements may be that hyperlipidemia increased fecal excretions of these elements as well as reduced absorption from gastrointestinal tract.

According to our results, serum Cu had significant positive correlations with cholesterol, and HDL-cholesterol. The elevation of the serum total cholesterol level during Cu deficiency and in association with high concentrations of high density lipoprotein cholesterol (HDL-cholesterol) has been proved in rats. This rise in serum concentration of cholesterol may be due to change in the metabolism of lipoproteins. Copper alters adipose tissue metabolism and decreases cholesterol synthesis, therefore deficiency of copper causes hypercholesterolemia [14]. The induced hyperlipidemia precipitated reduction in serum copper concentration observed in this study which in turn might have further aggravated the elevation of cholesterol concentration as seen in this study. Several studies reported an inverse relation between serum copper and cholesterol in rats during Copper deficiency [15,16], while [17] found no significant correlation between the serum copper and cholesterol levels in non copper deficient rats.

Serum concentration of manganese in animals given atherogenic diet was observed to be reduced. Although, the mechanism behind this reduction may be unclear, however, this may portend a grave implication taking into consideration the essential roles of manganese in the body physiology. Manganese is essential for normal skeletal growth and development, glucose utilization, lipid synthesis and metabolism, pancreatic function and development, protein and nucleic metabolism, enzyme function activation and thyroid hormone synthesis. However, its deficiency has been observed in a number of animal species. Signs of manganese deficiency include impaired growth, impaired reproductive function, skeletal abnormalities, impaired glucose tolerance, and altered carbohydrate and lipid metabolism. In humans, demonstration of a manganese deficiency syndrome has been less clear [18,19]. A child on long-term total parenteral nutrition (TPN) lacking manganese developed bone demineralization and impaired growth that were corrected by manganese supplementation

[20]. Young men who were fed a low-manganese diet developed decreased serum cholesterol levels and a transient skin rash [21].

The next assessed essential trace element is zinc. It takes part in various important body functions including protein synthesis, DNA synthesis, and cellular growth. It is found almost in every cell and plays a vital role in body's immune system affecting innate and acquired immunity. The increased serum concentration observed for zinc in group given atherogenic diet may be due to increased demand for zinc which possesses antioxidant property. Zinc has significant antioxidant properties thereby protecting the cells from damages due to free radicals. It is the active site for a number of metalloenzymes which are required for nucleic acid synthesis and also important for other host defense mechanisms like production of monocytes and macrophages and chemotaxis of granulocytes [22]. Hypercholesterolemia as observed in this study is associated with increased oxidative stress leading to peroxidation of lipids both in the blood and tissues. However, to counter oxidative processes, body has different defensive mechanisms which include increased synthesis of enzymic antioxidant and need for antioxidant elements such as zinc and selenium.

In the present study, serum calcium was affected by consumption of high lipid diet with increased serum concentration along with increasing concentration of serum cholesterol in the rabbits given atherogenic diet. The increase was almost 100% when compared with values observed in group that consumed normal diet. Also, strong association between serum calcium and cholesterol was found. Calcium exists in 3 major forms in plasma. Approximately 50% is in the free or ionized form, which is the physiologically important fraction, 40% is bound to albumin, and the remaining 10% is in soluble complexes with anions such as bicarbonate, phosphate, and lactate [23]. In epidemiological studies, serum calcium has been found to increase with increasing levels of systolic and diastolic blood pressure (BP) [24,25]. Furthermore, in patients with hyperparathyroidism and therefore chronic hypercalcemia, hypertension is prevalent and an increased mortality rate exists, particularly from cardiovascular diseases [26]. Serum calcium has also been found to correlate with serum cholesterol and blood glucose [25] and therefore is apparently associated with the metabolic syndrome. Recently, serum calcium has been reported to be an independent, prospective risk factor for myocardial infarction in middle-aged men [26].

Hypercalcemia usually occurs when the body dissolves bone at an abnormally fast rate, increasing both serum calcium and serum phosphate. Epidemiological evidence has established a link among hyperlipidemia, visceral obesity, osteoporosis, and cardiovascular diseases (CVD), suggesting some interactions between bone, adipose tissue and vessels. Studies have recently proposed the hypothesis that the associations of those disorders are based on interactions of the three organs, i.e. the bone, fat, and vascular tissues [27,28]. There are several possible mechanisms to explain those interactions. First, these interactions can be attributable to actions of several humoral factors, i.e. hormone in a broad sense. Second, it can also be explained by transcription factors, which are shared by several of the three organs. Third, from the viewpoint of developmental physiology, the fact that there exist interactions between the three organs may be fully reasonable, since the three organs share the cells of common origin, i.e. mesenchymal stem cells (MSCs); the osteoblasts in the bone, the vascular smooth muscle cells (VSMCs) in the vessel, and the adipocytes in the fat. Fourth, the interactions can also be attributable to the function of macrophages, which evolve into the osteoclasts in the bone, evolve into the foam cells in the vessel, and infiltrate into the fat, causing inflammation of the adipocytes [29,30,31]. Other researchers have proposed several hypotheses based on similar concept [32,33,34]. Burnett

and Vasikaran [32] suggested a link between lipids and bone. Hamerman [33] has suggested that osteoporosis and atherosclerosis have biological linkages.

Excessive elevation in serum calcium has, in addition to its pro-atherogenic tendency, has other negative consequences. Renal damage is the most serious clinical consequence of prolonged hypercalcemia. Because of the high plasma free ionized calcium concentration, the solubility of calcium phosphate may be exceeded and precipitate in extraosseous sites of which the kidneys are the most clinically important. The elevated serum concentrations of urea and creatinine in this study support the inimical effects of hypercalcemia which is a finding in this study. Another possible effect of this hypercalcemia is polyuria which is the characteristic of chronic hypercalcemia and may result from impairment of renal concentrating ability due to calcification of the tubular cells. Hypokalemia is another common finding in hypercalcemia often with metabolic alkalosis. Here, calcium may directly inhibit potassium reabsorption from tubular lumen. Other complications of hypercalcemia include depression, anorexia, nausea, vomiting, neuromuscular excitability, effects on stomach, blood pressure and heart. High levels of calcium ions (hypercalcemia) occur at free calcium ion concentrations over 5.2 mg/dL or total serum calcium above 10.4 mg/dL in humans. Sudden hypercalcemia can cause vomiting and coma, while prolonged and moderate hypercalcemia results in the deposit of calcium phosphate crystals in the kidneys which may be the cause of observed negative effect on renal.

In this study, elevated serum magnesium concentration was observed. This may, at first instance appeared contradictory to the general theory that hypomagnesemia is associated with atherogenecity. However, closer look at the general results showed that the elevation may be due to increased retention of magnesium due to decreased renal excretion which may be as a result of negative effect of diet-induced hyperlipidemic condition on kidney. One of the routes of magnesium excretion is kidney. Magnesium homeostasis is determined largely by the balance between intestinal absorption and renal excretion. Overall, very little is known about the factors that control magnesium homestasis, however, the kidneys play a pivotal role in controlling serum magnesium levels by modulating tubular reabsorption Although, the control of reabsorption is complex, however, dietary content is a major factor. However, reduced glomerular filteration may results in retention of magnesium leading to high serum magnesium concentration as seen in this study. Furthermore, continuous consumption of dietary magnesium in the presence of reduced glomerular filteration might have further aggravated the condition. The negative impact of the hyperlipidemic effect on kidney in this study is evident by elevated serum concentrations of urea and creatinine which are two prominent markers of renal disorder/impairment. Unfortunately, we do not have data of 24-hr urine concentration of magnesium to further explain the observed hypermagnesemia. Furthermore, in this study, it is interesting to note that the serum profile of magnesium is similar to that of calcium. Studies have shown that similar factors affect the two minerals in the metabolism of bone and that hypercalcemia is always accompanied by hypermagnesemia. It is very possible that the observed hyperlipidemia induced outward movement of magnesium from the bone causing elevation in its serum concentration.

5. CONCLUSION

Therefore, determination of minerals excretion in 24-h urine sample may be recommended in individuals suffering from dyslipidemia and atherosclerosis as this may improve management of the disorder and prevent occurrence of another grave consequences.

CONSENT

It is not applicable as we did not use human subjects!

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

ACKNOWLEDGEMENT

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COMPETING INTEREST

There was no conflicting interest as this study was not sponsored by any organization.

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