Atherogenicity, Oxidative Stress, Heavy Metals and Bioelements Status in Hypertensive Nigerians in an Urban Population

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: This study investigated levels of measured parameters; bio-elements (zinc, copper, selenium, chromium, and manganese), heavy metals (cadmium and lead), oxidative stress biomarkers (vitamin C, Vitamin E, reduced Glutathione-GSH and malondialdehyde-MDA) and lipid profile (cholesterol-CHOL, triglyceride- TG, high-density lipoprotein- HDL, low-density lipoprotein-LDL and VLDL) in primary hypertensive individuals.

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Materials and Methods: A total of 74 confirmed hypertensive individuals (30 males, 44 females) of 30-55 years (test subjects) and 46 (22males, 24 females) age-matched apparently healthy normotensive individuals (control) were involved in this study. Atomic absorption spectrophotometer was used to determine the concentrations of bio-elements and toxic metals while spectrophotometric methods were used for the concentrations of oxidative stress biomarkers and lipid fractions.
Results: Comparative analysis between test and control subjects showed significantly reduced concentrations of vitamins C and E, GSH, the bio-elements, HDL and Zinc/Copper ratio. Significantly elevated concentrations of the toxic metals, MDA, CHOL, TG, LDL, and VLDL, the atherogenic indices- LDL/HDL, TC/HDL, and TG/HDL as well as Cadmium/ Zinc ratio. There was indirect association between the bio-elements and atherogenic lipid fractions but a direct correlation with HDL. Also, negative correlations were observed between the heavy metals and the antioxidants biomolecules.
Conclusion: The concentrations of the measured bio-elements, vitamins C and E, GSH and HDL were significantly lower in hypertensive individuals while the levels of heavy metals, cholesterol, TG, LDL and MDA were significantly higher.

1. INTRODUCTION

Globally, non-communicable diseases constitute a pivotal cause of morbidity and mortality [1]. Hypertension being an example of the non-communicable diseases is a significant universal public health problem and is one of the biggest health challenges in the 21st century. Little or lack of attention has been observed about this disease and this has made it to be referred to as 'neglected disease' by the institute of medicine [2]. It has been reported to be the major contributor to premature death in both developed and developing countries [3]. Its prevalence is highest in the African among adults of 25 years and above but low prevalence has been reported in the American region [4]. Some recent studies had reported high prevalence of hypertension in Nigeria [5,6] and a major cause of morbidity and mortality among adults in the country [7]. "It is one of the leading causes of death and disability due to complications such as coronary heart disease, stroke, congestive heart disease, end-stage renal disease and peripheral vascular disease" [8].

"Bio-elements are naturally occurring inorganic metals which are present in very small amounts in the living tissues but are important for the vital processes of life" [9]. "They are involved in a wide range of physiological processes such as prosthetic groups of many proteins, water balance, cofactors of many enzymes and so on" [10]. "Therefore, the regulation of various metallic contents in the body is pre-requisite for their proper functioning" [11]. These elements which make up less than 0.01 per cent of the body's dry weight [12] have been found to individually react directly and indirectly in a variety of metabolic and structural activities known to participate in blood pressure regulation. "However, the role of these elements in the aetiology and control of blood pressure has not been fully elucidated" [13]. The involvement of bio-elements in hypertension has been reported by a few studies with contradictory findings. While Taneja and Mandal [14] demonstrated "high level of zinc in some hypertensives", Ekmekci et al. [15] "did not observe any significant difference between serum zinc level of subjects with essential hypertension and normal controls". Some other reports have shown that "deficiency of Cu, Se, Zn and Mn might be associated with an increased risk of hypertension" [16,17].

Oxidative stress (OS) occur when there is an imbalance between the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and the antioxidant defense systems. The ROS and RNS comprise many molecules with different effects on the cellular function. They are regularly formed as a result of normal organ functions and their excess is associated with various pathological conditions. Oxidative stress is believed to be involved in many types of disease processes and has been suggested to be a key player in the pathogenesis of hypertension.

Abnormalities in the lipid fractions concentrations have been found to be associated with the

incidence and severity of hypertension by some authors [18,19]. An additional cardiovascular risk factor that has been found in the majority of people with severe high blood pressure is the risky dangerous lipid fraction usually referred to as hyperlipidemia [20] which has been earlier reported by few authors. However, there are contradictions in the interpretations and application of atherogenicity to the severity of hypertensive cases in these earlier studies.

Many previous related studies have focused on the associations of hypertension separately with dyslipidemia and oxidative stress bio-markers. The current study is aimed at studying the combined associations of oxidative stress biomarkers, the artherogenicity of lipid fractions and trace metals concentrations in hypertensive individuals residing in urban community of a tropical country. This is to understand the interplay of these parameters together in hypertension which may enhance the management of this endemic disease.

2. MATERIALS AND METHODS

2.1 Participants

The study comprised of 120 individuals. This included 74 individuals with primary hypertension (30 males and 44 females) age range 30-55years who were recruited from general out-patient department, Mushin General Hospital, Lagos, Nigeria, who were the test subjects. Forty six 46 (22 males and 24 females) apparently healthy individuals recruited among the staff of the hospital with normal blood pressure, age and sex matched with the test subjects resident within Mushin local government area, Lagos were taken as control subjects.

Exclusion criteria for test subjects: Individuals who engaged in social habits like smoking, alcoholism, and drug addiction. In addition, individuals with hypertensive urgency, hypertensive emergency, secondary hypertension, liver and kidney diseases, diabetes mellitus, post-myocardial infarction, congestive heart failure, pregnancy were excluded from the study.

Exclusion criteria for control subjects: Individuals with blood pressure above or below expected normal, with history of smoking, alcoholism, and drug addiction.

2.2 Experimental Procedure

After overnight fast (12 hours) 10mL of blood sample was collected from the participants and placed into metal free plain bottles. The blood samples were allowed to clot and they were centrifuged at 3000 revolution per minute (1000g) for 10 minutes. The serum was harvested and kept frozen and used for the assay of various biochemical parameters.

Blood pressure was measured with a mercury sphygmomanometer in the sitting position after 5 minutes of rest in a quiet environment. Mean of 2 readings of systolic blood pressure (SBP) and diastolic blood pressure (DBP) (Korotkoff phase I and phase V, respectively) were taken at 5-minutes intervals. The blood pressure measurement was taken by trained and registered nurses working in the hospital.

2.2.1 Concentration of fasting lipid fractions

Fasting lipid profile including Total cholesterol, high-density lipoprotein cholesterol, and triglycerides were analyzed by standard enzymatic colorimetric methods respectively.

Friedewald formula was used to calculate the concentration of LDL following:

LDL Cholesterol = total serum cholesterol − (HDL + total TG/2.2)

VLDL was calculated by the formula VLDL = 1/5 of plasma triglyceride.

2.2.2 Assay of lipid peroxidation product

The lipid peroxidation was determined by estimation of thiobarbituric acid reactive substance/malondialdehyde (MDA) which is a bye product of lipid peroxidation. In brief, 0.1ml of plasma was treated with 2ml of (1:1:1 ratio) TBA-TCA-HCL reagent (TBA 0.37%, 0.25N HCL,15%TCA) and placed in water bath for 15 minutes, cooled and centrifuged and then clear supernatant was measured at 535nm against reference blank (using analar grade reagents from BDH, England).

2.2.3 Determination of concentration of ascorbic acid

Vitamin C (ascorbic acid) concentration was determined spectrophotometrically using Dinitrophenyl hydrazine reagent (25%DPNH)

and 4% thiourea in 9N sulphuric acid. The colour was read with spectrophotometer at 520nm. The assay procedure involved 0.5ml plasma added to 2.0ml of freshly prepared metaphosphoric acid (6.0g/100ml) and was mixed well on a vortex mixer. The plasma – metaphosphoric acid mixture was centrifuged for 10 minutes at 2500 rpm. To 1.2 ml of the clear supernatant 0.4ml of Dinitrophenylhydrazine-thiourea-copper sulphate (DTCS) reagent was added. The content was mixed well and incubated in a water bath at 37^0C for 3 hour. The tubes were removed from the water bath and chilled for 10 minutes in an ice bath. 2.0 ml of cold 12M sulphuric acid was slowly added into all the tubes and were capped and mixed with a vortex mixer (the temperature of the mixture was not allowed to exceed room temperature).

2.2.4 Determination of concentration of vitamin E

Vitamin E (α-tocopherol) concentration was measured by the extraction of α-tocopherol from serum by addition of 0.3 ml (95 %, v/v) ethanol and 0.7 ml petroleum ether to 1.6 ml of serum and centrifuged. The supernatant was separated and evaporated. To the residue, 0.2 ml of 2 % α, α-dipyridyl, 0.2 ml of 0.5 % ferric chloride was added and kept in dark for 5 min. An intense red coloured layer obtained on addition of 1 ml butanol was read at 520 nm against reagent blank.

2.2.5 Assay of the concentration of reduced glutathione (GSH)

Reduced glutathione concentration was determined using the method based upon the development of a relatively stable (yellow) colour when 5', 5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman reagent with the reduced glutathione, 2- nitro-5-thiobenzoic acid possesses a maximum absorption at 412 nm (the absorbance is proportional to its concentration).

2.2.6 Determination of bio-elements and heavy metals concentrations

The concentrations of bio-elements and heavy metals were assayed with Atomic Absorption Spectrophotometer by the assay method reported by Kaneka (1990). The metals were first released from the protein matrix using the nitric and hydrochloric acid (1:1) wet digestion method. The concentrations of the elements were determined by direct aspiration of the acidic filtrate into the atomic absorption spectrophotometer (Buck 210-VGI (Bulls Scientific, East Norwalk, CT) (AAS) flame. The wavelength for the determination of zinc was set at 214 nm and 247 nm for copper with a detection limit of 0.005 ppm for both elements selenium. That of Se was set at 196nm and cadmium (Cd) 226 nm for Cd, and detection limit of 0.15 ppm and 0.01 ppm, respectively.

2.3 Statically Analysis

Normally distributed continuous data were expressed as mean ± standard deviation. The independent variables were analyzed using the unpaired student "t' test for comparison of the mean. Pearson's correlation coefficient (r) was used to determine the relationship between the mean of the variables. All analyses were conducted using SPSS (IBM, SPSS version 22.0), and significant level was set at $P < .05$.

3. RESULTS

The result of this study is presented in Fig. 1 and Tables 1-6. Fig. 1 show the demographic details of the participants in the study. Table 1 showed serum concentrations of lipid fractions, Table 2 showed serum concentrations of antioxidants and lipid peroxidation product while Table 3 presents serum concentrations of bioelements and heavy metals in hypertensive and normotensive subjects studied. Table 4 present correlations of trace metals with lipid fractions in hypertensive subjects, Table 5, association of trace metals with oxidative stress biomarkers and Table 6 reports correlation of lipid fractions with oxidative stress biomarkers in hypertensive subjects.

The demographic results (Fig. 1) show no difference in the ages of test and control subjects but significant differences in the blood pressure. The results equally revealed a significantly reduced concentrations of antioxidants in hypertensive subjects; glutathione peroxidase ($P < 0.001$), vitamin C ($P < 0.001$), vitamin E ($P < 0.001$) while there was significantly increased ($P < 0.05$) concentration of malondealdehyde which is peroxidation product. These results indicate oxidative stress in hypertensive patients.

In addition, there were significantly low ($P < 0.001$) concentrations of the essential trace metals (manganese, zinc, copper, selenium and

chromium) in hypertensive subjects in comparison with the control subjects while there were significantly (P < 0.001) elevated concentrations of the toxic metals (lead and cadmium). The Zn: Cu ratio was found to significantly lower in the hypertensive subjects when compared with that of the control subjects.

Furthermore, the concentrations of total cholesterol, triglyceride, LDL, VLDL, were significantly higher in the hypertensive subjects in comparison with the control subjects. Conversely, the concentration of HDL- C was found to be significantly lower in the hypertensive subjects. The LDL/HDL which is the Coronary heart disease index; the TC/HDL which is the Ischemic disease index and the TG/HDL which is an index that indicates early risk detection for the development of insulin resistance which can help in detecting the

predisposition of an hypertensive individual to develop type 2 diabetes mellitus were all significantly higher in the hypertensive subjects when compared with those of the control subjects (Value above 2 is abnormal).

Finally, the Pearson correlation between the lipid fractions and bioelements showed significant inverse correlations between the artherogenic lipid fractions and direct correlation with HDL. There were indirect correlation between the heavy metals and the antioxidants biomolecules (reduced glutathione, vitamins C and E) but direct with malondealdehyde. The investigation on the association between trace metals and oxidative stress biomarkers showed that malondealdehyde had negative correlation with the bioelements but positive correlations existed between malondealdehyde and chromium (r=0.751, P<0.001) and the heavy metals.

Table 1. Serum concentrations of lipid fractions in test and control subjects

Analytes	Test subjects (n= 74)	Control subjects (n= 46)	T-value	P-value
Cholesterol (mmol/L)	8.75±0.19	3.6±0.12	13.16	< 0.000
HDL-Cholesterol (mmol/L)	1.06±0.08	2.0±0.06	12.05	< 0.001
Triglyceride (mmol/L)	3.41±0.15	1.15±0.07	13.68	< 0.001
LDL-Cholesterol (mmol/L)	4.06±0.14	1.21±0.09	17.02	< 0.001
VLDL-Cholesterol (mmol/L)	1.85±0.06	0.55±0.04	15.20	< 0.001
LDL/HDL (CHDI)	3.45	0.63	13.33	< 0.001
TC/HDL (IDI)	8.26	1.84	12.45	< 0.001
TG/HDL	3.22	0.57	10.78	< 0.001

Note: The results were expressed as mean ± standard deviation; CHDI= Coronary Heart Disease Index
IDI= Ischemic Disease Index

Table 2. Serum concentrations of antioxidants and lipid peroxidation product in test (TST) and control (CTL) subjects

Analytes	TST (N= 74)	CTL (N= 46)	t- Values	p-values
Reduced Glutathione (ng/ml)	0.42 ± 0.27	1.79 ± 0.32	12.694	< 0.001
Vitamin C (mg/dl)	15.3± 2.5	44.0± 3.9	18.117	0.001
Vitamin E (mg/dl)	12.4± 1.7	32.7± 4.0	12.110	< 0.001
Malondealdehyde (nmol/L)	0.45 ± 0.07	0.29 ± 0.06	6.713	< 0.05

Note: The results were expressed as mean ± standard deviation

Table 3. Serum concentrations of bioelements and heavy metals in test (TST) and control (CTL) subjects

Analytes	TST (N= 74)	CTL (N= 46)	t- Values	p- Values
Manganese (mg/L)	18.01±0.42	24.83±0.70	6.45	0.01
Zinc (mg/L)	6.27±0.19	9.97±0.66	8.79	0.01
Copper (mg/L)	9.24±0.35	13.04±0.32	11.9	0.00
Chromium (mg/L)	1.19±0.05	2.07±0.08	8.97	0.05
Selenium (mg/L)	3.47±0.16	5.62±0.25	7.36	0.04
Lead (mg/L)	1.23±0.08	0.42±0.03	13.96	0.01
Cadmium (mg/L)	1.50±0.07	0.55±0.04	18.69	0.00
Zn/ Cu Ratio	0.68	0.77	9.78	0.01
Cd/ Zn Ratio	0.24	0.09	8.76	0.02

The results were expressed as mean ± standard deviation

Table 4. Correlation of heavy metals and bioelements with lipid fractions in test subjects

	TC	HDL	TG	LDL	VLDL
Manganese	-0.500 [a]	0.565 [a]	-0.554 [a]	0.477 [a]	-0.554 [a]
Zinc	-0.870 [b]	0.874 [b]	-0.869 [b]	-0. 856 [b]	-0. 869 [b]
Copper	-0.602 [b]	0.637 [b]	-0.650 [b]	-0.674 [b]	-0.650 [b]
Selenium	-0.707 [b]	0.819 [b]	-0.718 [b]	-0.764 [b]	-0.718 [b]
Chromium	-0.815 [b]	0.729 [b]	-0.794 [b]	-0.676 [b]	-0.794 [b]
Cadmium	0.801 [b]	-0.782 [b]	0.739 [b]	0.936 [b]	0.739 [b]
Lead	0.686 [b]	-0.659 [b]	0.641 [b]	0.646 [b]	0.641 [b]

Note: a = statistically significant difference between hypertensive and normotensive groups at P < 0.05;
b = statistically significant difference between hypertensive and normotensive groups at P < 0.001

Table 5. Correlation of bioelements and heavy metals with oxidative stress biomarkers

	Manganese	Zinc	Copper	Selenium	Chromium	Lead	Cadmium
MDA	-0.615 [a]	-0. 807 [b]	-0.584 [a]	-0.675 [a]	0.751 [b]	0.467 [a]	0.694 [b]
Vitamin C	0.579 [a]	0. 745 [b]	0.507 [a]	0.710 [b]	-0.820 [b]	-0.623 [a]	-0.716 [b]
Vitamin E	0.499 [a]	0.856 [b]	0.626 [b]	0.826 [b]	-0.792 [b]	-0.682 [b]	-0.776 [b]
GPx	-0.477 [a]	-0.745 [b]	-0.546 [a]	-0.807 [b]	0.753 [b]	0.696 [b]	0.735 [b]

Note: a = statistically significant difference between hypertensive and normotensive groups at P < 0.05; b = statistically
significant difference between hypertensive and normotensive groups at P < 0.001; GPx- Glutathione Peroxidase;
MDA - Malondealdehyde

Table 6. Correlation of lipid fractions with oxidative stress biomarkers in hypertensive subjects

	TC	HDL	TG	LDL	VLDL
MDA	0.780 [b]	-0.815 [b]	0.832 [b]	0.737 [b]	0.831 [b]
Vitamine C	-0.832 [b]	0.821 [b]	-0.856 [b]	-0.746 [b]	-0.856 [b]
Vitamine E	-0.901 [b]	0.879 [b]	0.877 [b]	0.820 [b]	0.877 [b]
GPx	0.820 [b]	-0.871 [b]	0.739 [b]	0.936 [b]	0.739 [b]

Note: a = statistically significant difference between hypertensive and normotensive groups at P < 0.05; b = statistically
significant difference between hypertensive and normotensive groups at P < 0.001; Total Cholesterol (mmol/L)-TC;
HDL-Cholesterol (mmol/L)-HDL; LDL-Cholesterol (mmol/L); LDL; Triglceride-TG; Very Low Density Lipoprotein- VLDL;
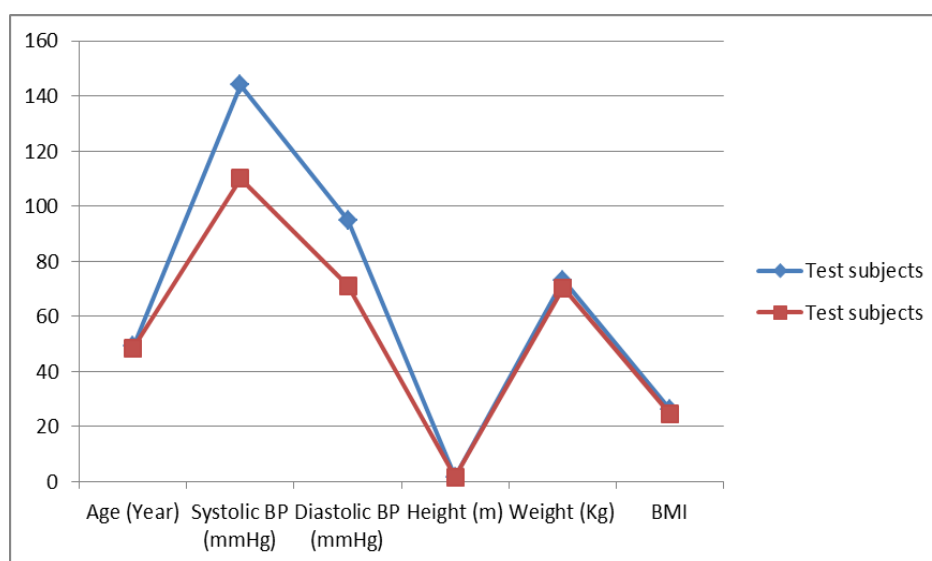*GPx- Glutathione Peroxidase; MDA - Malondealdehyde*

Fig. 1. Demographic characteristics and blood pressure e of the participants
Note: The line with blue colour for test subjects while the red is for test subjects
The first coordinates are for age, second for systolic BP, third for diastolic, the fourth for height, the fifth for
weight and the sixth for BMI

4. DISCUSSION

The etiology of primary hypertension and its association with the collective effects of lipid fractions, heavy metals, bioelements and oxidative stress parameters was studied. This study provides data on the status of bioelements, Zinc, Copper, Selenium, Chromium, manganese and Magnesium, toxic metals, oxidative stress biomarkers and lipid profile. Copper serve as a cofactor for key metabolic and redox related proteins. Zinc and Magnesium play important roles in normal cellular metabolism and assists several enzymatic catalytic reactions Selenium is a cofactor for the activities of glutathione peroxidases (GPx) and other selenoenzymes which are indispensable antioxidant enzymes to prevent the oxidation of lipids and atherosclerotic plaque formation [21,22]. This study reports significantly lower level of selenium in hypertensive subjects than in the control group. This finding is in consonant with earlier report of Salonen and his coworkers [23] where the selenium concentration was reported to be lower in hypertensive, but contrary to the findings of Jossa et al [24] where no difference was reported between hypertensives and normotensives and other studies where higher concentration of Se has been reported to be associated with higher blood pressure [25]. Se concentration corresponds to GPx activity in vivo. In this study glutathione peroxidase demonstrated direct correlation with Se at low concentration.

The significantly lower concentration of Zn found in the hypertensive subjects than that of the control subjects in this study agrees with the finding of Hajjar & Kotchen [17] but at variance with findings of Taneja & Mandal [14] who demonstrated high level of zinc in some hypertensive subjects and those of Ekmekci et al [15] who did not observe any significant difference between serum zinc level of subjects with essential hypertension and normal controls.

The significantly low concentration of copper observed in the hypertensive subjects when compared with the control subjects is in consonance with the findings of Asaolu et al [26] but in contrast with Pfeiffer & Mailloux [27] who reported a strong positive correlation between high serum copper and hypertension with the assumption that copper excess might be a strong factor in the aetiology of hypertension. The zinc/copper ratio in the hypertensive subjects was significantly lower than that of the normotensive subjects in this study. This result contradicts the findings of He et al [28] where higher ratio in the hypertensive subjects was reported.

The association of chromium and hypertension has been less studied. However, studies using animals had reported reduced serum chromium level in hypertensive rats when compared with non-hypertensive ones. The current study finds significantly higher chromium level in the control subjects when compared with the hypertensive subjects. A few earlier studies have indicated that chromium is essential for lipid metabolism and that chromium treatment is associated with a reduction in liver triglyceride, total cholesterol, LDL-cholesterol levels and lipid accumulation [29] with concomitant increased HDL-cholesterol levels [30]. Some studies have shown reduction of cholesterol, triglyceride and LDL with chromium treatment [31], while other findings report no effects [32]. The reduced chromium concentration may explain the significant increase in LDL-cholesterol, triglyceride and total cholesterol in hypertensive patients found in this study. Again, the present study observe an indirect correlation between chromium level and lipid fractions (cholesterol, triglyceride, LDL and VLDL), but a direct correlation between the bioelements and HDL.

The result of significantly lower plasma manganese level and negative correlation with cholesterol, triglyceride and LDL in hypertensive subjects observed in this study may be of special pathogenic importance. Some earlier studies have shown manganese to possess choline-like lipotrophic properties which is known to prevent atherosclerosis and therefore affect lipid metabolism in hypertensive patients [33,34,35].

Atherogenic process has been established to be in association with alterations in lipid particle sub-fractions. This study observed elevated cholesterol level and significantly high values in the atherogenic indices evaluated. The Coronary heart disease index (LDL/HDL), Ischemic disease index (TC/HDL) and TG/HDL (an index to establish the risk of insulin resistant development and predisposition to type 2 diabetes mellitus) were all higher than 2, which made them abnormal. These findings describe the severity of hypertension and its association with high morbidity, mortality and type 2 DM when poorly managed.

Cadmium interference with renin activity and subsequent production of angiotensin II and metabolism of catecholamine as well as sodium retention in the renal system has been indicated to be the basis of toxic metal association with increased blood pressure. The poor homeostatic control of the Cd and lead (Pb) results into their accumulation and significantly compete with the absorption of several bioelements in the gastrointestinal tract [36]. In this study significantly high concentrations of Cd and Pb were found in hypertensive subjects. Similar pattern was reported by Asaolu et al [26]. The toxicologically inverse relationship between cadmium and zinc because of their competition for the same binding targets in favour of cadmium explains the significantly low concentration of zinc in hypertensive subjects observed in this study. The high concentration of cadmium and its consequential reduction on zinc absorption may be an additional reason for increase in the concentration of lipid peroxidation product and reduced antioxidant enzymes and vitamins (vitamin C and E) observed in this study. The significantly lower concentrations of vitamins C and E, glutathione peroxidase with significantly high concentration of MDA in hypertensive patients were also reported by Pedro-Botet et al [37,38].

5. CONCLUSIONS

In conclusion the study reveals that oxidative stress due to increased lipid peroxidation and reduced anti-oxidant levels, bio-elements (Zinc, Copper and Selenium) coupled with significant increase in toxic metals (cadmium and lead) is associated with hypertension. Also this study shows that hypertensive individuals are predisposed to insulin resistance and type 2 DM because of elevated TG/HDL ratio which is an index to establish the risk of developing insulin resistance and predisposition to type 2 diabetes mellitus. Finally, significant atherogenecity was observed among the studied hypertensive subjects as shown by the significantly altered atherogenic indices LDL/HDL (Coronary heart disease index) and TC/HDL (Ischemic disease index) in these individuals.

CONSENT

As per international standard or university standard, Participants' written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

The procedures in this study were in accordance with the ethical standards on human experimentation. Ethical clearance was obtained from the biomedical research ethics committee of the Hospital, and the study was carried out in strict compliance with the guidelines for the care and use of human sample for research which is in line with that set by World Health Organization.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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