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Original Article

Biochemistry Section

Elevation of Oxidative Stress and Decline in Endogenous Antioxidant Defense in Elderly Individuals with Hypertension

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ABSTRACT

Introduction: Hypertension is becoming an important medical and public health problem all over the world and is the most common disorder of ageing. There is a growing evidence of involvement of vascular oxidative stress in the development of hypertension from animal studies. However, studies on humans with hypertension, particularly in elderly are least and data remained controversial. Moreover, studies in elderly people with hypertension are scarce.

Aim: To investigate the possible role of oxidative stress and antioxidant defense in the pathogenesis of hypertension in elderly.

Materials and Methods: A cross-sectional study was conducted on elderly males (n=60) with newly diagnosed hypertension and with normal blood pressure. Oxidative stress and antioxidant status were evaluated by assessing the following parameters: plasma Malondialdehyde (MDA), and antioxidants: Superoxide Dismutase (SOD) activity, reduced Glutathione (GSH), and vitamin C levels; and total Nitric Oxide concentration in plasma (NOx). Difference between groups was determined by using unpaired t-test/Mann-Whitney U test. Bivariate correlation and multiple regression analysis were used to determine the relationship between variables.

Results: A significant rise in plasma MDA (p-value=0.013) and lower levels of endogenous antioxidants: SOD (p-value≤0.001) and GSH (p-value≤0.001) were observed in elderly individuals with hypertension when compared to healthy controls. Though not significant, there was a mean decrease in plasma NOx in hypertensive subjects than normotensive ones. While vitamin C showed no significant difference between two groups. Decrease in GSH (β=-0.398; p-value=0.001) and SOD (β=-0.423; p-value≤0.001) were the significant determinants of hypertension in elderly individuals.

Conclusion: Above findings indicate that elevation in oxidative stress and decrease in endogenous antioxidant level may be involved in the pathogenesis of hypertension. However, it remains unclear whether oxidative stress causes or augments hypertension.

Keywords: Ageing, High blood pressure, Pathogenesis

INTRODUCTION

Hypertension is a major health problem and an important Cardiovascular (CV) risk factor in elderly individuals. The prevalence of hypertension in elderly ranges from 50-75% and it is estimated that two out of three individuals over 75 years of age suffer from hypertension [1,2]. Ageing along with hypertension is a major risk factor for CV morbidity and mortality [1]. Several age-associated physiological changes such as arterial stiffness, endothelial dysfunction, sympathetic over-activity, low-grade inflammation are attributed for the elevation of Blood Pressure (BP) in elderly individuals [2].

There is a growing evidence of involvement of vascular oxidative stress in the development of hypertension [3,4]. Oxidative stress is defined as an imbalance between the production of Reactive Oxygen Species (ROS) and antioxidant defense system of the body [3,5]. ROS are chemically reactive species containing oxygen that is not processed completely. Example of ROS includes superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and singlet oxygen. Several ROS are generated in multiple compartments and by multiple enzymes within the cell. But majority of ROS are produced within the mitochondria during Adenosine Triphosphate (ATP) production by oxidative phosphorylation [5]. If the level of ROS is not maintained at an optimum level, it can cause damage to the cell membrane and macromolecules like proteins, DNA and RNA. Our body has an ability to counteract or detoxify the harmful effects of ROS and keep them at optimum level by compounds known as antioxidants. Antioxidants are produced either in our body (endogenous antioxidants) or received from outside (exogenous antioxidants). Examples of antioxidants are enzymes like SOD, catalase, glutathione peroxidase, glutathione reductase; compound like reduced glutathione; minerals like selenium, manganese, copper and zinc and, vitamins like vitamin A, C and E [6].

Surprisingly, most of the data supporting the role of oxidative stress in the pathogenesis of hypertension is derived from animal studies [7-12]. However, studies on humans with hypertension are least and data remain controversial [13,14]. Moreover, studies on elderly people with hypertension are scarce. Hence, the present study was aimed to investigate the possible role of oxidative stress and antioxidant defense in the pathogenesis of hypertension in elderly.

MATERIALS AND METHODS

A cross-sectional study was conducted on elderly male subjects (n=60) between 60 to 80 years with hypertension and normal BP in Shri B M Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka, India, during June 2015 to May 2016. Newly diagnosed individuals with hypertension with Systolic Blood Pressure (SBP) ≥140 mmHg and Diastolic Blood Pressure (DBP) ≥90 mmHg were included in study group, Hypertension group (HT). Whereas subjects with SBP ≤139 mmHg and DBP ≤89 mmHg were included in the control group, Normotension group (NT) [15]. Subjects on any medications, with diabetes mellitus, cardiovascular diseases and subjects suffering from any acute or chronic diseases were excluded from the study.

Prospective ethical clearance for the study was obtained from the Institutional Ethical Committee. Participants were informed about the research objectives and protocol and written consent was taken

prior to their enrollment for the study. The declaration of Helsinki has been followed during the entire study.

Elderly people above 60 years were screened, thoroughly examined and selected for the study as per selection criteria. Brachial BP was measured thrice with an interval of one minute for three consecutive days [2] using mercury sphygmomanometer (Diamond, Industrial electronic and allied products, India) in a sitting posture. An average of nine BP measurements was considered [16].

Blood sample was collected in morning hours between 8.00 am to 10.00 am with overnight fasting. About 6 ml venous blood sample through venous puncture was collected under aseptic conditions. The sample was further divided as follows: 2 ml blood in EDTA vacutainer that was used for the estimation of erythrocyte reduced Glutathione; 4 ml blood in Heparin vacutainer that was used for estimation of vitamin C, Superoxide dismutase, MDA and NOx. The blood was centrifuged at 3000 rpm for 10 minutes, the plasma was separated and collected in polythene tube with cork and stored at -20^{0}C in deep refrigerator until the analysis was done.

Estimation of Plasma MDA

Plasma MDA, a marker of oxidative stress was estimated by Kei Satoh method [17]. Three hundred µl of plasma and 1.5 ml of 20% Tricholoroacetic Acid (TCA) was taken in a test tube and centrifuged for 10 minutes at 3500 rpm. The supernatant was decanted and the precipitate obtained was washed with 0.05 M sulphuric acid. To the precipitate, 1.5 ml of 0.05M sulphuric acid and 3 ml of Thiobarbituric Acid (TBA) reagent (670 mg of TBA in 100 ml of 2 M sodium sulphate solution) were added and the mixture was kept in a boiling water bath for 30 min. The mixture was cooled in cold water and 2.4 ml of n-butyl alcohol was added with vigorous shaking to extract the chromogen. Organic phase was separated by centrifuging at 3000 rpm for 10 min and absorbance was read at 530 nm using spectrophotometer.

Estimation of Erythrocyte GSH

GSH was estimated by Beutler E et al., method [18]. Whole blood (0.2 ml) and distilled water (1.8 ml) was taken in a test tube, mixed well and allowed to haemolyze. To this haemosylate, 3.0 ml of precipitating solution (1.67gm of glacial metaphosphoric acid, 0.2 gm of disodium or dipotassium Ethylene Diamine Tetra Acetic Acid (EDTA) and 30 gm of sodium chloride was dissolved in 100 ml of distilled water) was added. The test solution was kept for five minutes, filtered and then 2 ml of supernatant was taken out for further reaction. To this supernatant 8.0 ml of phosphate solution (0.3 M Na_2HPO_4) and 1.0 ml of DTNB reagent (40 mg 5, 5'dithiobis-2-nitrobenzonic acid in 100 ml of 1% sodium citrate) was added, mixed and absorbance were read against the blank at 412 nm within five minutes (after adding DTNB reagent).

Estimation of Plasma SOD

SOD activity was measured by Marklund and Marklund method [19]. Tris buffer (50 mM Tris buffer and 1 mM of ethylene diamine tetra acetic acid in 50 mM HCL at pH 8.2): 3.0 ml and 2.95 ml were added to two test tubes, labeled as Test (T) and Control (C) respectively. Plasma (0.05 ml) was added to tube (T). Immediately after addition of 0.3 ml of working pyrogallol solution (Stock solution: 2.5 mM Pyrogallol in 0.5 M HCL; Working solution: 0.01 ml of stock pyrogallol was diluted with 5 ml DW), absorbance was read continuously for about four minutes at 420 nm. The absorbance was read at 1.30, 2.30 and 3.30 minutes respectively. The difference in absorbance (at 1.30 minute and 3.30 minute) between Control and Test sample was multiplied with 40 to determine SOD activity (Units/ml). One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation.

Estimation of Plasma Ascorbic Acid (vitamin C)

Plasma vitamin-C concentration was estimated by 2,4-dinitrophenyl-hydrazine method [20]. A mixture of plasma (0.5 ml) and ice cooled 10% TCA (0.5 ml) was centrifuged for 10 minute. From this, 0.5 ml of supernatant was taken in another test tube to which 5% TCA (0.5 ml) and 2,4 Dinitrophenyl Hydrazine (DNPH)-Thiourea-Copper sulphate (DTC) solution (1.0 ml) (DTC solution: 0.4 gm Thiourea, 0.5 gm $CuSO_4$ and 3.0 gm DNPH in 9 N H_2SO_4 to make final volume of 100 ml) were added. Following incubation for three hours at 37^{0}C, 65% H_2SO_4 (0.75 ml) was added and absorbance was taken against blank after 30 minutes at 520 nm. Plasma ascorbic acid concentration (mg/dL) was calculated using the standard graph.

Estimation of Plasma Total NOx

Plasma NOx was determined by Kinetic Cadmium-Reduction method [21]. Nitric oxide is relatively unstable which is converted to nitrite and nitrate. Reduction of nitrate to nitrite was done by cadmium-reduction method after deproteinization with somogyi reagent. Nitrite was quantified using griess reagent by diazotization of sulphanilamide and coupling to naphthalene ethylene diamine. The color complex formed was measured at 540 nm wavelength using spectrophotometer.

Estimation of Other Biochemical Parameters

Fasting blood glucose (Trinder's method), plasma triglyceride level (glycerol phosphate-oxidase method), plasma cholesterol (cholesterol oxidase-peroxidase enzymatic method), HDL cholesterol (phosphotungstic acid method), plasma urea, and creatinine were estimated using commercial (ERBA-MANNHEIM) diagnostic kits.

STATISTICAL ANALYSIS

Data was expressed in mean and standard deviation. To determine the statistical significance between the two groups, an Unpaired t-test was used for normally distributed data and Mann-Whitney U test for non-normally distributed data. The Pearson's correlation coefficient (normal data) and Spearman's correlation coefficient (non-normal data) was used to determine an association between arterial stiffness and BP parameters. The predictors of high BP were determined by multiple linear regression analysis. Statistical significance was established at p-value<0.05. Data was analysed using SPSS software version 20.0.

RESULTS

The baseline characteristics of study participants are shown in [Table/Fig-1]. There was no significant change in age, Body Mass Index (BMI), blood glucose, total cholesterol, triglyceride, and creatinine between the HT and NT groups. Plasma urea was more in HT group than NT, but its value was within the normal range. As expected, there was a significant difference in the BP between the two groups. Mean SBP in HT group was 146.03±11.37 and NT group was 113.76±3.18. DBP was within normal range.

A significant increase in plasma MDA (p=0.013) in HT group compared to NT group was observed. Antioxidant capacity showed a significant decrease in SOD (p≤0.001) and GSH (p≤0.001) in HT group than NT group. While vitamin C showed no significant difference between two groups. Though there was mean decrease in NOx in HT than NT group, but it was not statistically significant [Table/Fig-2].

[Table/Fig-3] shows correlation between blood pressure and covariates. There was a weak positive correlation (but not significant statistically) between plasma MDA level and BP (SBP and MAP). Fall in plasma GSH and SOD level was significantly correlated with increase in SBP, PP and MAP. We could not find any correlation between changes in plasma vitamin C and plasma NOx with BP.

Further, multiple regression analysis has showed, the decrease in plasma GSH (β= -0.398; p-value=0.001) and SOD (β= -0.423; p-value≤0.001) as the significant determinants of hypertension.

Variables	HT group (Mean±SD)	NT group (Mean±SD)	t-value	p-value
Age (years)	68.47±4.86	68.5±4.26	-0.028	0.978
BMI (kg/m²)	24.52±3.66	24.64±3.65	-0.124	0.902
Systolic BP (SBP)	146.03±11.37	113.76±3.18	25.88	0.000***
Diastolic BP (DBP)	75.33±5.21	71.70±5.17	2.11	0.009*
Pulse Pressure (PP)	70.70±6.14	43.6±7.69	14.824	0.000***
Mean Arterial Pressure (MAP)	98.83±4.69	86.88±4.33	10.422	0.000***
Blood glucose	86.7±9.28	85.0±8.62	0.735	0.466
Total cholesterol	172.9±38.3	174.16±24.6	-0.152	0.88
Triglyceride	111.0±24.5	105.03±20.5	1.024	0.310
Plasma Urea	27.4±4.9	22.53±4.5	0.984	0.000***
Plasma Creatinine	1.03±0.2	1.35±2.21	-0.783	0.440

[Table/Fig-1]: Baseline characteristics of participants.
Unpaired t-test has been applied to find the difference between two groups;
*p<0.05, ***p<0.001.

S. No.	Variables	HT group (Mean±SD)	NT group (Mean±SD)	t-value	p-value
1	MDA (µmol/L)	1.69±0.45	1.44±0.30	2.558	0.013*
2	SOD (U/ml)	1.87±0.94	3.06±0.84	-5.136	0.000***
3	VIT C (mg/dl)	0.83±0.44	0.44±0.47	-1.468	0.148
4	GSH (mg/dl)	16.15±4.35	21.12±5.34	-3.953	0.000***
5	NOx (µmol/L)	33.46±9.69	36.9±8.6	-1.454	0.151

[Table/Fig-2]: Oxidant, antioxidants and nitric oxide concentration in elderly individuals with hypertension and normal blood pressure.
MDA-Malondialdehyde; SOD-Superoxide Dismutase; Vit C-Vitamin C; GSH-reduced Glutathione; NOx-total Nitric Oxide concentration;
*p<0.05, ***p<0.001.

Variables	SBP (mmHg)		DBP (mmHg)		PP (mmHg)		MAP (mmHg)	
	R	p-value	R	p-value	R	p-value	R	p-value
MDA (µmol/L)	0.247	0.057	0.232	0.074	0.126	0.339	0.249	0.055
GSH (mg/dl)	-0.471	0.000***	-0.323	0.12**	-0.375	0.003***	-0.454	0.000***
SOD (U/ml)	-0.536	0.000***	-0.128	0.331	0.499	0.000***	-0.422	0.001***
VIT C (mg/dl)	-0.112	-0.196	0.059	0.657	0.115	0.381	0.041	0.576
NOx	-0.196	0.113	0.066	0.617	-0.188	0.151	-0.077	0.561

[Table/Fig-3]: Bivariate correlation between blood pressure and other variables (n=60).
MDA-Malondialdehyde; GSH-reduced Glutathione; SOD-Superoxide Dismutase; Vit C-Vitamin C; NOx-total Nitric Oxide concentration;
*p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

The present investigation on level of oxidative stress and antioxidant status in elderly individuals with hypertension and normal BP showed a significant elevation in plasma MDA (marker of oxidative stress) and a significant decrease in endogenous antioxidants SOD and GSH. These findings indicate a possible involvement of oxidative stress in mechanism of hypertension.

Oxidative stress is involved in inactivation of Nitric Oxide (NO) and its reduction in bioavailability. Nitric oxide is a key molecule of endothelium that is involved in multitude of vascular function. It is a potent vasodilator and regulates vascular tone, vascular permeability and antithrombotic properties [22]. Sufficient bioavailability of NO is essential for endothelial integrity and function. Insufficient NO bioavailability results in endothelial dysfunction and decreased vasodilator capacity that is associated with all major CV risk factors such as hypertension, diabetes, hyperlipidemia and atherosclerosis [23]. We have observed a mean decrease in plasma NOx in hypertensives than normal controls. A study has shown an increase in vascular endothelial oxidative stress in healthy elderly individuals compared to younger one. They have also shown that increased expression of NADPH oxidase and NF-κB contributes to endothelial oxidative stress [24]. Studies have also shown an association of oxidative stress with inflammation, cell migration, and angiogenesis in hypertension [25]. Oxidative stress promotes smooth muscle cell proliferation and hypertrophy with collagen deposition causing thickening of the vascular media and stiffening of artery [26].

Our study has found an elevation of MDA by 17.36% in hypertensive subjects compared to healthy controls suggesting that oxidative stress is associated with hypertension in elderly. Though plasma MDA levels were elevated in hypertensive subjects, there was a weak association between plasma MDA level and BP. There are few studies on humans showing an elevation of markers of oxidative stress such as H_2O_2 [27,28] superoxide anions, lipid peroxides [28] and thiols [29] in hypertensive subjects. However, these studies have not included elderly hypertensive subjects. On the contrary, other studies have showed no contribution of oxidative stress in the pathogenesis of hypertension [30,31]. Cracowski JL et al., demonstrated no increase in lipid peroxidation in subjects with untreated hypertension compared to healthy controls [30]. Ward NC et al., measured urinary and plasma F_2-isoprostanes in treated and untreated hypertensives and healthy controls. They did not find any significant difference in oxidative stress level between hypertensive subjects and healthy controls [31]. We could find only one study on elderly individuals showing an elevation in oxidative stress markers in hypertensive subjects than normal controls [32] and our findings are in accordance with this study.

The release of biologically active NO is determined by the endogenous antioxidant SOD. SOD detoxifies superoxide radical by converting it into H_2O_2. Reduced activity of SOD increases the superoxide level that inactivates the biologically active NO [33]. Reduction in antioxidants is also implicated in the development of hypertension and other CV risk factors. In our study, we found a significant lower level of plasma SOD and GSH in hypertensives than normal elderly individuals suggesting that decreased antioxidant defense is associated with hypertension. Other studies in middle-aged subjects have shown a negative correlation of SOD activity with systolic and diastolic BP [34]. Moreover, we found a reduction in SOD ($p \leq 0.001$) and GSH ($p \leq 0.001$) as significant determinants of increasing systolic BP.

Studies have shown that oxidative stress increases with age and is implicated in the development of age-related diseases including hypertension [35-37]. However, it has been observed that all the elderly individuals will not suffer from hypertension. Lacy F et al., have investigated the heritability of H_2O_2 production in a family based cohort and found H_2O_2 production as heritable [27]. This data reflects that oxidative stress is heritable and therefore this may be one of the reasons for the existing discrepancies in the documented reports on relationship between oxidative stress and hypertension. Studies have shown an improvement in the antioxidant defense [38] and lowering of oxidative stress in hypertensive subjects following a treatment with antihypertensive drugs [31]. A reduction in oxidative stress was associated with decrease in BP with life-style intervention in elderly with hypertension has been noticed in our earlier study [39]. It remains unclear from the available data whether increased production of ROS contributes to the development of hypertension or rise in BP increases oxidative stress. We presume that the rise in BP triggers the ROS production and increases oxidative stress which in turn inactivates NO and reduces its bioavailability leading to further elevation in BP. To detoxify this increased BP-induced ROS, excess antioxidants (SOD and GSH) might have been utilized. Hence, the antioxidants levels were lesser in hypertensive patients than healthy controls. Therefore, probably oxidative stress may not be the underlying cause for hypertension in elderly, but it may be associated with augmentation of BP by modulating the arterial wall structure and endothelial function.

LIMITATION

Inclusion of only male participants, small sample size and selective evaluation of antioxidant system are the limitations of the study.

Further longitudinal and interventional studies on humans with large sample size are needed to derive a definite conclusion on role of oxidative stress in the pathogenesis of hypertension in elderly.

CONCLUSION

The data of the study showed a significant elevation in oxidative stress and significant decrease in endogenous antioxidants SOD and GSH in elderly individuals with hypertension when compared with healthy controls. There was no significant change in exogenous antioxidant level (vitamin C) between hypertensive and healthy subjects. These findings indicate that elevation in oxidative stress and decrease in endogenous antioxidant level may be involved in the pathogenesis of hypertension. However, role of oxidative stress in the pathogenesis of hypertension remains unclear, whether oxidative stress is the cause of hypertension or high BP induces the production of ROS that augments hypertension.

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