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ORIGINAL ARTICLE

Oxidative Stress Markers and Antioxidant Status in Human Hypertension

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Abstract: *Background:* There is growing evidence that oxidative stress contributes to hypertension justified by observations, predominantly in animal models. *Objective:* The aim of the present study was to observe the relationship, if any, between systemic arterial hypertension and biochemical markers of oxidative stress, inflammation and aging. *Materials & Methods:* 50 subjects with hypertension and 50 age and sex-matched controls were included in the study. The nitrate-nitrite ratio, malondialdehyde (measured as thiobarbituric acid reactive substances, superoxide dismutase activity, and high sensitive C-Reactive Protein were assayed on samples from these subjects by standardized methodology. The data obtained was statistically analyzed. *Results:* Statistically significant mean values of serum nitrate-nitrite ratio and plasma superoxide dismutase activity were lower and that of serum malondialdehyde was higher in hypertensive subjects when compared to the controls. Serum nitrate-nitrite ratio, malondialdehyde and superoxide dismutase activity exhibited statistically significant multivariate correlation with each other while High Sensitive C-Reactive Protein was not significantly correlated. Nitrate-nitrite ratio was significantly lower with aging both in normotensive and hypertensive subjects. *Conclusion:* The present study confirmed the role of oxidation mediated tissue damage in generation of hypertension and suggested the usefulness of examining nitrate:nitrite ratio as a surrogate marker of the pathophysiologic process leading to generation of human hypertension. The study also confirmed an exacerbation of oxidative stress with advancement of age in both study groups.
Key Words: Hypertension, Aging, Nitric oxide, Antioxidants, Inflammation.

Introduction

There is growing evidence that oxidative stress contributes to hypertension. This has been justified by observations that in almost all models of hypertension, there was significant oxidative stress which, if corrected, reduced the blood pressure (BP). On the other hand, other studies have demonstrated that creation of oxidative stress in normal animals may lead to development of hypertension. Four lines of evidence, mostly derived from experiments done on animal models, have indicated that ROS can cause hypertension. It has been observed that rats given lead in their drinking water develop oxidative stress and nitrotyrosine deposition in their blood vessels and organs and hypertension showing that interventions designed to cause oxidative stress lead to development of hypertension [1-2]. Secondly, there have been observations that oxidative stress can precede hypertension. Studies in 4-wk-old spontaneously hypertensive stroke-prone rats (SHRs) showed higher plasma levels of lipid peroxidation products, whereas BP did not rise until after this age [3].

Thirdly, it has been observed that deletion of extracellular superoxide dismutase gene in mice led to development of oxidative stress and higher basal BP [4-5]. Finally are many reports that the correction of the oxidative stress that accompanies a variety of hypertensive models, for example, by administration of the superoxide dismutase mimetic Tempol, also corrected hypertension [6-7]. Thus the causative effect of oxidative stress on hypertension seems to be well established, predominantly on animal models. Consequently, a few definitive markers of oxidative status have gained importance. In humans, circulating nitrite level have been documented to represent an estimate of regional endothelial Nitric Oxide (NO) formation, whereas nitrate, with some caution, has appeared to be useful in estimating overall nitrogen / NO turnover. Moreover, the blood nitrate-nitrite ratio (NO_x) approximately reflects the NO**.** bioavailability status [8]. Other established markers of oxidative status include Malonyldialdehyde (MDA), also called thiobarbituric acid reacting substances (TBARS), which arises as a secondary product of lipid peroxidation and the enzyme superoxide dismutase (SOD), which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide.

A typical marker of inflammation has traditionally been C-reactive protein (CRP), a nonglycosylated protein produced by human hepatocytes in response to infection, inflammation, or tissue damage. Following the onset of the inflammatory stimulus, CRP levels begin to rise within a few hours and peak within 48 hours. Depending on the type and chronicity of the stimulus or treatment, levels may fall rapidly or remain elevated. Thus CRP level may be useful as an unequivocal marker of tissue damage. Measurement of CRP is sensitive and reproducible. The level does not have diurnal variation, and is not affected by age, sex, or hematocrit. Using high sensitivity assays for CRP, recent observations indicate that slightly elevated CRP levels which would be in the normal range of conventional assays are a novel marker for a higher risk for cardiovascular events. Prospective epidemiologic studies have demonstrated High sensitive CRP (hs-CRP) to be a strong predictor of future vascular disease, and several other studies have confirmed that hs-CRP adds prognostic information in the metabolic syndrome and in the prediction of type 2 diabetes [9].

The aim of the present study was to observe the relationship, if any, between systemic arterial hypertension and biochemical markers of oxidative stress and inflammation. In addition, it had also been the authors' intention to observe the effect of age on such subjects. Thus, it was imperative to assess the nitrate-nitrite ratio as a surrogate marker of $NO^{\cdot}$ bioavailability, measure MDA as a definitive marker for oxidative stress and estimate the activity of the major antioxidant enzyme SOD while hsCRP was assessed to serve as marker of inflammation and find out whether any of these factors were associated with the hypertensive state.

Material and Methods

This hospital-based, non-interventional, cross-sectional study was undertaken in the Department of Biochemistry in collaboration with the Department of Cardiology, Medical College, Kolkata, India.

Subjects: The cases were selected from the patients attending the Department of Cardiology, Medical College, Kolkata, who were newly diagnosed to be suffering from hypertension (BP > 140/90). The controls were selected from apparently healthy age and sex matxched individuals from amongst the attenders and relatives accompanying these patients. Written informed consent was obtained from all participants in the study. The study was approved by the institutional ethical committee.

A detailed history was obtained from all participants of the study followed by a thorough physical examination including general examination, systemic examination with emphasis on cardiovascular system and routine diagnostic evaluation which included assessment of Blood Glucose, Serum Creatinine and Serum Lipids (Total Cholesterol, HDL-Cholesterol & Triglycerides). All subjects suffering from diabetes mellitus, having history of stroke or kidney disease, family history of coronary artery disease, addiction to smoking, chronic alcoholism and other substance abuse, conditions associated with free radical mediated tissue injury or secondary hypertension and metabolic syndrome were summarily excluded from the study. 50 subjects with hypertension and 50 age and sex-matched controls were finally included in the study. Each of these two groups was further sub-divided into two age-based subgroups comprising of 25 subjects in each. Individuals between 20 and 40 yrs of age were classed as Young subjects and those between 60 – 70 years of age were classed as Elderly subjects.

Samples: Venous blood samples were collected from all participants of the study observing standard aseptic procedures, Such blood samples were divided into separate suitable vials for estimation of different analytes viz. fluoride-oxalate vials for glucose, Li-heparin vials for SOD and plain vials without anticoagulants or additives for the other tests. Glucose and SOD activity were measured on the same day, while clotted samples were promptly separated and serum stored at -20^0C for 1 week or until tests were completed, whichever was earlier. In order to minimize the effect of diurnal variation, if any, in the value of the analytes, all the samples were routinely collected in the morning between 10 to 11:30 AM.

Methods: The nitrate-nitrite ratio was determined by the Cadmium reduction method (Cortas & Wakid), whereby the nitrate was reduced to nitrite by metallic cadmium granules and nitrite determined by diazotization of sulphanilamide and coupling it to N-naphthylethelene diamine [10]. Malonyldialdehyde was determined by the Thiobarbituric acid reaction, as described by Dahle, Hill et al [11]. The SOD activity was measured by the phenazinemethosulphate reaction as elaborated by Kakkar, Das et al [12]. The hsCRP was measured by a standard kit-based Immunoturbidimetric assay [13].

Statistical Analyses: The data obtained was statistically analyzed. The data were first segregated into four groups: young normotensives, young hypertensives, elderly normotensives & elderly hypertensives. Group statistics (mean, SD, bivariate & multivariate correlation, logistic regression analysis by backward stepwise method to

determine the likelihood ratio of the parameters as predictors of hypertension) were then determined. p value of less than 0.05 was predefined to be a statistically significant observation. Coefficient of Variation, expressed as a percentage (CV%) for each method was also derived.

Results and Discussion

A statistically significant lower mean value of serum nitrate-nitrite ratio (NO_x) ($p < 0.05$) and plasma superoxide dismutase (SOD) activity ($p < 0.05$) and a statistically significant higher mean value of serum thio-barbituric acid reactive substances (TBARS) ($p < 0.05$) was observed in hypertensive subjects when compared to the controls. However, there was only a statistically insignificant elevation in the mean level of hsCRP in the hypertensives in comparison to the control subjects. These findings have been summarized in Table 1. The Precision for each assay method was evaluated by calculating the Coefficients of Variation (CV) from 20 data points obtained by testing a pooled human serum sample. The calculated CVs expressed as a percentage for Nitirate-Nitrite Ratio, TBARS, SOD and hsCRP were 4.07%, 4.89%, 5.92% and 1.97% respectively.

Table-1: Comparison of Mean and Standard Deviation of parameters between hypertensive (cases) and normotensive (controls) groups

Parameters	Cases (n=50) Mean (± 2SD)	Controls (n=50) Mean (± 2SD)	Level of significance	CV (%)
Serum Nitrate-Nitrite Ratio	2.1 (±0.7)	4.8 (±1.5)	p =0.021	4.07
Serum TBARS (μM/L)	16.9 (±3.4)	9.6 (±3.2)	p =0.009	4.89
Plasma SOD (mUnits/L)	4.9 (±1.5)	10.3 (±2.9)	p =0.018	5.92
Serum hsCRP (mg/L)	5.6 (±4.4)	6.5 (±5.3)	p =0.358	1.97

Several mechanisms contributing to a lower $NO^{\cdot}$ bioavailability in hypertension has been suggested. Stroes E et al have observed that under certain conditions, eNOS can produce superoxide rather than NO [14]. This may be coupled with the ability of superoxide to quench NO, resulting in the formation of the potent oxidant peroxynitrite. One major product of peroxynitrite attack on proteins is the nitration on the 3 position of tyrosine. Indeed, several data have shown that there is a significant increase in the nitrotyrosine levels in the kidneys of hypertensive rats. From these data, it may be suggested that there indeed is a higher formation of peroxynitrite in hypertension that reduces the $NO^{\cdot}$ bioavailability and hence in turn may induce vasoconstriction of the vasculature. Also, it has been postulated by Jung O et al that extracellular SOD (EC-SOD) is a major determinant of $NO^{\cdot}$ bioavailability [15]. $NO^{\cdot}$ bioavailability is lowered due to peroxynitrite formation in presence of $O_2^{\cdot-}$. SOD has the opposite effect of quenching the $O_2^{\cdot-}$. Hence a lower SOD level may be accompanied by a simultaneous lower $NO^{\cdot}$ bioavailability.

A significant finding of the present study was lower SOD levels in hypertensive cases as compared to controls. Several other studies have reported similar findings [16-17]. This may seem paradoxical in the view of SOD activity being expected to be augmented in conditions of higher oxidative stress. However, Didion and Faraci[35] have postulated that under activity of SOD may be the primary defect, which in turn contributes to the development of hypertension [18]. Indeed, this has been confirmed by studies on gene knockout animal models and transgenic animals [19-20]. Further, these findings have been corroborated from studies involving human beings. Zhou, Xiang et al have demonstrated low SOD acivity in African-American hypertensive individuals [21]. Simić, Perunicić et al have shown low RBC SOD activity in different stages of hypertension [22].

In spite of numerous reports indicating an association between hsCRP and hypertension per se or with cardiovascular risk factors in general, no such association was observed in the present study (Table 1) [23-24]. Numerous prospective studies conducted in the general population, as well as in selected groups have reported a positive association between hsCRP and higher CVD risk, or surrogates of vascular risk. However, in such studies, when the emphasis was put on the predictive power of hsCRP over other CVD risk factors, it became evident that the available data on hsCRP were not consistent [25]. Indeed, several large prospective studies did not find a significant additive prognostic value of hsCRP over traditional CVD risk factors. Thus, it might be logical to suggest hsCRP as quite a non-specific marker which may vary from the effect of a number of confounders including acute infections, chronic diseases, smoking, hormone replacement therapy, obesity, age, diabetes, and atrial arrhythmias, and it is associated with the metabolic syndrome that is strongly linked to a proinflammatory state [26]. Hence it remains unclear whether hs-CRP is a marker for a low-grade inflammatory systemic state, a marker for the metabolic syndrome, or a marker for both. Moreover, Levinson et al has argued that hs-CRP may be a statistical predictor of heart disease because it reflects ongoing arteriosclerosis rather than serving as a predictor of a disease that has not yet arisen, such as dyslipidemia and hypertension [27]. They have further concluded that hs-CRP exhibits a very low Bayesian positive predictive value when used alone as a marker for predicting CAD and the positive predictive value for hs-CRP was calculated to be only 0.86%. In the light of these observations, it may not be entirely surprising that no significant difference in mean levels of hsCRP between the cases and control groups.

Table 2 shows the results of multivariate correlation analysis between NO_x, TBARS and SOD in Cases and Controls groups. It was observed that NO_x, TBARS and SOD showed statistically significant multivariate correlation with each other ($p < 0.05$). However, no statistically significant correlation was found between hsCRP and any of the other three parameters. Logistic regression analysis of the case vs. control data in 20-40 yrs. age group by the backward stepwise method to determine the likelihood ratio for the parameters as predictors of hypertension mediated tissue destruction revealed that TBARS and SOD were good predictors for hypertension mediated tissue destruction in the 20-40 yrs. age group with β-values of 5.893 and -9.936

respectively while NO_x and TBARS have a significant likelihood ratio in the 60-70 yrs. age group with β-values of -22.037 and 4.418 respectively (statistically significant β-values are those between > +1 or < -1). This observation that TBARS has consistently emerged as a good predictor of hypertension mediated tissue destruction in both young and the elderly individuals may be expected. But SOD as a reliable predictor for hypertension in the younger age group may deserve a plausible explanation. Since several lines of evidence, as already discussed, suggests that an inherent defect in SOD may result in hypertension, it may not be impertinent to suggest that such disabling mutations in the SOD genes may be more likely to affect individuals very early in their lives. Also, the observation that NO_x was a good predictor of hypertension in the elderly age group may be justified by the fact that among the parameters included in the present study, only NO_x has been described to have a significant negative correlation with age. Since, in addition, NO_x is also negatively correlated with hypertension, it may be inferred that age and hypertension may have combinatorial influence on NO_x.

Table-2: Multivariate Correlation Analysis between different parameters in hypertensive (cases) and normotensive (controls) groups
X_1 = Serum Nitrate-Nitrite Ratio, X_2 = Serum TBARS (μM/L), X_3 = Plasma SOD (mU/L)

		$R_{1.23}$	$R_{2.13}$	$R_{3.12}$
Cases (n = 50)	R value p value	0.816 p =0.019	0.901 p =0.012	0.773 p =0.030
Controls (n = 50)	R value p value	0.877 p =0.017	0.932 p =0.009	0.809 p =0.018

Note: $R_{1.23}$ is the multivariate correlation coefficient when considering X_1 as the dependent variable and X_2 and X_3 as independent variables. $R_{2.13}$ & $R_{3.12}$ denote similar relative measurements.

Table-3: Comparison of Mean and Standard Deviation of parameters between Young (20–40 yrs.) and Elderly (60 – 70 yrs.) subjects

Parameters	Young (n=50) Mean (±2SD)	Elderly (n=50) Mean (±2SD)	Level of significance
Serum Nitrate-Nitrite Ratio	3.7 (±2.9)	3.2 (±2.8)	p =0.099
Serum TBARS (μM/L)	12.7 (±7.7)	13. 9 (±8.2)	p =0.139
Plasma SOD (mUnits/L)	7.8 (±6)	7. 4 (±5.8)	p =0.457
Serum hsCRP (mg/L)	5.4 (±3.8)	6. 7 (±5.7)	p =0.201

When the data obtained by analysis of blood samples collected from the study population was segregated into two age-based groups viz. young and elderly subjects, it was observed that though NO_x and SOD levels were lower and TBARS and hsCRP were higher in the elderly individuals, none of the results were

statistically significant. These findings have been depicted in Table 3. However, when data for normotensive subjects (controls) were stratified into the two age-based groups, it was found that NO_x was significantly lower with aging (p = 0.023). This finding, presented in Table 4, has been corroborated by the work of Tschudi et al, who have observed a very strong correlation between NO_x and age [28]. A similar observation was noted in hypertensives between the two age-based groups (Table 5). Thus study confirmed an exacerbation of oxidative stress, as represented by reduced NO_x, with advancement of age in both the cases and control population.

In as far as TBARS and SOD is concerned, there was no significant deviation observed between the mean levels of these two parameters among the two age-based population subgroups (Table 3). In the context of the present study, it may be noted that a predominant section of the study population was from an urban area, which may have been exposed to high levels of oxidant stress due to prevalent air pollution, thus exhibiting discordant levels of these two analytes irrespective of the age variations. This finding has been corroborated by the results of a study by Niwa, Iizawa et al, who have demonstrated an absence of variation in the basal activity of all three isoforms of SOD with aging [29]. The absence of significant alteration in hsCRP levels may be justified by other studies demonstrating no significant variation of hsCRP with age [30].

Table-4: Comparison of Mean and Standard Deviation of parameters between young (20-40 years) and elderly (60-70 years) normotensive individuals (Controls)

Parameters	Young (n = 25) Mean (±2SD)	Elderly (n = 25) Mean (±2SD)	Level of significance
Serum Nitrate-Nitrite Ratio	5.0 (± 0.9)	4.5 (± 0.5)	p =0.023
Serum TBARS (µM/L)	9.2 (± 1.6)	10.1 (± 1.6)	p =0.057
Plasma SOD (mUnits/L)	10.5 (± 1.6)	10.1 (± 1.3)	p =0.280
Serum hsCRP (mg/L)	5.8 (± 3.9)	7.2 (± 6.5)	p =0.363

Table-5: Comparison of Mean and Standard Deviation of parameters between young (20-40 years) and elderly (60-70 years) hypertensive individuals (Cases)

Parameters	Young (n = 25) Mean (±2SD)	Elderly (n = 25) Mean (±2SD)	Level of significance
Serum Nitrate-Nitrite Ratio	2.4 (± 0.3)	1.9 (± 0.3)	p = 0.012
Serum TBARS (µM/L)	16.2 (± 1.8)	17.7 (± 1.3)	p = 0.005
Plasma SOD (mUnits/L)	5.1 (± 0.5)	4.7 (± 0.9)	p = 0.039
Serum hsCRP (mg/L)	0.6 (± 0.5)	0.5(± 0.4)	p = 0.373

Thus, the present study confirmed the role of oxidation mediated tissue damage in generation of hypertension in human, as evidenced by the lower serum nitrate-nitrite ratio and plasma superoxide dismutase activity and higher serum thiobarbituric acid reactive substances. However, an association of hsCRP with hypertension was not observed. Further, the study suggested the usefulness of examining nitrate:nitrite ration as a surrogate marker of the pathophysiologic process leading to generation of human hypertension. The study also confirmed an exacerbation of oxidative stress, as represented by reduced NO_x, with advancement of age. However, other indicators of oxidative damage were not significantly altered with age, probably due to the effects of other confounders. These findings of the present study may find usefulness if these results can be reproduced in larger population studies which may then establish serum nitirate: nitrite ratio as a surrogate marker in hypertension and probably examining the efficacy of antioxidant therapy based treatment modalities in human hypertension.

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