Biomarkers of antioxidant status and lipid peroxidation in elderly patients with hypertension

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ABSTRACT

Objective: The objective of the present study was to examine selected parameters of the blood redox system in elderly patients with hypertension. Methods: We analyzed differences in redox-associated molecules and enzymes among elderly hypertensive subjects (age above 65 years, n = 49) and two groups of normotensive subjects (<65 years old – Control group I; n = 27, and >65 – Control group II; n = 30). Results: Decreased activity of antioxidant enzymes, increased lipid peroxidation and reduced production of nitric oxide were observed in hypertensive subjects, compared to healthy younger controls, or those of the same age. In healthy controls, an age-related decrease in the production of nitric oxide and the activities of SOD-1 and GPx-1 was also evident. The pathology of hypertension was characterised by further, significant decreases in the values of these parameters. When the subgroups of females and males were compared to their respective controls, a compromised redox balance was observed that was more evident in female hypertensives. Discussion: Hypertension in elderly patients is accompanied by changes in biomarkers of antioxidant status and lipid peroxidation status, which significantly differ from those observed in healthy ageing subjects. Our study also suggests that the relationship of gender and changes in redox balance with regard to hypertension should be further explored.

Introduction

Raised blood pressure (BP) represents a global problem, affecting around 40% of 25-year-old adults in 2008. Moreover, approximately 12.8% of the total global mortality is ascribed to high BP. The situation is worsening due to aging populations, resulting in substantial rises in the number of people with uncontrolled hypertension. This number rose from 600 million to nearly one billion between 1980 and 2008 [1]. These statistics highlight that hypertension is a worldwide epidemic and the risk of high BP increases with age. In addition, epidemiological evidence suggests differences in BP-related pathology between men and women.

Understanding interdependencies between age, sex, and BP, and the biological mechanisms underpinning these relationships, is crucial for the development of effective preventive and therapeutic strategies for controlling BP and management of BP-related diseases. Research into age- and sex-dependent associations between genetic and nongenetic factors and hypertension has been performed in animals and humans [2,3]. For example, Salazar et al. [3] studied the role of angiotensin II (Ang II) in hypertension and renal changes during the nephrogenic period. They also investigated whether BP and renal changes are age and sex dependent. The results indicated an age- and sex-dependent role of Ang II in maintaining hypertension and renal changes. Moreover, it has been demonstrated that oxidative stress (OS) is involved in the activity of Ang II. Other alterations in regulatory systems that affect hypertension include upregulation of the renin–angiotensin–aldosterone system, activation of the sympathetic nervous system, perturbed G protein cell signaling, inflammation, and altered T-cell function [4]. All these processes share the common feature of increased OS.

OS occurs due to increased Reactive Oxygen Species generation, decreased nitric oxide (NO) bioavailability, or reduced antioxidant capacity of cells and tissues. It alters redox signaling, which can play a role in pathogenesis and progression of hypertension. Indeed, there is accumulating evidence that OS is associated with altered function of the endothelium, and has direct effects on contractility of vascular smooth muscle [5]. Both OS and the risk of hypertension are known to increase with age [6].

The role of reactive oxygen and nitrogen species in the pathology of hypertension has been extensively analyzed in various biological systems, including animals and human and in vitro experiments. However, the relationship between OS, age, and hypertension remains unclear. Epidemiological studies have shown that advancing age is associated with an increased prevalence of hypertension [7] and gender-related effects on BP [8]. These studies do not clarify the relationship between OS, hypertension, and age. In our study, we hypothesize that OS and hypertension are independent in a sense that there is no absolute causal link between them, considering that the former is also present in ‘healthy aging’. Therefore, to explain the relationship between OS and hypertension in general terms, mechanisms of action and biomarkers of antioxidant and oxidants status, specific to hypertension, need to be clarified. This information can be used to distinguish between healthy aging and...
pathologies, such as hypertension, for which age is an independent risk factor. OS can then be confirmed as the mechanism behind this relationship. Moreover, to our knowledge, no studies have been published on gender differences in the redox systems of healthy aging and hypertensive populations.

In this study, we have compared the biomarkers of antioxidant status and lipid peroxidation in elderly patients with hypertension, with those of healthy controls. The differentiation between younger (<65 years old) and older (≥65 years old controls) subjects allowed us to identify age-related differences in antioxidants and lipid peroxidation in healthy aging, and compare them with the pathology of hypertension. Comparisons of these parameters were also made between genders. Our results indicate that age-dependent OS, and OS associated with hypertension, are distinct phenomena which require further investigation.

Patients and methods

Subjects

Participants in this study were elderly patients (aged ≥65 years) who had been diagnosed with hypertension, according to ICD-10 diagnostic criteria. Normotensive subjects were divided into two groups according to age (<65 years – Control I, and >65 years – Control II). Patients were recruited from the Clinic of Geriatrics, Collegium Medicum UMK, Bydgoszcz, Poland, whereas normotensive volunteers were also recruited in the Nursing Home ‘Jesień Zycia’ and the Department of Biochemistry, Collegium Medicum UMK, Bydgoszcz, Poland. In total, there were 49 hypertensive subjects (36 females, 13 males), 27 normotensive subjects aged <65 (19 females, 8 males), and 30 normotensive subjects aged ≥65 (23 females, 7 males).

Based on the results from our previous research, a minimum sample size of 30 is required to achieve 80% power to detect differences between mean values of analyzed parameters [9]; the confidence level was set as 95%. The sample size was computed using the Minitab 17 software.

The specific inclusion criteria for patients were stage one and two hypertension, diagnosed ‘de novo’ according to the guidelines of the Polish Society of Hypertension (2011). All subjects were evaluated by standard physical examination and routine laboratory tests. Table 1 shows the clinical and biochemical characteristics of the controls and patients.

We excluded from this study subjects who were abusing alcohol, smoked, suffered from a condition relating to OS (e.g. diabetes and cancer), had been diagnosed with cardiovascular comorbidities, or were supplemented with medicines that affect redox balance.

Written informed consent was obtained from all study participants. The Nicolaus Copernicus University in Torun Human Ethics Committee approved the study protocol and the study was conducted under the tenets of the Declaration of Helsinki.

Blood sample collection

Fasting venous blood was collected into EDTA, heparin, and clot-activated tubes in the morning (08:00am). The remaining blood was centrifuged and, after careful removal of plasma or serum and buffy coat, the erythrocytes were washed with cold isotonic saline (0.89% NaCl, pH 7.4). The sediment was then lysed by the addition of distilled water, followed by three cycles of freezing and thawing to obtain erythrocyte hemolysate. The hemolysate was suspended in bi-distilled water.

After blood processing, the serum and plasma samples were aliquoted and immediately stored at −80°C for further analyses.

Laboratory methods

Reduced glutathione (GSH) concentration was determined spectrophotometrically in whole blood, using the method of Beutler [10], with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB).

GSH reacts non-enzymatically with DTNB to generate oxidized GSH, and 5-thio-2-nitrobenzoic acid (peak absorbance at 420 nm). The concentration of Hb in the hemolysate was determined using a commercial reagent at 540 nm.

Glutathione peroxidase (GPx-1), superoxide dismutase (SOD-1), and catalase (CAT) activities of erythrocytes were determined in hemolysates, according to Paglia and Valentine [11], Misra and Fridovich [12], and Beers and Sizer [13], respectively. GPx activity was determined by measuring the oxidation of NADPH, by the decrease in absorbance at 340 nm at 25°C, in coupled reactions with t-butylhydroperoxide, GSH, and Glutathione Reductase. SOD activity was determined in ethanol/chloroform-treated samples, by measuring the inhibition of autoxidation of adrenaline at 37°C and pH 10.2. The activity of CAT was determined by following the absorbance of H₂O₂ decomposition at 240 nm, pH 7.0, and 25°C.

Erythrocyte concentration of thiobarbituric acid reactive substances (TBARS) was determined using the 2-thiobarbituric acid (TBA) method [14]. The samples were added to a mixture of TBA and trichloroacetic acid in HCl to precipitate

| Parameters | Patients | Control I (<65) | Control II (≥65) |
|------------|----------|----------------|----------------|
| n          | 49       | 30             | 30             |
| Age (years)| 75.0 ± 9.2 | 32.5 ± 9.1    | 79.3 ± 9.6     |
| Smoking    | No       | No             | No             |
| BMI (kg m⁻²) | 25.0 ± 0.2 | 26.2 ± 4.4    | 25.7 ± 3.1     |
| Systolic BP (mmHg) | 161.9 ± 11.9*** | 118.2 ± 7.5 | 118.4 ± 10.7 |
| Diastolic BP (mmHg) | 90.3 ± 6.9**   | 78.1 ± 5.4#    | 69.7 ± 6.3     |
| Plasma glucose concentration (mg dL⁻¹) | 86.4 ± 10.1    | 79.7 ± 5.4    | 95.7 ± 14.9     |
| Plasma total cholesterol (mg dL⁻¹) | 193.4 ± 20.2   | 176.8 ± 14.2  | 196.3 ± 40.3 |
| Plasma HDL cholesterol (mg dL⁻¹) | 52.5 ± 8.8     | 46.4 ± 6.9    | 57.6 ± 12.8    |
| Plasma LDL cholesterol (mg dL⁻¹) | 143.1 ± 17.9   | 136.3 ± 16.1  | 140.3 ± 37.9   |
| Plasma triglycerides (mg dL⁻¹) | 161.7 ± 39.7** | 126.7 ± 11.5  | 119.8 ± 26.4  |
| Serum creatinine concentration (mg dL⁻¹) | 0.863 ± 0.18   | 0.718 ± 0.11  | 0.989 ± 0.12   |

Values are expressed as mean ± SD. p < 0.05 was considered statistically significant.

Patients vs. Control I (p < 0.05, **p < 0.01, ***p < 0.001); Patients vs. Control II (p < 0.05, i i ip < 0.01, i i i ip < 0.001); Control I vs. Control II (ip < 0.05, # # ip < 0.01, # # # ip < 0.001).
the protein. The reaction was performed at pH 2–3 at 90°C for 20 min. The precipitate was pelleted by centrifugation at 4000 g at room temperature for 15 min. Absorption of supernatants was measured at a wavelength of 532 nm.

Production of NO (concentration of nitrite/nitrate in plasma) was determined by the Griess reaction, according to Marletta et al. [15]. The nitrite concentration in plasma was measured as an indicator of NO production, using the Griess reaction. Briefly, the sample was mixed with the Griess reagents (sulfanilamide and naphthalene-ethylene diamine dihydrochloride), and the absorbance at 540 nm was measured.

Oxidase activity of ceruloplasmin (Cp) was assessed according to the method of Ravin [16]. Cp activity against aromatic amines was measured by the action of Cp on p-phenylenediamine at 530 nm.

**Statistical analyses**

The results of the measurements are presented as the mean ± standard deviation of the mean. The comparisons between the groups were conducted using the one-way ANOVA with the post hoc Tukey HSD (honestly significant difference) test. Results are reported as statistically significant when p < 0.05.

**Results**

Comparisons of redox status between elderly hypertensive subjects (aged ≥65) and two control groups of subjects with normal BP divided by age (<65 and ≥65 years) are shown below. The comparison between the control groups is also illustrated. Moreover, we analyzed the differences between males and females separately.

We observed significant differences between the patients and control groups for all study parameters. Significantly, sex-dependent differences were also evident between hypertensive patients and the controls.

The mean concentration of GSH was significantly different between the patients, control group I, and II (p < 0.01). Post hoc comparison using the Tukey HSD test indicated that the mean GSH concentration in erythrocytes was significantly decreased in hypertensive patients, when compared with younger or older normotensive subjects 2.50 ± 0.38 vs. 2.73 ± 0.21 mmol/L (p < 0.01), and vs. 2.75 ± 0.33 mmol/L (p < 0.01), respectively (Table 2). An assessment of the gender-dependent differences between the patients and controls revealed that the mean glutathione concentration of female hypertensive patients was different from non-hypertensive female controls aged <65 and non-hypertensive female controls aged ≥65; both p < 0.05 (Table 3).

With regard to the activity of antioxidant enzymes, we observed significant differences in the activity of glutathione peroxidase (GPx-1) between the study groups, p < 0.001. The activity of GPx in hypertensive subjects (13.00 ± 2.46 U/gHb−1) was significantly lower than that in healthy controls I (17.39 ± 3.22 U/gHb−1, p < 0.001) and healthy controls II (15.19 ± 2.88 U/gHb−1, p < 0.01). The difference between younger and older controls was also significant, p < 0.01 (Table 2). Furthermore, the mean level of activity of glutathione peroxidase in female hypertensive patients was different from non-hypertensive female controls aged <65 and from non-hypertensive female controls aged ≥65, p < 0.001 and p < 0.05, respectively (Table 3).

The activity of SOD-1 was significantly different between the groups, p < 0.001. The activity of this enzyme in hypertensive patients was significantly lower than control I (2293 ± 177.2 vs. 3258 ± 362.4 U/gHb−1, p < 0.001) and control II (2926 ± 371.8 U/gHb−1, p < 0.001). A significant difference was also observed between control I and II, p < 0.001 (Table 2). Comparisons between subgroups based on gender

**Table 2.** Comparison of study parameters between the study and control groups by ANOVA.

| Parameters                  | Patients (n = 49) | Control I (<65) (n = 30) | Control II (65) (n = 30) | P-value |
|-----------------------------|------------------|--------------------------|--------------------------|---------|
| GSH (mmol/L−1)              | 2.50 ± 0.38**    | 2.73 ± 0.21              | 2.75 ± 0.33              | <0.01   |
| Glutathione peroxidase (GPx) | 13.00 ± 2.46**   | 17.39 ± 3.22#            | 15.19 ± 2.88             | <0.001  |
| SOD1 (U/gHb−1)              | 2293 ± 177.2***  | 3258 ± 362.4#            | 2926 ± 371.8             | <0.001  |
| CAT (U/gHb−1)               | 21.8 ± 1.7**     | 23.8 ± 3.60              | 23.4 ± 3.91              | <0.01   |
| TBARS (mol/gHb)             | 0.277 ± 0.03**   | 0.230 ± 0.03             | 0.238 ± 0.03             | <0.01   |
| NO (mol/L)                  | 1.34 ± 0.39      | 1.80 ± 1.16#             | 3.62 ± 1.48              | <0.001  |
| Cp (U/L)                    | 1116.77 ± 318.6*** | 756.1 ± 229.54         | 840.30 ± 156.71          | <0.001  |

One-way ANOVA, *significant at 5% level of significance (p < 0.05), values of parameters are expressed as mean ± SD.

**Table 3.** Comparison of study parameters between the study and control gender-based subgroups by ANOVA.

| Parameter                  | Group I Male | Group II Female | Group III Control I (<65) Male | Group III Control I (<65) Female | Group IV Control II (65) Male | Group IV Control II (65) Female | Group V Control I (65) Male | Group V Control I (65) Female |
|----------------------------|--------------|-----------------|-------------------------------|---------------------------------|-------------------------------|---------------------------------|----------------------------|-----------------------------|
| GSH (mmol/L−1)             | 2.63 ± 0.42  | 2.46 ± 0.34**   | 2.68 ± 0.42                   | 2.75 ± 0.21                     | 2.84 ± 0.27                   | 2.73 ± 0.34                     |
| Glutathione peroxidase (GS-Px) (U/gHb−1) | 13.68 ± 2.44  | 13.00 ± 2.48***  | 14.81 ± 3.05                  | 17.39 ± 3.39                    | 15.63 ± 2.88                   | 15.19 ± 2.96                    |
| SOD1 (U/gHb−1)             | 2333 ± 190.3*** | 2279 ± 164.1**  | 3275 ± 333*                   | 3542 ± 391.8                    | 2753 ± 360.2                   | 2985 ± 383.4                    |
| CAT (U/gHb−1)              | 21.6 ± 1.5   | 21.8 ± 1.98     | 24.3 ± 4.10                   | 23.8 ± 3.10                     | 24.8 ± 4.20                   | 23.0 ± 3.62                     |
| TBARS (mol/gHb)            | 0.271 ± 0.04 | 0.280 ± 0.02***  | 0.240 ± 0.02                  | 0.238 ± 0.04                     | 0.242 ± 0.04**                 | 0.212 ± 0.02                    |
| NO (mol/L)                 | 1.34 ± 0.37** | 1.37 ± 0.41**    | 2.55 ± 1.15                   | 1.57 ± 1.10*                    | 3.84 ± 1.26                   | 3.57 ± 1.56                     |
| Cp (U/L)                   | 1149 ± 495.2* | 1135 ± 262.0**  | 797 ± 269.2                   | 796 ± 162.3                     | 869 ± 250.7                   | 846 ± 62.7                      |

One-way ANOVA, *significant at 5% level of significance (p < 0.05), values of parameters are expressed as mean ± SD.
revealed significant differences between hypertensive male subjects and their respective controls aged <65 years, p < 0.001, or aged ≥65, p < 0.05. The difference in the mean activity of SOD-1 between male control groups I and II was also significant, p < 0.05. A significant difference in SOD activity between hypertensive patients and female controls I and II was also detected, both p < 0.001. In addition, the differences in the mean activity of SOD-1 between female controls aged <65 and those age ≥65 was also significant, p < 0.05 (Table 3).

Significant differences in the activity of CAT were observed between the study groups, p < 0.01. The measurements for CAT activity in hypertensive patients and control group I were 21.8 ± 1.74 and 23.84 ± 3.60 U/gHb, respectively; p < 0.05 (Table 2). No significant differences between the patients and controls divided into gender subgroups were evident.

We also measured the product of lipid peroxidation, TBARS. Statistically significant differences in TBARS levels were detected, p < 0.001. The erythrocyte concentration of TBARS was significantly increased in patients compared to control group I (0.277 ± 0.03 vs. 0.230 ± 0.03 μmol/gHb; p < 0.001), and also when compared with control group II (0.277 ± 0.03 vs. 0.238 ± 0.03 μmol/gHb; p < 0.001).

Significant differences in mean TBARS concentration were observed between men and women in the patient and two control groups. First, there was a significant difference in the mean concentration of TBARS in female hypertensive patients when compared with non-hypertensive control females age <65 and with non-hypertensive control females age ≥65, both p < 0.001. Second, the comparison between controls revealed a significant difference in TBARS between males and females age ≥65, p < 0.01 (Table 3).

There were significant differences in the production of NO between the study groups. The NO level was lower in the patients with hypertension than the control group II, 1.34 ± 0.39 vs. 3.62 ± 1.48 μmol/L, respectively; p < 0.001. A significant difference between control group I and II (p < 0.001) was also observed. There was also a significant difference between hypertensive and non-hypertensive males (age ≥65, control II); p < 0.01, as well as between hypertensive and non-hypertensive females (age ≥65, control I); p < 0.01. In women, NO production varied between control groups I and II, p < 0.001 (Table 3).

Significant variations in the activity of Cp, p < 0.001, were observed between the groups. Measurements for the patient and control group I are 1116.77 ± 318.66 U/L vs. 756.18 ± 229.54 U/L, respectively; p < 0.001, and between patient and control group II are 1116.77 ± 318.66 U/L vs. 840.30 ± 156.71 U/L, respectively; p < 0.01. In the male subgroup of the hypertensive patients, the mean activity of Cp differed from the control group I, p < 0.05. Similarly, in a female subgroup of the patients, the mean activity of Cp differed from control groups I and II, both p < 0.001.

**Discussion**

In this study, we have observed changes in the normal redox balance in the blood of hypertensive patients. Decreased activity of antioxidant enzymes, increased lipid peroxidation, and reduced production of NO were found in hypertensive subjects, compared to healthy younger controls, and those of the same age. Age-related decreases in the production of NO and the activities of SOD-1 and GPx-1 were also observed in healthy subjects.

The influence of gender in the changes of redox balance in relation to hypertension and aging was also revealed in our study. In men, differences between the hypertensive and control group were evident for SOD-1, NO, and Cp, whereas healthy males age <65 and healthy males age ≥65 differed only with regard to SOD-1 activity. In women, differences between hypertensive and control females were detected for GSH, GPx-1, SOD-1, TBARS, NO, and Cp. Moreover, mean SOD-1, TBARS, and NO values were significantly different between healthy females aged <65 years and ≥65 years.

Our findings are supported by previous studies which demonstrated redox imbalances associated with aging and hypertension. For example, we have reported a decrease in SOD-1 activity related to age, both in healthy controls and hypertensive subjects [17]. We concluded that this strong association between age and SOD activity cannot be easily altered, although it can be modified by pathological conditions, such as hypertension and pharmaceutical treatment. SOD activity may decrease in patients with untreated hypertension, but be enhanced by antihypertensive medication [18–20]. Similarly, CAT activity decreases in untreated hypertensive patients, but can also be enhanced by antihypertensive medication [18–20]. Overexpression of CAT has been shown to prevent hypertension and to normalize the expression of renal angiotensin-converting enzyme-2 expression in Akita mice [21]. Published results on the link between GPx activity and hypertension are inconsistent, with reports of increases and decreases, as well as no changes in enzyme activity [20,22]. Similarly, there is conflicting evidence regarding the effect of age on GPx activity.

In this study, the activity of Cp, a regulator of NO activity, was also evaluated. Increased serum activity has been linked to long-term adverse cardiovascular events. This relationship was strong even after multivariable model adjustment for traditional clinical and biological risk factors [23]. Antihypertensive treatment has been shown to decrease Cp activity [24]. Pro-antioxidant imbalance in hypertensive patients was also reflected in our study by the increased level of lipid peroxidation (TBARS). A similar association between TBARS and hypertension has been reported previously [25,26].

The published literature fails to explain the sex-based differences in redox parameters between hypertensive and healthy aging. Furthermore, the impact of gender in hypertension also remains poorly clarified [27]. Animal studies have demonstrated considerable differences in the expression of antioxidant and pro-oxidant enzymes, levels of markers of OS, and functional responses to pro- and antioxidant agents in male and female spontaneously hypertensive rats. For example, the activity of glutathione peroxidase was found to be higher in the kidneys of males [28]. A recent study by Horvathova et al. [29] investigated the contribution of blood OS to the development of hypertension, using the genetic models of hypertension. They also examined gender differences in the antioxidant defense system, and found no differences in SOD, CAT, and GPx between males and females. Here, we also observed no differences between males and females, although a compromised redox balance of the measured parameters was more evident in female hypertensives.

One limitation of this study is that its design differs from other studies [28] in terms of clinical vs. preclinical, or comparing genetic predisposition with development due to non-genetic factors. The small number of subjects in the groups and uneven gender distribution are another limitation of
our study; results on gender differences should be viewed as preliminary. Moreover, regarding the measurements, the results for nitrite/nitrate concentration need to be interpreted with the caution since we measured it by Griess reaction without using adequate control measures for the confounding effects of dietary nitrate ingestion.

The lack of a consensus on the relationship between hypertension and the parameters measured in this study hinders the interpretation of the results. Unequivocal results from different studies may be partly explained by the fact that cellular antioxidants are under homeostatic control. Hence, a decrease in one antioxidant can be compensated for by an increase in another. This is a limitation of the majority of studies involving the selective assessment of redox parameters. Comprehensive studies into age-related changes in the whole antioxidant system, in hypertensive patients, are needed. Moreover, there is still no consensus on whether OS is a cause or consequence of hypertension; further studies are needed to clarify the mechanisms behind, and fulfill the potential of, redox medicine [29,30].

Conclusions and further research

Based on the results from this study, we conclude that the redox balance changes in the blood of hypertension elderly patients. This imbalance is represented by changes in the activities of antioxidant enzymes, increases in lipid peroxidation and decreases in the production of NO. These changes differ from the redox imbalance associated with healthy aging. Our results, therefore, highlight the need for more research to clarify the difference between OS linked to age and hypertension, and other age-related pathological conditions. Our study also demonstrated, for the first time, that redox metabolism is compromised in female hypertensive patients compared to non-hypertensive subjects. Further studies into the interaction between gender and the pathology of hypertension would be highly beneficial.

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