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High serum levels of reactive nitrogen species and low total antioxidant capacity in patients with resistant hypertension compared to those in age- gender matched healthy controls, controlled hypertension and follow up with propranolol treatment in the extended APPROPRIATE trial

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Abstract

Objectives To perform a comparative analysis of the extended APPROPRIATE trial of measures of reactive nitrogen species and antioxidant capacity in patients having resistant hypertension with controlled hypertension and healthy controls.

Results Mean serum NO_2- and NOx levels were significantly lower and mean AOC was significantly higher in patients with controlled hypertension ($n=38$) and healthy controls ($n=38$) compared to resistant hypertension (RHTN) patients ($n=40$) at the pre-intervention stage ($p<0.001$). The serum NO_2-, NOx and AOC levels of both controlled hypertension and healthy controls were comparable to those of the RHTN patients following treatment with propranolol ($n=18$). Considering all samples ($n=114$) we noted that there were significant weak and moderate positive correlations between NO_2^- levels with systolic blood pressure (SBP) and diastolic blood pressure (DBP) ($r=0.396$, $p<0.001$ and $r=0.292$, $p=0.004$) as well as total NOx levels with SBP and DBP ($r=0.636$ and $r=0.480$ respectively, $p<0.001$). Conversely, there was a significant negative correlation between AOC levels with SBP and DBP (r= -0.846 and r = -0.626 respectively, $p<0.001$).

Keywords Oxidative stress, Reactive nitrogen species, Anti-oxidant capacity, Resistant hypertension, Sri Lanka

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Introduction

Oxidative stress is defined as the imbalance between the rate and magnitude of oxidant formation (reactive oxygen species; ROS) and the rate of oxidant elimination. Oxidative stress is a significant contributor to hypertension [1], enhancing renal vascular tone and increasing vasoconstrictors due to increased superoxide levels [2]. This leads to impaired endothelium dependent vasodilatation [1, 3, 4] and disrupts the balance of endothelium-derived vasoactive factors [5, 6]. ROS stimulate intracellular Ca^{2+} concentration, leading to vascular dysfunction and remodelling [7].New evidence suggests a strong relationship between renal oxidative stress and hypertension development and maintenance [8]. Lower levels of superoxide dismutase(SOD) and glutathione peroxidase (GPX) in hypertensive patients make them more susceptible to damage [9–11].

The role of oxidative stress in the setting of RHTN has not been clearly defined. We previously reported trend to lower nitrate and nitrite levels and higher antioxidant capacity following treatment with propranolol compared to placebo in patients with resistant hypertension [12]. An extension of this data is provided with this study where we conduct an evaluation of oxidative stress using reactive nitrogen species and total antioxidant capacity in patients with resistant hypertension compared to those in age and gender matched healthy controls and controlled hypertension (HTN). The oxidative stress metrics in these experimental groups are also compared to those from the post propranolol arm in the APPROPRIATE trial [12].The study was conducted in compliance with the Declaration of Helsinki.

Methodology

This study was conducted as an extension to the APPROPRIATE trial which was a prospective, randomized, double-blind placebo controlled clinical trial to evaluate the safety and efficacy of propranolol in patients with resistant hypertension RHTN [12].

Thirty-eight consecutive patients with controlled HTN were recruited from medical clinics of the National Hospital of Sri Lanka. These patients were age and gender matched to existing recruits with RHTN enrolled in the APPROPRIATE trial (n=40) [12]. Age and gender matched 38 healthy volunteers, without hypertension [13] and other chronic illnesses were recruited. For comparative analysis we included samples obtained from the pre-intervention RHTN group (n=40) and the post-propranolol arm (n=18) of the APPROPRIATE trial [12].

For purpose of this study, hypertension was defined by office systolic blood pressure values 140 mmHg and/or diastolic blood pressure 90 mmHg [14] while resistant hypertension (RHTN) is blood pressure (BP) exceeding 140/90 mmHg in patients under the age of 60 or above 150/90 mmHg in patients above 60 years of age despite being treated with 3 anti-hypertensive of which one is a diuretic at maximum tolerable dose.

All recruited participants were administered a structured questionnaire to document socio demographic and clinical data (Supplementary file: S1 Table). Office blood pressure readings over the last 3 consecutive clinic visits were documented. Patients and healthy volunteers were invited for 30 minutes' clinic visits with an informed date to collect their blood samples.The recruitment period was1st May 2022- 28th June 2023.

Ten milliliters of blood was collected into a sterile plain tube and serum was separated by centrifugation at 900 *g* for 10 min, and clear sera were stored at −20 °C. Since serum nitrate is dependent on dietary factors, patients and healthy individuals were informed to have their last meal ~ 8 h prior to the time of blood collection to minimize the dietary effect.

Estimation of the serum levels of reactive nitrogen species and antioxidant capacity in patients with resistant hypertension and evaluation of its role in the pathogenesis

NO is highly unstable and it converts to nitrite and then nitrate rapidly [15]. Nitrite (NO_2^-) and total nitrite and nitrate levels (NOx) separately, are shown to be a surrogate marker to estimate serum NO levels [16–18].

Blood samples are first deproteinized to avoid the interference from blood proteins in the Griess assay [19]. $NO_2^-$is directly measured using Griess assay while NOx is measured following conversion of $NO_3^-$to NO_2^- using Vanadium chloride as described below [20].

Estimation of the nitric oxide activity by assessment of serum levels of reactive nitrogen species (nitrites and nitrates)

The collected blood samples were centrifuged, and the separated sera were kept in storage at −20° C. Zinc sulphate was added to the thawed serum samples to deproteinize during the analysis. Ten microliters of 1.5 g/mL zinc sulphate solution was added to 1mL of serum.

Mixture was thoroughly vortexed for 1 min and centrifuged at 10,000 g for 10 min at room temperature (RT, 25 °C). The supernatant was centrifuged again for 10 min, and the clear deproteinized serum from each sample (100 µL) was applied in duplicate to a 96-well Enzyme-linked immunosorbent assay (ELISA) plate, 100 µL of Vanadium (III) chloride (8 mg/mL) was added to each well (for reduction of nitrate to nitrite) followed by the addition of 100 µL of Griess reagent (equal mixture of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine hydrochloride in distilled water). The plates were incubated for 30 min at RT and the optical density measured at 540 nm using the ELISA reader (Bio-Tek Instruments INC, USA). Two -fold dilution series of

Sodium nitrite $NaNO_2$ (0.193–100 μM) was used to plot the standard curve.

Estimation of serum total antioxidant capacity with the ABTS decolourization method

Serum AOC levels were tested using 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) decolourization method, expressed as serum6-Hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC) [21]. Radical cations were generated by the oxidation of ABTS with potassium persulfate ($K_2S_2O_8$). The freshly prepared ABTS stock solution was diluted in 40- fold with 5 mM phosphate buffered saline (PBS) to prepare the ABTS working solution. Regent blank was prepared by mixing equal volumes of distilled water and $K_2S_2O_8$ (10 µL in each) were mixed with 800 µL of 5 mM PBS. Test serum from each sample was mixed with ABTS working solution in 1: 9 rations and kept exactly for 1 min to complete the scavenging process in dark. Samples were analyzed in duplicates and absorbance were measured at 734 nm against the reagent blank using spectrophotometer (UV Spectrophotometer, Shimadsu, Japan) [22, 23]. A two-fold dilution series of 6-Hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid (Trolox) (12.5–400 µM) was mixed at the same ration with ABTS working solution and the standard curve was plotted using absorbance values. TEAC was calculated using the Trolox standard curve.

Statistical analysis

Data was analyzed using the SPSS version 21 (SPSS Inc., Chicago, IL, USA).

Nitric oxide activity measures and AOC were compared between the defined groups using the *t* test and ANOVA for means corrected for multiple comparisons with the Bonferroni method.

Extreme outliers on NO_2^-, NOx and AOC, measures were identified using the 1.5 IQR rule and removed during the data analysis [24]. Shapiro Wilk test was conducted to test the normality. Pearson correlation was applied to test the correlation.

Results

The mean age of participants in RHTN group was 57±9.9 years and most (73%) were females. The mean age of controlled hypertensive subjects and healthy controls were 58±10.3 and 54±9.7 years respectively.

As expected, the mean systolic blood pressure (SBP) 158.9±10.9 mmHg and Diastolic blood pressure (DBP) 91.8±11.3 mmHg of the baseline RHTN group were significantly higher than those having controlled hypertension (124.9±11.1/ 77.7±8.6 mmHg) ($p<0.0001$) and the healthy controls (119.7±9.2 / 76.8±6.8 mmHg) ($p<0.0001$). The mean differences of office SBP and DBP between the groups were analysed at baseline to end point (Fig. 1).

Comparison of NO_2^- and NOx levels

Mean serum NO_2^- and NOx levels in patients with controlled hypertension and healthy controls were

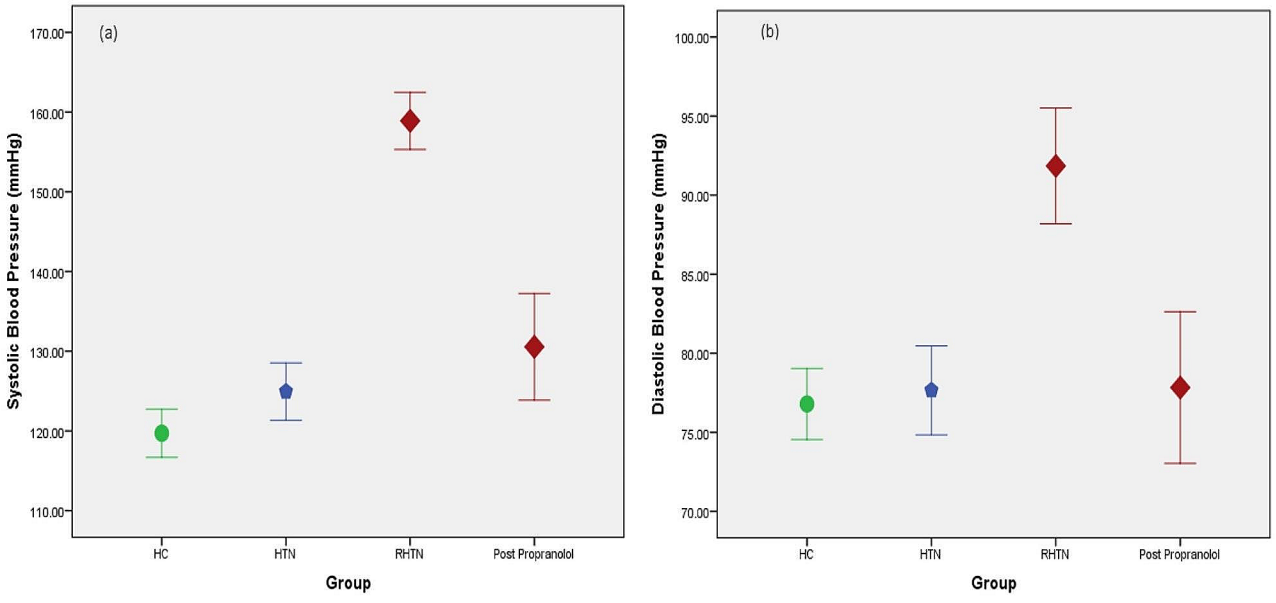

Fig. 1 Office blood pressure readings of study groups – Systolic blood pressure (**a**) and Diastolic blood pressure (**b**). Values represent mean ± SD; $n = 38$, 38, 40 and 18 for HC, HTN, RHTN and post-propranolol RHTN groups

significantly lower than the mean NO_2^- and NOx levels at the pre-intervention stage of the RHTN patients ($p<0.001$) (Supplementary file: S2 Table, Fig. 2.a and 2.b).

Comparison of AOC levels

The mean serum AOC in patients with controlled hypertension and healthy controls were significantly higher than the mean AOC at the pre-intervention stage of the RHTN patients ($p<0.001$) (Supplementary file: S2 Table, Fig. 2.c).

Comparison of NO_2^-, NO_X and AOC levels in post-propranolol RHTN group with those in the study groups

The analysis of NO_2^-, NOx and AOC levels in post - propranolol RHTN group ($n=18$) was statistically comparable to values obtained for normotensives and controlled hypertension groups, $p=1.000$ (Supplementary file: S2 Table, Fig. 2.a, 2.b and 2.c).

Correlation between blood pressure and oxidative stress parameters

We then examined correlation between measured blood pressure in our total samples ($n=114$) and parameters of oxidative stress. There was a significant correlation between total NOx levels with SBP and DBP ($r=0.636$ and $r=0.480$ respectively; $p<0.001$) (Fig. 3.a and 3.b) as well as between NO_2^- levels with SBP and DBP ($r=0.396$, $p<0.001$ and $r=0.292$, $p=0.004$) (Fig. 3.c and 3.d). Conversely, there was significant negative correlation between AOC levels with SBP and DBP (r= -0.846 and r= -0.626 respectively; $p<0.001$) (Fig. 3.e and 3.f).

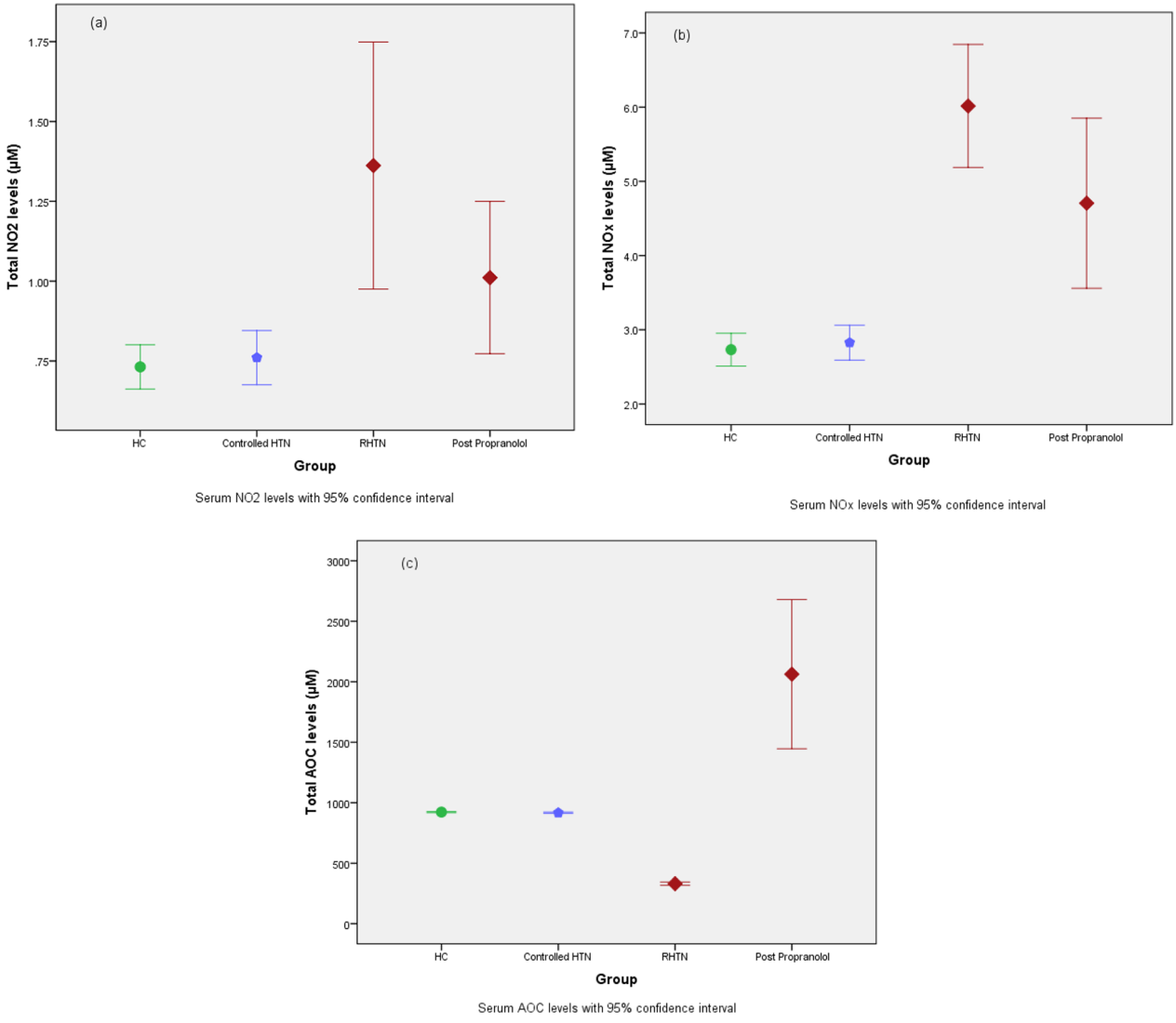

Fig. 2 Comparison of Oxidative Stress. The mean levels (± SD) of NO_2^-(**a**), NOx (**b**) and AOC (**c**) between the study groups

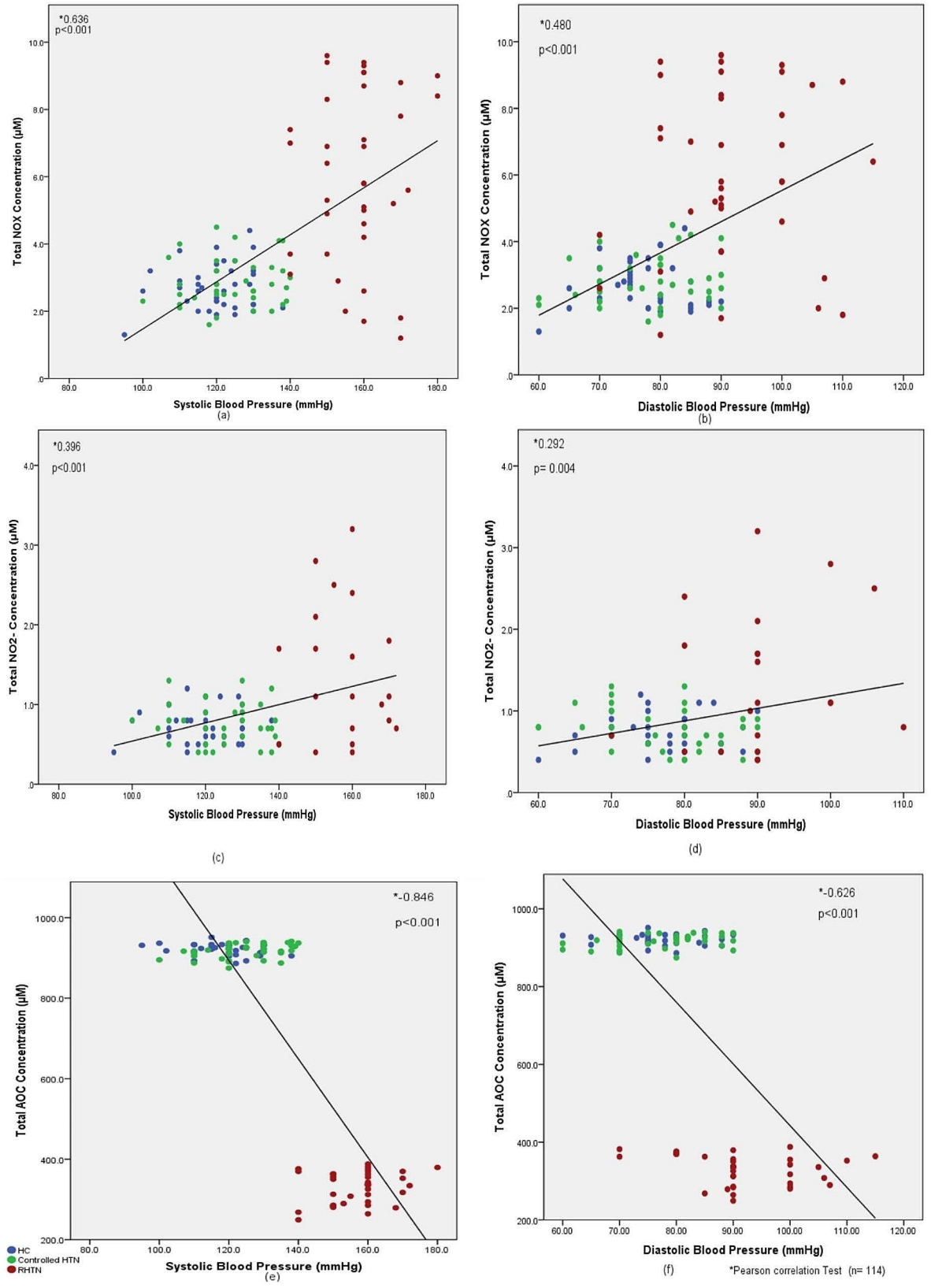

Fig. 3 Correlation between blood pressure and oxidative stress parameters, NOx, NO_2^- and AOC levels with systolic blood pressure respectively (**a**), (**c**) & (**e**) and with diastolic blood pressure (**b**), (**d**) & (**f**) respectively

Discussion

Our findings show significantly lower AOC and higher NO_2- and NOx in patients with resistant hypertension compared to healthy controls and those with controlled hypertension. Furthermore AOC, NO_2- and NOx reached comparable levels to healthy controls and those with controlled hypertension following treatment of resistant hypertensives with propranolol. We also noted significant positive correlations between NO_2-, NOx, SBP and DBP as well as significant negative correlations between AOC, SBP and DBP.

Our finding of lower antioxidant capacity in resistant hypertension, coupled with a negative correlation with systolic and diastolic blood pressure are consistent with prior observations in animal and human models of hypertension [25–28]. Important endogenous antioxidant systems include superoxide dismutase (SOD), glutathione peroxidise (GPX), catalase and thioredoxin [29, 30]. Superoxide dismutase is a major superoxide scavenger in humans and convert the superoxide anion to hydrogen peroxide which is a substrate for both catalase and glutathione peroxidate [31]. Lower levels of SOD and GPX cause to overproduction of superoxide anion and hydrogen peroxide, reduced nitric oxide synthesis and the impair the bioavailability of antioxidants [9, 32]. Zhou et al. showed that compared to normotensive controls, hypertensive patients had significantly reduced plasma SOD activity [33]. Plasma SOD activity was also shown to be reduced in elderly patients with essential hypertension [34]. Thus, increased oxidative stress or reduced the production of free radical scavengers lead to more susceptible to damage [9, 35]. Glutathione (GSH) has direct antioxidant activity by stabilizing free radicals and impaired activity has been previously shown in animal models of hypertension [36]. Reduced glutathione S-transferase mu type 1 expression was noted in stroke-prone spontaneously hypertensive rats when compared with the normotensive control rats [25]. In human studies, Rodrigo et al. report reduction of erythrocyte GSH/Glutathione disulphide (GSSG) ratio in hypertensives compared to normotensives [27].

We demonstrate [37] higher NOx and $NO_{2\_}$ levels in patients with resistant hypertension compared to controlled hypertension and healthy controls as well as a positive linear correlation with blood pressure values. NOx and NO_2- are surrogate measures of NO activity [16–18]. In contrast to our findings, NO has previously been shown to be reduced in hypertensive patients [37]. NO is produced by three major nitric oxide synthase isoforms of which endothelial NOS (eNOS) is mainly expressed in the cardiovascular system [38]. Endothelially derived NO is an important paracrine regulator of vascular tone. It also regulates vascular smooth muscle proliferation and migration and platelet and leucocyte aggregation to endothelial cells. iNOS is activated by inflammatory stimuli and in contrast NO derived from this isoform is involved in the rise in sympathetic tonus and ROS production [39].

We postulate that the observation of increase in NOx and NO_2- in RHTN compared to HC and controlled hypertension is a result of persistent eNOS activation, continually generating NO. However, the beneficial vasodilatory effects of this excess NO are potentially counteracted in the setting of enhanced oxidative stress (which we demonstrate by showing reduction of AOC) promoting formation of peroxynitrite which uncouples eNOS leading to further ROS formation and a vicious positive feedback loop [40, 41]. Peroxynitrite can also impair the activity of PGI2 synthase resulting in lower PGI2 [29] alleviating the effect of PGI2 on vascular dilation. It is also possible that the NO activity in RHT is mainly derived from the iNOS isoform which further perpetuates ROS production and sympathetic overactivity resulting in vasoconstriction.

The dysregulation of NOx with resultant increase in ROS generation, altered redox signalling and oxidative injury may play a significant role in pathophysiology of elevated blood pressure, primarily through effects on endothelial function, vascular tone, arterial remodelling and vascular inflammation [42].

Based on the preliminary findings of this study, we propose further mechanistic studies to finely dissect pathways involved in the reduction of AOC and increase in NO in patients with resistant hypertension.

Limitations

The key limitation of this work is the small sample size. However, despite this we believe that the preliminary observations detailed above warrant further evaluation. Due to resource limitations we could only assess three parameters of oxidative stress and anti – oxidant capacity. Further work will be undertaken in future studies to evaluate these mechanisms in finer detail. This will include measurement of ROS and DNA/RNA damage, lipid peroxidation, protein oxidation and nitration as well and parallel evaluation of glutathione and AOC for anti – oxidant potential. This analysis will also be extended to examine the unique effects of other antihypertensives on these mechanisms.

Abbreviations

NO	Nitric oxide
NOx	Total nitrites and nitrates
NO_2^-	Nitrite
ELISA	Enzyme-linked immunosorbent assay
ROS	Reactive oxygen species
iNOS	Inducible nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
SOD	Superoxide dismutase
GPX	Glutathione peroxidise
GSH	Glutathione

GSSG	GSH/Glutathione disulphide
AOC	Anti-oxidant capacity
RHTN	Resistant hypertension
HTN	Hypertension
HC	Healthy controls
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
$NaNO_2$	Sodium nitrite
TEAC	Trolox equivalent antioxidant capacity
$K_2S_2O_8$	Potassium persulfate
PBS	Phosphate buffered saline
ABTS	2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
IQR	Interquartile range
SPSS	Statistical Package for Social Sciences

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-024-06884-8.

Supplementary Material 1

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Author contributions

Study concept and design: GRC, PG, SMH, PK and SR. Data collection and carried out the research: HNR. Qualitative analysis and interpretation of data: PNW, HNR, NF and SMH. The first draft of the manuscript: PNW and HNR. Critical revision of the manuscript for important intellectual content and interpretation: GRC, SMH and PNW. Administrative, technical and material support: HNR. Review and approval of the manuscript: GRC, SMH, PG, PNW and NF. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declaration

Ethics approval and consent to participate

The study was approved by the Ethics Review Committee (ERC) of the Faculty of Medicine, University of Colombo (EC/15/152), (EC/21/002) and the Ethics Review Committee of National Hospital Sri Lanka. The trial was also registered at the Sri Lanka Clinical Trials Registry (SLCTR/2016/002). Date of Registration 27th Jan 2016, https://slctr.lk/trials/slctr-2016-002. Informed written consent was obtained from all subjects prior to recruitment to the study. Participants were recruited on voluntary basis and they could leave the study at any time they wished.

Competing interests

The authors declare no competing interests.

Consent for publication

Not applicable.

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