Endocrine Journal 2011, **58** (9), 789-799

ORIGINAL

Impaired endothelium mediated vascular reactivity in endogenous Cushing's syndrome

Dinu S Chandran[1)], Ashok Kumar Jaryal[1)], Viveka P Jyotsna[2)] and Kishore Kumar Deepak[1)]

[1)]*Department of Physiology, All India Institute of Medical Sciences, New Delhi, India*
[2)]*Department of Endocrinology & Metabolism, All India Institute of Medical Sciences, New Delhi, India*

Abstract. Endogenous Cushing's syndrome (CS) is associated with a high incidence of cardiovascular morbidity and mortality resulting from the co-existence of multiple cardiovascular risk factors which probably induce a state of endothelial dysfunction. Recently, studies conducted *in vitro* as well as in normal human subjects suggest a direct role of cortisol in the causation of vascular dysfunction in this disorder. We non-invasively assessed the vascular reactivity and its potential association with elevated cortisol levels in patients of CS. A single time point observational study was conducted in 18 patients of CS and 15 age and gender matched healthy subjects. Vascular reactivity was assessed non-invasively by measuring the peripheral pulse wave form changes during reactive hyperemia (RH) using digital Photoplethysmography (PPG). Parameters measured were pulse wave form amplitude (PWA), slope and pulse transit time (PTT). Maximal percentage changes in PWA during RH with reference to baseline were significantly lower in the patients as compared to controls [23.19% (13.19-53.54) *vs* 61.71% (38.21-95.36); p=0.0079]. Normalized PTT responses during the 1st minute of RH were blunted in the patients as compared to controls (1.036 ± 0.026 *vs* 1.056 ± 0.029; p=0.0425). Percentage changes in PTT during the 2nd minute of RH were negatively correlated to the morning cortisol levels in patients (r = -0.6328; p=0.0064). The present study showed that endothelium mediated vascular reactivity along with myogenic regulation of vascular tone is impaired in CS. Hypercortisolism possibly plays an important role in the causation of impaired vascular reactivity and endothelial dysfunction in CS.

Key words: Cushing's syndrome, Vascular reactivity, Photoplethysmography, Endothelial function

ENDOGENOUS Cushing's syndrome (CS) is a state of chronic endogenous hypercortisolism characterized by long term inappropriate exposure of tissues to excess circulating free cortisol [1, 2]. Abdominal obesity, systemic arterial hypertension, insulin resistance, dyslipidemia and thrombotic diathesis are common features of patients with CS which are well known cardiovascular risk factors [3, 4]. Patients with untreated or non remitted CS have been shown to have 3 to 4 times higher mortality due to cardiovascular complications than expected in the general population [5, 6]. Patients in the active phase of CS have been shown to have a higher intima media thickness and increased prevalence of atherosclerotic plaques in the carotid arteries along with biochemical and clinical features of metabolic syndrome which were found to persist even after successful cure of hypercortisolism [7, 8]. Based on all these evidence, it has been postulated that, the existence of metabolic syndrome in the form of abdominal obesity and decreased insulin sensitivity during the active phase of CS and its persistence even after therapeutic normalization of cortisol levels, probably induce a state of endothelial dysfunction leading to premature atherosclerosis and consequent morbidity and mortality in these patients [3, 7, 8].

High plasma cortisol levels have been linked to the presence and severity of coronary atherosclerosis in patients who presented with clinical and electrocardiographic evidence of coronary artery disease [9]. Recently, a direct causative role of cortisol in the induction of vascular endothelial dysfunction has been suggested by studies conducted in patients treated with exogenous glucocorticoids [10] and normal human subjects administered with short term oral hydrocortisone

Submitted May 19, 2011; Accepted Jul. 4, 2011 as EJ11-0030
Released online in J-STAGE as advance publication Jul. 21, 2011
Correspondence to: Ashok Kumar Jaryal, Additional professor, Department of Physiology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India.
E-mail: ashok.jaryal@gmail.com

acetate [11], based on forearm blood flow responses to reactive hyperemia and intra-arterially infused acetylcholine respectively. In both these studies patients and subjects were shown to be free of any of the clinical or biochemical features of glucocorticoid induced metabolic syndrome suggesting that excess glucocorticoid levels can per se affect vascular endothelial function. Pathological changes in vascular structure and function in patients with excess circulating glucocorticoid levels due to an endogenous or exogenous cause have also been linked to an increased oxidative stress in them [10, 12].

Even though literature review suggests a high likelihood of derangements in vascular reactivity and endothelial function in CS associated endogenous hypercortisolism, only very few studies have directly addressed this question by *in vivo* assessment of vascular functions [13-15]. We hypothesized that vascular reactivity and endothelial function would be deranged in CS and these pathophysiological changes would be related to the levels of hypercortisolism and/or oxidative stress in these patients.

Multiple studies in the past have shown monitoring of peripheral pulse wave form changes during reactive hyperemia as a well validated technique to assess vascular reactivity and endothelial function [16-18]. Photoplethysmography (PPG) is an optical measurement technique used to record digital volume pulse by measuring the pulsatile changes in blood volume in the finger microvasculature using light of infrared wavelength [19, 20, 21]. Photoplethysmographic measurement of peripheral pulse waveform changes is a highly objective and operator independent technique used to assess changes in both the calibre and tone of blood vessels during reactive hyperemia [17, 22]. In this study we assessed and compared the vascular reactivity and endothelial function in patients of CS with those of healthy human subjects by studying the peripheral pulse wave form and pulse transit time (PTT) responses during reactive hyperemia using R wave gated photoplethysmography.

Materials and Methods

Subjects

The study was conducted in 18 patients of endogenous hypercortisolism and 15 age and gender matched healthy controls. The study protocol was approved by Ethics committee for research on human subjects, All India Institute of Medical Sciences, New Delhi. A written informed consent was obtained from all the subjects before enrolment.

Consecutive cases of endogenous Cushing's syndrome admitted in the endocrine ward of All India Institute of Medical Sciences were included in the study. In patients with clinically apparent Cushing's syndrome, diagnosis of endogenous hypercortisolism was confirmed by low dose dexamethasone suppression test (LDDST). Patients were given 0.5mg of dexamethasone six hourly for 48 hrs starting at 8:00am on day 1 and plasma cortisol was measured at 8:00am after 48hrs. A plasma cortisol value of more than or equal to 2 μg/dL was considered confirmatory for endogenous hypercortisolism. Patients diagnosed with coexisting diabetes mellitus based on fasting plasma glucose levels ≥ 126 mg/dL as per American Diabetes Association criteria were excluded from the study to avoid the potential confounding effect of diabetes on vascular functions. Exclusion criteria also included other disease conditions which can independently affect vascular functions including coronary artery disease, congestive heart failure and chronic kidney disease based on clinical and biochemical criteria. Patients who were under antihypertensive medications (Calcium channel blockers, Beta blockers) were asked to continue the same during the course of assessment of vascular functions. Controls included age (± 5years) and gender matched non-overweight (BMI<25 as per WHO criteria), non-smoking healthy volunteers who were reported to be free from any acute or chronic illness and not under any kind of medications. Measurement of morning (8:30 am to 9:30 am) fasting serum cortisol levels were done in all recruited patients and controls on the day of vascular function assessment.

Assessment of vascular function

Vascular reactivity and endothelial function were assessed by measuring the pulse wave form and pulse transit time responses during reactive hyperemia using simultaneously acquired lead Ⅱ ECG and finger PPG signals. MP150 (BIOPAC Systems Inc., CA, USA), a computer based digital data acquisition system with Ethernet interfacing and software Acqknowledge® 3.8.2 was employed to record ECG and PPG signals at a sampling rate of 1kHz. PPG acquisition unit essentially comprised of an infrared (860±6 nm), reflection type photoelectric transducer (TSD200) and a biopotential amplifier (PPG100C) with a preset of gain and

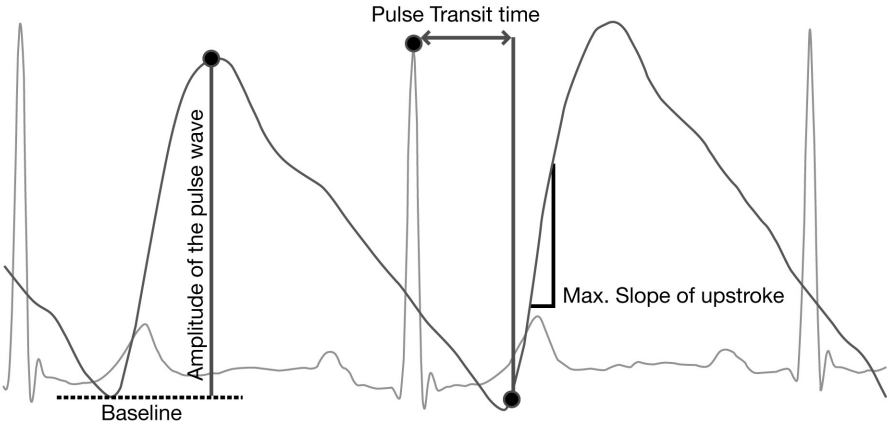

Fig. 1 Parameters used for assessing vascular function Figure shows the overlapped and magnified image of simultaneously acquired lead II ECG and PPG signals labelled with various vascular function parameters.

cut-off frequencies as 100 and 0.05-10 Hz respectively. Another differential, biopotential amplifier (ECG100C) with gain 1000 and cut-off frequencies 0.05-35 Hz was used to record ECG signal in the bipolar limb lead II mode with disposable Ag-AgCl electrodes. Offline analysis of both ECG and PPG signals for the extraction of pulse wave form parameters was done using the software LabChart Pro 7® (AD Instruments, Australia).

All recordings were done in controlled ambient temperature and luminance. Patients and control subjects were asked to report in the vascular function lab after overnight fasting at 8:30 am in the morning. Patients who were smokers were asked to abstain from smoking 24 hrs prior to the recording. Subjects were given 15 minutes of supine rest after which baseline blood pressure was taken. Sphygmomanometer cuff was kept fastened to the arm for subsequent use when arterial occlusion had to be produced. Disposable Ag-AgCl electrodes were placed for recording standard bipolar limb lead II ECG. Photoplethysmograph probe was fixed to the right middle finger with the help of an attached Velcro strap. The entire recording period comprised of 5 minutes of baseline, 5 minutes of arterial occlusion and 5 minutes post release of occlusion during the phase of reactive hyperemia. After 5 minutes of baseline recording of ECG and PPG signals, arterial occlusion was produced in the right arm by a supra-systolic cuff pressure 50 mm Hg above the subject's baseline systolic BP [17, 31] and signal acquisition was continued during the period of occlusion. Completeness of occlusion was ensured by constantly verifying the absence of pulse wave form signal from the computer display. Cuff pressure was released completely after 5 minutes of arterial occlusion and recording was continued during the phase of reactive hyperemia.

All signals recorded and saved in the Acqknowledge® format were analysed offline using LabChart Pro 7® software for the extraction and calculation of the following parameters (Fig. 1) with appropriate peak detection algorithms for ECG and PPG signals. 1) *Amplitude of the PPG pulse wave form*: Defined as the magnitude of the difference between maximal signal voltage and baseline signal voltage for each PPG pulse wave form. 2) *Maximum slope of the upstroke (here after mentioned as slope of the pulse waveform)*: Maximum positive value of the first derivative of PPG signal for each pulse wave form. 3) *Pulse transit time (PTT)*: Time interval between each R wave peak and the foot (defined as point at which signal voltage is 10% above the preceding baseline value) of the corresponding PPG pulse wave form. Beat to beat values of all the above mentioned parameters were extracted for the baseline and the RH data. Mean values of each parameter were computed for the entire baseline recording and for every one minute period of RH. Further analysis was done to study the time course of responses during RH with in each group in comparison to their respective baseline means. To compare the responses between groups, normalization with respect to respective baseline means was done for the RH data of each subject. To assess the peak response and its latency, maximum percentage change in each parameter and time to reach the peak were calculated and compared between groups.

Estimation of cortisol levels and oxidative stress markers

Blood samples were collected from all the subjects in the morning (8:30 am – 9:30 am) on the day of vascular function assessment after an overnight fast. For the diagnosis of endogenous hypercortisolism in patients, blood samples were collected at 8:00 am following LDDST and plasma cortisol was estimated by electrochemiluminescence immunoassay (ECLISA) with a measuring range of 0.018µg/dL to 63.4µg/dL (intra & inter assay coefficient of variance 6%) using Elecsys® autoanalyzer (Roche, USA). Morning fasting serum cortisol levels were estimated in all the subjects on the day of vascular function assessment by a direct immunoenzymatic colourimetric method (Diametra, Italy) with a measuring range of 1.0 µg/dL to 50.0 µg/dL (Intra-assay and inter-assay coefficient of variances of 7% and 9.32% respectively). Measurement of absorbencies were done using microplate reader Benchmark plus (BIORAD, USA).

To assess the oxidative stress, serum malondialdehyde (MDA) and vitamin C levels were estimated in the morning fasting serum samples of both the study groups. Serum MDA was estimated spectrophotometrically using modified Yagi's method [23, 24] which is reported to accurately estimate serum MDA levels within a range varying from 1nmol/mL to 100nmol/mL (Intra-assay and inter-assay coefficient of variances of 4% and 11% respectively). Serum Vitamin C levels were measured spectrophotometrically by ascorbate oxidase method [25] with a reported measuring range of 0.1mg/dL to 10mg/dL (Intra-assay and inter-assay coefficient of variances of 2.8% and 5.2% respectively). Chemicals required for both the estimations were purchased from 'Sigma' or 'Qualigens'. Absorbencies were measured at the required wavelengths using a double beam UV-VIS spectrophotometer ELICO SL-210 (ELICO, India).

Statistical analysis

Data are expressed as mean±SD or median with interquartile range (1^{st} quartile – 3^{rd} quartile) depending on the distribution of data. Each parameter was tested for normality in the distribution of data using standard normality tests (D' Agostino-Pearson omnibus normality test and Shapiro-Wilk test), based on which appropriate parametric or non-parametric tests were applied. To assess the significant trends in each parameter during RH, Repeated measures ANOVA or Friedman test with appropriate post hoc tests were applied within each group. For intergroup comparison of parameters unpaired t test or Mann Whitney tests were applied as appropriate. The relationship between two parameters was evaluated using Pearson's correlation coefficient or Spearman's rank correlation coefficient if they were appropriate. The level of statistical significance was set at $p<0.05$. All statistical analyses were done using GraphPad prism version 5.00 for Windows (GraphPad Software, Inc., USA).

Results

The objectives of the present study were to non-invasively assess the vascular reactivity and endothelial function in patients of endogenous hypercortisolism and study its relationship with serum cortisol levels and markers of oxidative stress. During the period from October 2008 to December 2010, 28 consecutive cases of CS admitted in the endocrine ward were recruited to the study. Out of these 10 had to be excluded (8 due to diabetes, 1 due to CKD with diabetes and 1 due to septicaemia). Eighteen patients of CS and fifteen age and gender matched healthy controls participated in the study. Of the 18 patients, 10 were hypertensives and 8 were normotensives. Thirteen of these patients (72%) had ACTH dependent Cushing's syndrome of which 12 had pituitaty lesion and 1 had an unknown source of ACTH. Patients with ACTH independent CS were 5 (28%), and all of them had adrenal cancer. Baseline characteristics of both the study groups are represented in Table 1. Patients had a significantly higher BMI, systolic and diastolic BP and morning fasting serum cortisol levels as compared to controls.

Photoplethysmographic pulse wave amplitude and slope responses during reactive hyperemia were studied to assess the vascular reactivity and endothelial function in both the groups. In the control group it was observed that, averaged pulse wave amplitudes during the 2^{nd} and 3^{rd} minute post release of occlusion [3.11 (2.28-4.11) volts and 2.45 (1.81-3.75) volts respectively] were significantly higher than the baseline values [2.22 (1.59-3.44) volts; $p<0.001$ and $p<0.05$ respectively]. In the patient group there was no significant change in the averaged pulse wave amplitude during any of the observed time periods following release of occlusion, in comparison to the baseline. On intergroup comparison after normalization (Fig. 2), it was observed that the rise in pulse wave amplitude was significantly lower in the patient group during the 2^{nd} and

Table 1 Baseline characteristics of patients and controls

Parameter	Patients (n=18)	Controls (n=15)	*p* value
Age (years)	21.22 ± 8.77	21.93 ± 4.69	N.S [a]
*Male : Female (n)	8 : 10	8 : 7	N.S [b]
Body mass index (Kg/m^2)	26.82 ± 04.76	23.42 ± 01.42	0.0122 [a]
Systolic blood pressure (mm Hg)	136.6 0± 21.86	123.60 ± 08.32	0.0383 [a]
Diastolic blood pressure (mm Hg)	88.56 ± 12.11	76.93 ± 04.89	0.0015 [a]
Morning fasting serum cortisol (µg/dL)	31.96 ± 14.53	13.51 ± 03.58	0.0001 [a]
Plasma cortisol post LDDST (µg/dL)	23.07 ± 15.34	–	–
Fasting plasma glucose (mg/dL)	86 ± 13.86	–	–
Postprandial plasma glucose (mg/dL)	123 ± 15.99	–	–
Total cholesterol (mg/dL)	235 ± 12	–	–
LDL cholesterol (mg/dL)	128 ± 10	–	–
HDL cholesterol (mg/dL)	55 ±7	–	–

Table shows the baseline characteristics of both the study groups in terms of clinical and biochemical parameters with their intergroup comparison. All values are expressed as Mean ± SD (*expressed as ratio of actual numbers). [a] indicates independent '*t*' test and [b] Fisher's exact test. Abbreviations: N.S-Not significant, LDDST – Low dose dexamethasone suppression test).

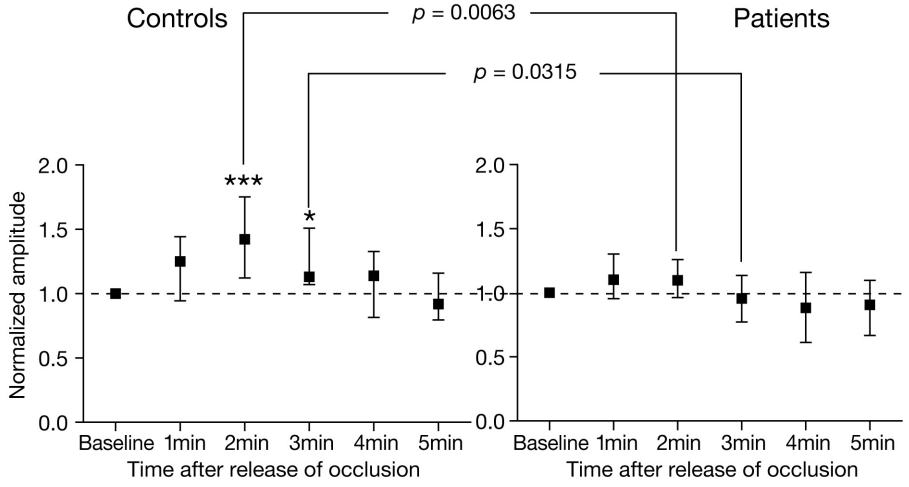

Fig. 2 Normalized pulse wave amplitude during RH in controls and patients Figure shows the graphical representation of normalized mean pulse wave amplitudes (y-axis) during baseline and different time periods during RH (x-axis) in controls (left) and patients (right). Values are plotted as median with inter-quartile range. Broken horizontal line represents the normalized baseline. *p* values shown represents those for intergroup comparison (Mann Whitney test). *** represents $p<0.001$ and * represents $p<0.05$ for intra-group comparison with respect to mean baseline values (Friedman test with Dunn's post hoc multiple comparison).

3rd minutes of RH when compared to controls [1.09 (0.96-1.26) *versus* 1.42 (1.12-1.75) for the 2nd minute and 0.96 (0.77-1.13) *versus* 1.13 (1.07-1.51) for the 3rd minute respectively; $p=0.0063$ and $p=0.0315$ respectively). Similar findings were also observed in the pulse wave slope responses during RH. Following the release of arterial occlusion in the control group, averaged pulse wave slopes during the 2nd and 3rd minutes [35.11 (28.30-54.58) volts/s and 33.31 (17.20-52.58) volts/s respectively] went significantly higher than the baseline values [26.64 (16.08-38.41) volts/s; $p<0.001$ and $p<0.01$ respectively]. In the patient group there was no significant change in the averaged pulse wave slope during any of the observed time periods following release of occlusion, in comparison to the baseline. On intergroup comparison after normalization (Fig. 3), it was observed that the rise in pulse wave slope was significantly lower in the patient group during the 2nd and 3rd minutes of RH when compared to controls [1.12 (0.93-1.22) *versus* 1.41 (1.12-1.73) for the 2nd minute and 0.96 (0.79-1.16) *versus* 1.17 (1.06-1.52) for the 3rd minute respectively; $p=0.0051$ and $p=0.0179$ respectively). To assess the magnitudes and latencies of peak responses in pulse wave amplitude and slope, maximal

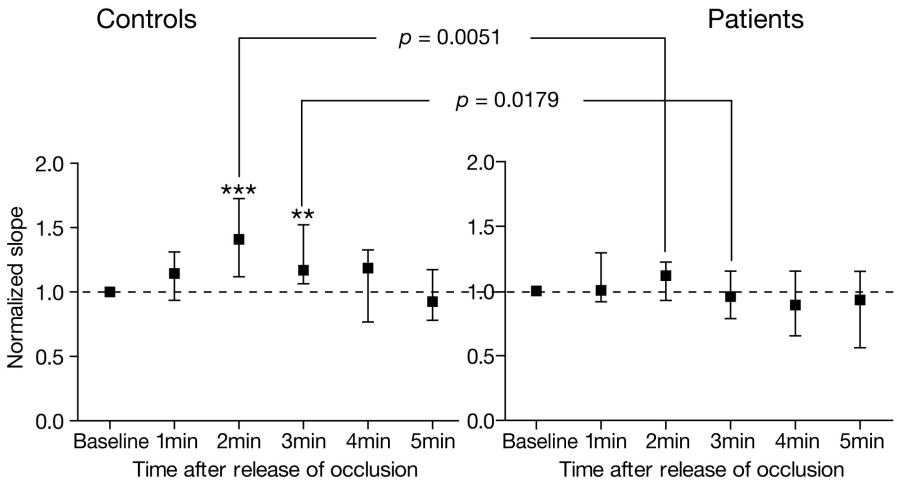

Fig. 3 Normalized pulse wave slope during RH in controls and patients Figure shows the graphical representation of normalized mean pulse wave slopes (y-axis) during baseline and different time periods during RH (x-axis) in controls (left) and patients (right). Values are plotted as median with inter-quartile range. Broken horizontal line represents the normalized baseline. *p* values shown represents those for intergroup comparison (Mann Whitney test). *** represents $p<0.001$ and ** represents $p<0.01$ for intra-group comparison with respect to mean baseline values (Friedman test with Dunn's post hoc multiple comparison).

Table 2 Maximum percentage change in pulse wave amplitude & time to reach the peak amplitude during reactive hyperemia

Parameter	Controls (n=15)	Patients (n=18)	*p* value
Maximum % change in amplitude	61.71 (38.21-95.36)	23.19 (13.19-53.54)	0.0079 [a]
Time to peak (sec)	100.50 (56.30-113.2)	63.28 (51.70-93.89)	N.S [a]

All values are expressed as median with inter-quartile range (1st quartile – 3rd quartile).
[a] Mann Whitney test. Abbreviations: N.S-Not significant.

Table 3 Maximum percentage change in pulse wave slope & time to reach the peak slope during reactive hyperemia

Parameter	Controls (n=15)	Patients (n=18)	*p* value
Maximum % change in slope#	69.73 (30.93-86.24)	24.06 (5.78-50.68)	0.0045[a]
Time to peak (sec)*	89.64 ± 25.96	69.66 ± 25.31	0.0330[b]

Values expressed as median with inter-quartile range. * Values expressed as mean±SD.
[a] Mann Whitney test and [b] independent '*t*' test.

percentage changes with reference to baseline means and time to reach the peak magnitude were computed for both these parameters (Tables 2 and 3). Maximal percentage changes in both pulse wave amplitude and slope were significantly lower in the patient group and time to reach the peak slope was significantly shorter in the patient group as compared to controls.

To assess the vascular tone responses during reactive hyperemia, beat to beat pulse transit times were obtained from simultaneously acquired ECG and PPG signals. Following the release of occlusion in both control and the patient group it was observed that the averaged pulse transit times were significantly higher than their respective baseline means. This initial rise was followed by a recovery to near baseline values by 4th minute post release of occlusion in the control group where as the values remained significantly higher than the baseline in the patient group for the entire recording period (Table 4 and Fig. 4). On intergroup comparison after normalization (Fig. 4), it was observed that the rise in normalized pulse transit time during the 1st minute post release of occlusion was significantly lower in the patient group as compared to controls (1.036 ± 0.026 *versus* 1.056 ± 0.029; $p = 0.0425$).

To study the state of oxidative stress in patients of CS *versus* normal healthy subjects we measured the serum levels of malondialdehyde and vitamin C in both study groups. Levels of both these oxidative stress markers

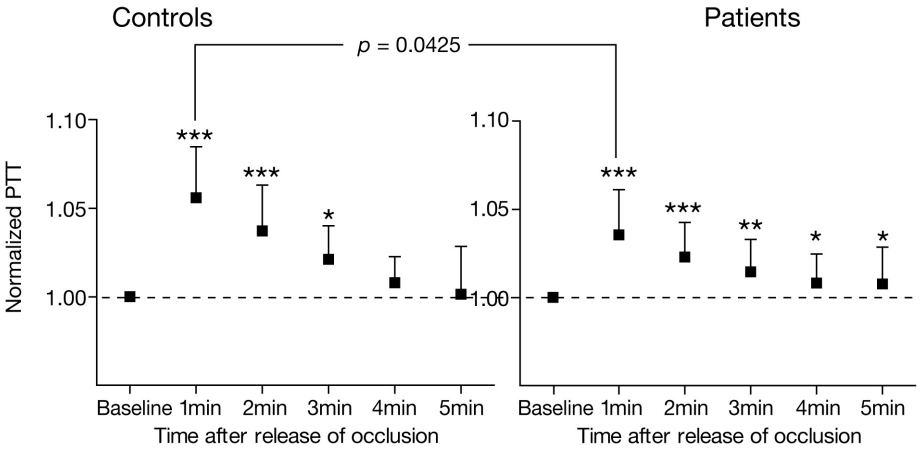

Fig. 4 Normalized pulse transit time during RH in controls and patients Figure shows the graphical representation of normalized mean pulse transit times (y-axis) during baseline and different time periods during RH (x-axis) in controls (left) and patients (right). Values are plotted as mean with SD. Broken horizontal line represents the normalized baseline. *p* values shown represents those for intergroup comparison (Independent '*t*' test). *** represents $p<0.001$, ** represents $p<0.01$ and * represents $p<0.05$ for intra-group comparison with respect to mean baseline values (Repeated measures ANOVA with Dunnett's post hoc multiple comparison test).

Table 4 Averaged pulse transit time (in milliseconds) during baseline and reactive hyperemia in controls and patients

Time period	Controls (n=15)	Patients (n=18)
Baseline	230.80 ± 23.22	220.50 ± 26.97
PRO-1st minute	243.60 ± 24.43 ($p<0.001$)	229.50 ± 27.53 ($p<0.001$)
PRO-2nd minute	239.30 ± 24.47 ($p<0.001$)	226.40 ± 26.58 ($p<0.001$)
PRO-3rd minute	235.60 ± 23.19 ($p<0.05$)	224.60 ± 27.17 ($p<0.01$)
PRO-4th minute	232.60 ± 23.24 (N.S)	223.50 ± 27.87 ($p<0.05$)
PRO-5th minute	230.80 ± 20.12 (N.S)	223.50 ± 28.92 ($p<0.05$)

All values are expressed as Mean±SD (units: milliseconds). *p* values indicated represent those for intra-group comparison versus respective baseline values (test applied: Repeated measures ANOVA with post hoc Dunnett's multiple comparison test). Abbreviations: PRO-post release of occlusion, N.S-not significant.

Table 5 Serum malondialdehyde and vitamin C levels in controls and patients

Parameter	Controls (n=15)	Patients (n=15)*	*p* value
Malondialdehyde (nmol/mL)	1.870 ± 0.208	1.633 ± 0.306	N.S [a]
Vitamin C (mg/dL)	0.286 ± 0.052	0.237 ± 0.024	N.S [a]

All values are expressed as Mean±SD. [a] Independent '*t*' test. * Serum samples from 3 patients were not used for estimation due to unacceptable hemolysis. Abbreviations: N.S-Not significant.

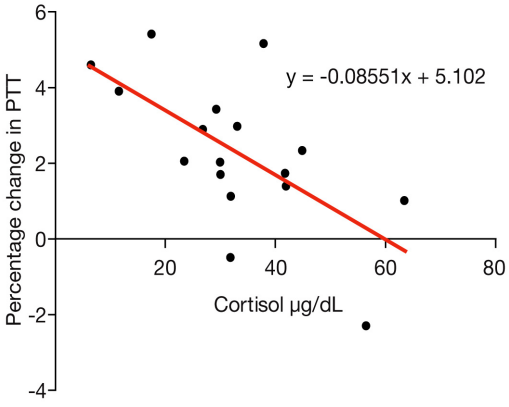

Fig. 5 Correlation between serum cortisol levels and percentage changes in PTT during the second minute post release of occlusion in patients Figure shows the scatter plot of percentage change in pulse transit time during the second minute post release of occlusion (y-axis) *versus* morning fasting serum cortisol levels (x-axis). Pearson r = -0.6328, $R^2 = 0.4004$ and $p = 0.0064$.

were neither significantly different between the study groups (Table 5) nor they were correlated to any of the vascular function parameters or serum cortisol levels in the patient group.

We also evaluated the relationship between various vascular function parameters and the levels of hypercortisolism in the patient group. On univariate correlation analysis (Fig. 5) it was observed that percentage changes in pulse transit time during the 2nd minute post release of occlusion had a significant negative correlation with morning fasting serum cortisol levels in the patient group (Pearson r = -0.6328, $R^2 = 0.4004$ and $p = 0.0064$). None of the other vascular function parameters were correlated to serum cortisol levels in the patient group. No significant correlation was found between any of the vascular function parameters with

systolic and diastolic blood pressures, BMI or duration of illness in the patient group.

Discussion

The present study was conducted in eighteen patients of CS and fifteen age and gender matched healthy subjects, to non-invasively assess the vascular reactivity and endothelial function. Peripheral pulse waveform changes during reactive hyperemia were studied using digital photoplethysmography in both the study groups for non invasive assessment of vascular functions.

The patient and control groups were matched for age and gender distribution of the subjects. Baseline systolic and diastolic blood pressures, body mass index (BMI) and fasting morning cortisol levels were significantly higher in the patients as compared to controls. These findings clearly portrayed the existence of typical clinical manifestations of endogenous hypercortisolism in our patient group which have already been reported in the literature [1-3].

We had excluded patients with coexisting diabetes mellitus as it has already been proven to directly affect the vascular endothelial function by previous studies [26, 27]. This confounding effect of diabetes mellitus has not been taken care of, while selecting the patient population in all of the previous studies which had assessed vascular function in endogenous hypercortisolism [13-15]. Approximately 50% of the patients who participated in these studies had coexisting diabetes mellitus. Absence of the diabetic state in our patient population makes this study a better model to investigate the role of hypercortisolism in the pathogenesis of vascular dysfunction in CS.

We have used reactive hyperemia as a physiological model to assess the reactivity of blood vessels to a temporary arrest of circulation. Transient arterial occlusion results in vasodilation of the resistance vessels and a decrease in the tone of both the resistance and conduit vessels due to metabolic and myogenic mechanisms [28-30]. Restoration of perfusion pressure at the release of arterial occlusion is followed by an increase in blood flow to the ischaemic tissues and a flow mediated vasodilatory response in the proximal conduit vessels [31]. These vascular responses are accompanied by simultaneous changes in peripheral pulse waveform characterised by a gradual increase in the pulse waveform amplitude and slope which tend to maximise around the time point of peak dilatation of proximal conduit arteries [16, 17, 22]. This rise in digital pulse waveform amplitude during reactive hyperemia has been shown to be dependent on endothelial nitric oxide synthesis [18]. Vascular tone changes in conduit and resistance vessels during reactive hyperemia are mainly due to myogenic vasorelaxation taking place during the period of arterial occlusion as well as due to the vasorelaxation induced by flow mediated release of NO during the phase of RH [22, 32, 33].

In the present study, on analysing the time course of pulse wave amplitude and slope responses in the healthy subjects, it was observed that both these parameters remained significantly higher than baseline during the second and third minutes of reactive hyperemia indicating the normal flow mediated vasodilatation of the proximal conduit vessels. These findings were in close agreement with the results obtained by Selvaraj *et al* in normal subjects [22].

On intergroup comparison after normalization, it was observed that CS patients had a significantly lower pulse wave amplitude and slope during the second and third minutes of reactive hyperemia as compared to controls. Similarly, the maximum percentage changes in pulse wave slope & amplitude during RH from the baseline means were significantly lower in the patient group in comparison to controls. Both these findings clearly indicated a significant impairment of FMD of conduit vessels in CS which is in accordance with the previous reports based on ultrasonographic method [13, 15]. The impaired FMD in CS could be either due to a decreased bioavailability of endothelial NO [15] or a diminished response of vascular smooth muscle to the available NO. The design of this study limits us from localizing the exact site of this impairment specifically to endothelium or vascular smooth muscle.

To the best of our knowledge, this is the first study to assess the vascular tone responses during reactive hyperemia in CS patients. On analysing the time course of changes in pulse transit time, it was observed that, in both the control and the patient groups there was a significant rise in the PTT values above the respective baseline values subsequent to the release of occlusion which was followed by a recovery towards the baseline values in the control group where as in the patient group, PTT values remained significantly higher than the baseline till the end of the recording period. On intergroup comparison it was observed that normalised PTT values for the first minute of reactive hyperemia was significantly lower in the patient group when compared to

controls. Both these observations when taken together would indicate an impaired myogenic regulation of vascular smooth muscle tone in CS leading to a diminished myogenic relaxation during the period of occlusion and a delay in the recovery of myogenic vasoconstrictor tone following the release of arterial occlusion and restoration of stretch on the vessel walls. This impairment in myogenic regulation of vascular tone could possibly be a pathophysiologic manifestation of the hypertrophic remodelling of blood vessels in patients with CS which has been shown by previous micromyographic studies [12]. The diminished vasorelaxation during the period of occlusion could also be due to endothelial dysfunction as it has been shown by *in vitro* studies that, deformation of endothelial cells resulting from the collapse of vessel intima during the period of arterial occlusion induces endothelial nitric oxide release and contributes to the maximal vasorelaxation response observed at the time of release of occlusion [30].

In the light of previous studies, we hypothesized and investigated the presence of oxidative stress as a putative cause of vascular dysfunction in patients of CS. The results of the present study showed that there was no significant difference in the levels of oxidative stress markers between the patient and control group. This was in contrast to the observation made by Prázny *et al*. [14] as they had showed a significantly higher oxidative stress in patients of CS in comparison to controls. A probable reason for this disparity could be the high prevalence of coexisting diabetes mellitus (52%) in their patient population (while we excluded it), as diabetes mellitus has already been proven to induce a state of oxidative stress by itself [34, 35].

To investigate the role of excess cortisol levels in the causation of vascular dysfunction in CS, we looked for correlation between pulse waveform parameters and morning cortisol levels in the patient group. On univariate correlation analysis it was observed that, percentage changes in PTT during the second minute of RH, had a strong and significant negative correlation with morning fasting cortisol levels in the patient group. PTT/pulse wave velocity changes during the second minute of reactive hyperemia would possibly represent the flow mediated, nitric oxide dependent vasorelaxation of the conduit vessels which has been reported as a marker of endothelial function [32, 33]. The results of our correlation analysis are in accordance with findings of Akaza *et al*. [15] who showed a significant negative correlation between percentage changes in brachial artery diameter during flow mediated dilatation and morning cortisol levels in patients of CS. Although morning serum cortisol level is not considered to be a disease marker in CS due to its inherent inter-individual variations independent of the disease status, our findings indicate the probable pathophysiologic role excess cortisol levels can play in the causation of vascular endothelial dysfunction as already been shown by previous studies [9-11].

The possible limitations of our study are the lack of insulin sensitivity data in the patient group and our control group not matched for BMI with respect to the patient group.

Multiple mechanisms have been proposed by which cortisol can affect the vascular endothelial function by reducing the bioavailability of endothelium derived NO using different in-vitro experimental models [36, 37]. Diminished bioavailability of nitric oxide resulting from all these mechanisms can very well explain the impaired vascular reactivity, accelerated rate of atherosclerosis and high incidence of vascular morbidity and mortality seen in CS.

Declaration of Interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References

1. Newell-Price J, Bertagna X, Grossman AB, Nieman LK (2006) Cushing's syndrome. *Lancet* 367: 1605-1617.
2. Boscaro M, Barzon L, Fallo F, Sonino N (2001) Cushing's syndrome. *Lancet* 357: 783-791.
3. Pivonello R, Faggiano A, Lombardi G, Colao A (2005) The metabolic syndrome and cardiovascular risk in Cushing's syndrome. *Endocrinol Metab Clin North Am* 34: 327-339.
4. Whitworth JA, Williamson PM, Mangos G, Kelly JJ (2005) Cardiovascular consequences of cortisol excess. *Vasc Health Risk Manag* 1: 291-299.
5. Etxabe J, Vazquez JA (1994) Morbidity and mortality in Cushing's disease: an epidemiological approach. *Clin Endocrinol* 40: 479-484.

6. Plotz CM, Knowlton AI, Ragan C (1952) The natural history of Cushing's syndrome. *Am J Med* 13: 597-614.
7. Colao A, Pivonello R, Spiezia S, Faggiano A, Ferone D, Filippella M, et al. (1999) Persistence of increased cardiovascular risk in patients with Cushing's disease after five years of successful cure. *J Clin Endocrinol Metab* 84: 2664-2672.
8. Faggiano A, Pivonello R, Spiezia S, De Martino MC, Filippella M, Di Somma C, et al. (2003) Cardiovascular risk factors and common carotid artery caliber and stiffness in patients with Cushing's disease during active disease and 1 year after disease remission. *J Clin Endocrinol Metab* 88: 2527-2533.
9. Troxler RG, Sprague EA, Albanese RA, Fuchs R, Thompson AJ (1977) The association of elevated plasma cortisol and early atherosclerosis as demonstrated by coronary angiography. *Atherosclerosis* 26: 151-162.
10. Iuchi T, Akaike M, Mitsui T, Ohshima Y, Shintani Y, Azuma H, et al. (2003) Glucocorticoid excess induces superoxide production in vascular endothelial cells and elicits vascular endothelial dysfunction. *Circ Res* 92: 81-87.
11. Mangos GJ, Walker BR, Kelly JJ, Lawson JA, Webb DJ, Whitworth JA (2000) Cortisol inhibits cholinergic vasodilation in the human forearm. *Am J Hypertens* 13: 1155-1160.
12. Rizzoni D, Porteri E, De Ciuceis C, Rodella LF, Paiardi S, Rizzardi N, et al. (2009) Hypertrophic remodeling of subcutaneous small resistance arteries in patients with Cushing's syndrome. *J Clin Endocrinol Metab* 94: 5010-5018.
13. Baykan M, Erem C, Gedikli O, Hacihasanoglu A, Erdogan T, Kocak M, et al. (2007) Impairment of flow-mediated vasodilatation of brachial artery in patients with Cushing's Syndrome. *Endocrine* 31: 300-304.
14. Prázný M, Jezková J, Horová E, Lazárová V, Hána V, Kvasnicka J, et al. (2008) Impaired microvascular reactivity and endothelial function in patients with Cushing's syndrome: influence of arterial hypertension. *Physiol Res* 57: 13-22.
15. Akaza I, Yoshimoto T, Tsuchiya K, Hirata Y (2010) Endothelial dysfunction aassociated with hypercortisolism is reversible in Cushing's syndrome. *Endocr J* 57: 245-252.
16. Kuvin JT, Patel AR, Sliney KA, Pandian NG, Sheffy J, Schnall RP, et al. (2003) Assessment of peripheral vascular endothelial function with finger arterial pulse wave amplitude. *Am Heart J* 146: 168-174.
17. Zahedi E, Jaafar R, Ali MAM, Mohamed AL, Maskon O (2008) Finger photoplethysmogram pulse amplitude changes induced by flow-mediated dilation. *Physiol Meas* 29: 625-637.
18. Nohria A, Gerhard-Herman M, Creager MA, Hurley S, Mitra D, Ganz P (2006) Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. *J Appl Physiol* 101: 545-548.
19. Allen J (2007) Photoplethysmography and its application in clinical physiological measurement. *Physiol Meas* 28: R1-39.
20. Shelley KH (2007) Photoplethysmography: beyond the calculation of arterial oxygen saturation and heart rate. *Anesth Analg* 105: S31-36.
21. Deanfield JE, Halcox JP, Rabelink TJ (2007) Endothelial function and dysfunction: testing and clinical relevance. *Circulation* 115: 1285-1295.
22. Selvaraj N, Jaryal AK, Santhosh J, Anand S, Deepak KK (2009) Monitoring of reactive hyperemia using photoplethysmographic pulse amplitude and transit time. *J Clin Monit Comput* 23: 315-322.
23. Conti M, Morand PC, Levillain P, Lemonnier A (1991) Improved fluorometric determination of malonaldehyde. *Clin Chem* 37: 1273-1275.
24. Knight JA, Pieper RK, McClellan L (1988) Specificity of the thiobarbituric acid reaction: its use in studies of lipid peroxidation. *Clin Chem* 34: 2433-2438.
25. Liu TZ, Chin N, Kiser MD, Bigler WN (1982) Specific spectrophotometry of ascorbic acid in serum or plasma by use of ascorbate oxidase. *Clin Chem* 28: 2225-2228.
26. De Vriese AS, Verbeuren TJ, Van de Voorde J, Lameire NH, Vanhoutte PM (2000) Endothelial dysfunction in diabetes. *Br J Pharmacol* 130: 963-974.
27. Henry RMA, Ferreira I, Kostense PJ, Dekker JM, Nijpels G, Heine RJ, et al. (2004) Type 2 diabetes is associated with impaired endothelium-dependent, flow-mediated dilation, but impaired glucose metabolism is not; The Hoorn Study. *Atherosclerosis* 174: 49-56.
28. Carlsson I, Sollevi A, Wennmalm A (1987) The role of myogenic relaxation, adenosine and prostaglandins in human forearm reactive hyperaemia. *J Physiol* 389: 147-161.
29. Imms FJ, Lee WS, Ludlow PG (1988) Reactive hyperaemia in the human forearm. *Q J Exp Physiol* 73: 203-215.
30. Koller A, Bagi Z (2002) On the role of mechanosensitive mechanisms eliciting reactive hyperemia. *Am J Physiol Heart Circ Physiol* 283: H2250-2259.
31. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, et al. (2002) Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 39: 257-265.
32. Kamran H, Salciccioli L, Ko EH, Qureshi G, Kazmi H, Kassotis J, et al. (2010) Effect of reactive hyperemia on carotid-radial pulse wave velocity in hypertensive participants and direct comparison with flow-mediated dilation: a pilot study. *Angiology* 61: 100-106.
33. Naka KK, Tweddel AC, Doshi SN, Goodfellow J, Henderson AH (2006) Flow-mediated changes in pulse

wave velocity: a new clinical measure of endothelial function. *Eur Heart J* 27: 302-309.
34. Giugliano D, Ceriello A, Paolisso G (1996) Oxidative stress and diabetic vascular complications. *Diabetes Care* 19: 257-267.
35. Maritim AC, Sanders RA, Watkins JB 3rd (2003) Diabetes, oxidative stress, and antioxidants: a review. *J Biochem Mol Toxicol* 17: 24-38.
36. Wallerath T, Witte K, Schäfer SC, Schwarz PM, Prellwitz W, Wohlfart P, et al. (1999) Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension. *Proc Natl Acad Sci U S A* 96: 13357-13362.
37. Johns DG, Dorrance AM, Tramontini NL, Webb RC (2001) Glucocorticoids inhibit tetrahydrobiopterin-dependent endothelial function. *Exp Biol Med (Maywood)* 226: 27-31.