

## Non-invasive evaluation of endothelial function in healthy Indian participants using tonometry

Chinthaparthi Prabhakar Reddy\*, MUR Naidu

Department of Clinical Pharmacology and Therapeutics, Nizam's Institute of Medical Sciences, Punjagutta, Hyderabad, Telangana, India

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**\*Correspondence to:**

Dr. Prabhakar Reddy  
Chinthaparthi,  
Email: [drreddycp@hotmail.com](mailto:drreddycp@hotmail.com)

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### ABSTRACT

**Background:** Vascular endothelium releases number of biological active mediators, including nitric oxide (NO) that regulates vessel tone. Endothelial dysfunction is independent risk factor for cardiovascular disease. The study was done to assess endothelial function by augmentation index (AIx) calculated from derived aortic wave forms using radial pulse wave analysis (PWA) with provocative pharmacological testing, measured in response to endothelium independent, direct stimulus with nitrovasodilator nitroglycerine (NTG) and endothelium dependent activation of L-arginine-NO-pathway by salbutamol.

**Methods:** Eighteen healthy subjects participated in the study. Radial artery wave forms were recorded with a high-fidelity tonometer (Sphygmocor, AtCor medicals, Australia). Recordings were taken at baseline and after 3, 5, 10, 15 and 20 minutes of NTG (0.5 mg) sublingual administration, and at baseline and after 5, 10, 15 and 20 minutes of salbutamol (400 µg) inhalation.

**Results:** Mean age was 25±2.4 years and body mass index 24±2.5 kg/m<sup>2</sup> of study subjects. Salbutamol and NTG significantly reduced AIx at all-time points (salbutamol: baseline 7.6%, 5 minutes -3.6%, 10 minutes -5.8%, 15 minutes -7.8% and 20 minutes -3.6%, p ≤0.05 and NTG baseline 8.4%, 3 minutes -3.2%, 5 minutes -7.7%, 10 minutes -9.7%, 15 minutes -11.2% and 20 minutes -8.6%, p ≤0.001). The effect of provocative pharmacological testing on heart rate, aortic and radial pressures, mean arterial pressure and pulse pressure was not altered (p ≥0.05).

**Conclusions:** This method of assessing endothelial function, which appears before onset of clinical signs and symptoms and is a simple, convenient, quick method.

**Keywords:** Endothelial function, Nitric oxide, Pulse wave analysis, Augmentation index, Salbutamol, Nitroglycerine

### INTRODUCTION

Endothelium is a dynamic, multifaceted organ with autocrine, paracrine and endocrine functions and is composed of a single layer of mitotically quiescent and metabolically active cells. Vascular endothelium lines entire cardiovascular system forming a biological interface between the flowing blood and other tissues and is strategically located and responds to locally generated mediators to alter its structure, and functions appropriately.

Endothelial dysfunction is a systemic vascular process that not only mediates the development of the

atherosclerotic plaque but may also modulate its clinical course and serves as a marker of the inherent atherosclerotic risk in an individual.<sup>1</sup> Dysfunction of either the coronary or peripheral vascular endothelium is shown to constitute an independent predictor of cardiovascular events, providing valuable prognostic information additional to that derived from conventional risk factor assessment and is regarded as an integrated index of both the overall cardiovascular risk factor burden and the vasculoprotective factors in any given individual.<sup>1</sup> Endothelial dysfunction is characterized by an impaired response to endothelial vasodilator agents, such as acetylcholine and bradykinin and is a common mechanistic link between risk factors and the

development of atherosclerosis. This process goes undetected. Detection of this process prior to any overt disease is important as this can be modified so as to reduce cardiovascular complications.

In recent years, several novel techniques have been devised to explore different facets of endothelial function *in vivo*, including endothelial expression of inflammatory markers, adhesive properties of the endothelium with respect to the interactions with leukocytes and platelets, and factors involved in regulation of thrombosis and fibrinolysis, endothelial progenitor cells and their possible role in endothelial repair and maintenance of vascular homeostasis.<sup>2</sup> During the last 2 decades the standard test of endothelial function are those that evaluate the vascular responsiveness to these endothelium-dependent stimuli. Both invasive and non-invasive methods can be used to assess endothelial dysfunction.

The invasive techniques include angiographic measurements of coronary vessels diameter and examination of microcirculation cannulating with a Doppler wire to measure blood flow velocity after infusion of different concentrations of acetylcholine and the endothelium-independent vasodilator nitroglycerin into the coronary arteries and measurement of flow-mediated dilation (FMD) by infusing either adenosine or papaverine into the mid-portion of the coronary artery.<sup>3-6</sup> And to further assess endothelial function of coronary microvasculature and macro-vasculature, investigators have used more physiological stimuli, such as the cold pressor test and dynamic exercise which were reported to induce epicardial vasodilation and an increase in coronary blood flow in angiographically normal coronary arteries. This method is considered a standard for measuring endothelial function but several limitations restrict its widespread use. Most importantly, the invasive nature of these studies not only confines their use to the patients undergoing coronary angiography for clinical reasons (introducing a selection bias) but also limits the possibility of repeated evaluations and measurements of coronary diameter and is also limited by the accuracy of coronary angiography and may pose technical difficulties in patients with atherosclerosis and potentially eccentric plaques.

Therefore, non-invasive techniques were developed to allow more widespread use of endothelial function testing. Importantly, impaired endothelial responses, characteristically found in coronary arteries of patients with cardiovascular risk factors, have also been confirmed in different peripheral circulatory territories of these patients. This has led to the concept of a generalized nature of endothelial dysfunction and has facilitated endothelial function testing in more accessible vascular beds.

Endothelial function can be assessed in peripheral vasculature as it responds dynamically to stimulus (stress) by releasing NO, causing flow-mediated vasodilation and assessment of FMD was developed using ultrasound imaging which involves measurement of change in the diameter of a conduit artery (typically brachial, although radial and femoral arteries are used) in response to the increased blood flow induced by a period of ischemia applied to distal part of the limb.<sup>7,8</sup>

Another method where endothelial function is assessed in peripheral vasculature is forearm perfusion technique; this has been a well-established tool for the study of the human microcirculation for decades, because it permits the investigation of the peripheral vascular bed without the confounding effects resulting from the activation of systemic counterregulatory mechanisms.<sup>9,10</sup> Laser doppler flowmetry has been implemented to evaluate endothelial function of the skin microvasculature using post occlusive hyperemia, local thermal hyperemia, and acetylcholine iontophoresis.<sup>11</sup>

Both the biomarkers of thrombosis and inflammation can be considered as indirect markers for endothelial function.<sup>12</sup> And recently endothelial progenitor cells and genetic markers are included to also assess endothelial function.<sup>13-15</sup> The markers of thrombosis (von Willebrand factor, coagulation markers (tissue plasminogen activator (t-PA) and plasminogen activator inhibitor PAI-1) are considered as indirect markers of endothelial function, however, prospective epidemiological studies aiming to evaluate the association between plasma levels of different hemostatic markers and the risk for cardiovascular disease are relatively sparse and often with inconclusive results.<sup>16-19</sup> Markers of inflammation (adhesion molecule-1, E-selectin, high-sensitivity C-reactive protein, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, cytokines (interleukin-8 and monocyte chemoattractant protein-1), and down regulating NO and PGI<sub>2</sub> release) can also be considered as indirect markers of endothelial function but these may reflect a generalized inflammatory state and further investigations.<sup>20,21</sup>

Recently, alternative non-invasive techniques, based on the analysis of the arterial pulse waveform, have been implemented in the assessment of endothelial function.<sup>22,23</sup> Applanation tonometry is a method that involves positioning the tonometer over the maximal arterial pulsation of the artery under study (radial, brachial, and femoral) to minimally flatten or appanate the arterial wall. The obtained pulse-waveform shape provides information about arterial compliance and serves as the basis for the calculation of the augmentation index (AIx a ratio between the pulse pressure at the second systolic peak and the pulse pressure at the first systolic peak), which is commonly used as a measure of arterial stiffness.

In these studies, systemically given  $\beta$ -2 receptor agonists (inhaled albuterol and salbutamol or subcutaneously injected terbutaline) diminished the reflected wave (or second systolic peak) of the pulse-wave contour and reduced the augmentation index in a similar fashion to sublingual nitroglycerin.<sup>24</sup> Importantly,  $\beta$ -2 agonist induced, but not nitroglycerin-induced, changes in the arterial pressure waveform could be blunted by the NG-monomethyl-L-arginine infusion, suggesting that  $\beta$ -2 agonists, in part, mediate their effects on wave reflection through the endothelial NO release and that pulse wave methodology might be applied to the assessment of endothelial function.<sup>24</sup>

Among various methods for assessing endothelial function and by considering their limitations it can be considered that evaluation of endothelial function using pulse wave analysis coupled with the administration of the endothelium-dependent  $\beta$ -2 adrenoceptor agonist salbutamol and the endothelium-independent vasodilator nitroglycerin (NTG) can be an easy, simple, quick and convenient method performed with precision. So this study was performed to test the hypothesis in a group of healthy individuals in Indian population.

## METHODS

The study was conducted at the department of clinical pharmacology and therapeutics, Nizam's Institute of Medical Sciences, Hyderabad, India after the approval of institutional ethics committee, following the good clinical guidelines established by declaration of Helsinki. Healthy participants were enrolled from community. Each subject gave written informed consent for participating in the study.

Participants were studied in the morning after an overnight fast. Healthy participants aged between 24-36 years were defined on the basis of normal laboratory investigation values, physical and clinical examinations done three days before the day of the study. Participants with history of cardiovascular, renal, hepatic dysfunction and use of any drugs 30 days prior to the day of procedure were excluded.

Tests of endothelial vasomotor function Studies was conducted in a quiet, temperature-controlled room ( $22\pm 2^\circ\text{C}$ ). All hemodynamic recordings were made in triplicate. Radial artery wave forms were recorded with a high-fidelity tonometer (Sphygmocor, AtCor medicals, Australia). On the day of study after subject has acclimatized for a period of 30 minutes, after three sets of recordings were made during a 15 minutes period of supine rest, and the last was taken as a baseline.

Salbutamol (Ventrolin inhaler, GlaxoSmithkline Pharmaceuticals Ltd) was given by inhalation with a spacer device (2 X 200 $\mu\text{g}$ ). A 500  $\mu\text{g}$  tablet of NTG was placed under the tongue for 3 minutes and then removed.

Endothelial dependent (ED) and endothelial independent (EID) vascular reactivity were assessed by the maximum changes in AIX following challenges with inhaled salbutamol (SAL) and sublingual nitroglycerin (GTN), respectively, as described by Wilkinson et al.<sup>25</sup> In these previous studies, the  $\beta$ 2-agonist and GTN both significantly and consistently reduced AIX. Inhalation of a  $\beta$ 2-agonist was consistent with an ED activation of the L-arginine NO pathway, since it was substantially inhibited by L-NMMA and correlated to that of intra-arterial acetylcholine.

AIX calculation radial arterial waveforms (20 cardiac cycles) were recorded by applanation tonometry (SphygmoCor, AtCor Medicals, Australia). BP was measured in the non-dominant hand by sphygmomanometry (mean of three readings taken after 10 minutes semirecumbency).

An averaged composite radial waveform was calculated from which specially designed software (SphygmoCorTM; PWV Inc., Australia) derived an aortic BP waveform, in real time, using a validated transfer function algorithm. A second series of measurements was obtained following pharmacological stimuli (similar to Wilkinson et al).<sup>25</sup> Recordings were made 3, 5, 10, 15 and 20 minutes following 500  $\mu\text{g}$  of sublingual GTN administration and the lowest value was considered as post-GTN AIX. As demonstrated also by Wilkinson et al.<sup>32</sup> 30 minutes were sufficient for the hemodynamic changes after GTN administration to completely return to baseline. Therefore, NTG was always administered first, followed by Salbutamol on the next day.

Salbutamol (SAL) was given by inhalation with a spacer-inhaler device (after careful patient instruction). Recordings were obtained starting 5, 10, 15, 20 and 25 minutes following administration, and the lowest obtained value was considered as post-SAL AIX.

## Data analysis

The response to Salbutamol, and NTG was defined as the maximum change in each parameter after drug administration and the time taken to achieve this change. Data were analysed using paired Student's t tests with GraphPad prism software version 4 and. All changes represent absolute differences, rather than percentage. All values represent mean $\pm$ SD and a p value  $<0.05$  was considered significant.

## RESULTS

Baseline clinical characteristics A total of 18 healthy participants participated in this study conducted at department of clinical pharmacology and therapeutics, Nizam's Institute of Medical Sciences, Hyderabad, India. The baseline clinical characteristics of our participants are summarized in Table 1.

**Table 1: Baseline characteristics of study participants.**

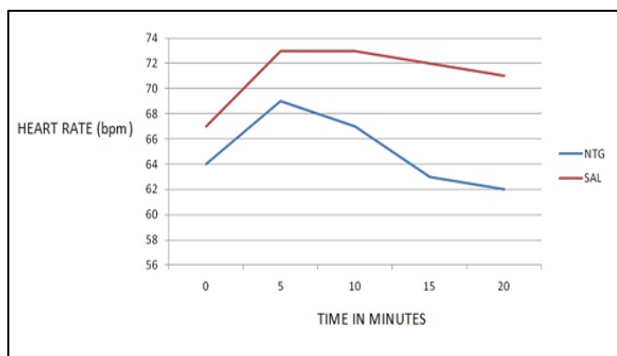
Age, years	24±2.40
Height cms	167±8.08
Weight kgs	67±11.05
BMI kg/cm <sup>2</sup>	24.5±2.49
Total cholesterol, mg/dL	176.1±56.83
LDL cholesterol, mg/dL	108.8±27.82
HDL cholesterol, mg/dL	42.0±19.00
Triglycerides, mg/dL	139.7±54.50

(Values are mean ± STDEV).

**Table 2: Hemodynamic changes in study.**

	NTG	Salbutamol
Augmentation index %*	-9.7±3.99	-7.8±3.61
Heart rate bpm	62±8.10	73±3.90
<b>Radial pressure</b>		
Mean pressure mm of Hg*	66±6.40	73±6.77
Pulse pressure mm of Hg*	48±5.46	54±3.24
<b>Aortic pressure</b>		
Mean pressure mm of Hg*	64±7.18	72±8.32
Pulse pressure mm of Hg*	30±2.54	30±1.82

(Values are mean ±STDEV). \*the maximal observed change with drug.

**Figure 1: Effect of salbutamol and NTG administration on heart rate (bpm).**

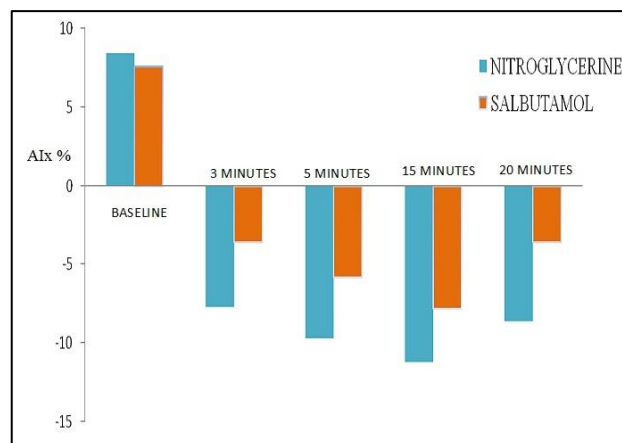
### Hemodynamic changes in study

The hemodynamic changes due to administration of Salbutamol and NTG are summarized in Table 2. The maximal reduction of augmentation index AIx (%) from a baseline value of 8.4±3.50 to -11.2±4.95 occurred at 15 minutes after administration of NTG. (133% reduction from baseline). The reduction of AIx with Salbutamol inhalation is from a baseline value of 7.6±0.87v to -7.8±3.61 at 15 minutes, a reduction of about 103% from baseline. The reduction seen with both NTG and Salbutamol is significant ( $p < 0.05$ ) from baseline to all time points. The effects of both SAL and NTG on heart rate, systolic blood pressure, diastolic blood pressure, mean arterial pressure and pulse pressure (both radial and aortic) were not significant ( $p > 0.05$ ).

**Table 3: Hemodynamic changes in study.**

Time (minutes)	NTG		Salbutamol	
	AIx (%)	HR (bpm)	AIx (%)	HR (bpm)
0	8.4±3.50	64±8.83	7.6±0.87	67±6.44
3	-3.2±2.46	71±8.60		
5	-7.7±4.21	69±8.58	-3.6±2.18	73±6.50
10	-9.7±3.99	67±9.96	-5.8±2.58	73±3.90
15	-11.2±4.95	63±8.24	-7.8±3.61	72±4.85
20	-8.6±4.29	62±8.10	-3.6±4.48	71±7.16

(Values are mean ± STDEV).

**Figure 2: Hemodynamic changes in study.**

### DISCUSSION

Since the first description of endothelial dysfunction in atherosclerotic epicardial coronary arteries in 1986 by Ludmer and colleagues, invasive assessment of coronary endothelial function by quantitative coronary angiography and coronary doppler flow measurements, along with graded intracoronary infusions of endothelium-dependent vasodilators such as acetylcholine, may be considered the “gold standard” for endothelial function testing.

Since the actions of the endothelial cell (EC) are multiple and involve several systems, alterations in EC function may affect one or more of these systems, either simultaneously or at distinct time periods. Thus, no single definition of EC dysfunction covers the whole array of possible disruption in normal function. In consequence, endothelial dysfunction has been defined pragmatically. It basically involves either an increase (or a decrease) in any of the EC-related chemical messenger and/or by alteration in any of the functional changes.

However, during the last decade, other less-invasive or non-invasive techniques for the assessment of endothelial function, including strain gauge forearm plethysmography in conjunction with intra-arterial infusion of endothelium-dependent vasodilators, such as methacholine or acetylcholine, and high-resolution external vascular ultrasound to measure flow-mediated endothelium-

dependent dilation (FMD) of the brachial artery during reactive hyperemia, have been developed. These techniques are based on the fact that endothelial dysfunction is not confined to the coronary arteries but rather represents a systemic disorder that also affects peripheral vascular beds, including both conduit arteries and small resistance vessels in the extremities.

Our study examined in detail endothelial vasomotor dysfunction in healthy Indian population as there are no studies done until now, using the simple, reproducible and non-invasive (3, 5, 7, 11) pulse-wave analysis methodology, combined with provocative pharmacological testing, recently validated both in normal participants and in participants with endothelial dysfunction.<sup>5</sup> Here we were able to define the changes of endothelial function that is seen in healthy individuals both by EID and ED vasomotor function.

Our main novel findings are that salbutamol and NTG produce qualitatively and quantitatively similar effects on AIx. Effect of salbutamol is, in part, NO and endothelium dependent and are consistent with the presence of endothelial dysfunction. Moreover, they suggest that PWA and administration of Salbutamol and NTG provide a simple, reliable, non-invasive method for assessing endothelial function, as we and others have previously hypothesized. As expected, inhalation of Salbutamol at the dose used reduced AIx without any accompanying alteration in heart rate or MAP, which is important because AIx is influenced by both. The magnitude of the response to both drugs was comparable. The test has shown values that are similar to what was previously reported for PWA and to values quoted for other techniques of assessing endothelial function, such as intra-arterial infusion of ACh and flow-mediated dilatation. Both drugs had similar effects on AIx, heart rate, MAP and pulse pressure. Previous studies have shown similar changes in the arterial pressure waveform after NTG, but the effect of endothelium-dependent NO dilators have not been reported.

## CONCLUSION

The results from this study indicate that PWA coupled with administration of SAL and NTG provides a simple, non-invasive method for assessing endothelial function. We believe that this technique provides a suitable means for assessing endothelial function in large numbers of patients and thus, answers the important question of the predictive value of endothelial function.

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