Non-invasive measurement of human endothelium dependent arterial responses: accuracy and reproducibility

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Abstract

Objective—To assess a non-invasive test for endothelial dysfunction, an important early event in the atherogenic process.

Methods—Using high resolution ultrasound, the accuracy of detecting small changes in vessel diameter was assessed using phantom "arteries", and the same equipment was then used to measure flow mediated dilatation in the brachial artery of 40 healthy adults aged 22–51 years, studied on four occasions; intervals between scans were 1–2 days, 1–2 weeks, and 2–4 months.

Results—Differences between pairs of phantom "arteries" with diameters 0.1–0.2 mm apart were correctly estimated in 162 of 264 cases (61%); no measurement by any of four independent observers was > 0.1 mm in error, and the mean error was 0.04 mm. For in vivo scans, the overall coefficient of variation for flow mediated dilatation was 1.8% (1.6% for women, 1.9% for men, $P = 0.18$). In 34/40 subjects (85%), all values for flow mediated dilatation were within 2.5% of the overall mean for each subject. A nested analysis of variance showed the expected between patient variability, and also significant day to day variation, but little between weeks or months. Using these data to generate power function analyses, we calculated that for individuals, an improvement in flow mediated dilatation of 4–8% is significantly greater than natural variability. In clinical trials, a mean improvement in flow mediated dilatation of at least 2% would usually be required to detect a treatment benefit, with much larger subject numbers needed for a parallel group compared to a crossover trial design.

Conclusions—Vascular responses to endothelium dependent and independent stimuli in systemic arteries can be studied non-invasively in man. Subjects should be studied on at least two occasions before and after any intervention, to optimise the chance of showing a significant effect from any potentially beneficial therapy.

(Br Heart J 1995;74:247–253).

Keywords: endothelium; atherosclerosis; ultrasound

Endothelial dysfunction is an early event in atherogenesis,^{1,2} and is also important in established coronary artery disease, where loss of endothelium dependent relaxation may cause dynamic narrowing at sites of arterial stenosis.³ Healthy endothelium produces relaxing factors which control local arterial tone, and this function may be lost in the presence of known atherogenic risk factors such as hypercholesterolaemia,⁴ cigarette smoking,⁵ and hypertension.⁶ Endothelium dependent arterial responses to a variety of pharmacological and physiological stimuli have been studied, usually using invasive catheter based techniques.^{7–9} These are, however, suitable neither for the study of the earliest preclinical phases of atherosclerosis, nor for serial evaluation of arterial physiology in response to risk factor modification or other potentially anti-atherogenic strategies. We have recently developed a non-invasive method designed to test endothelial function in the systemic arteries of children and young adults at risk of atherosclerosis, using high resolution external ultrasound imaging.¹⁰ In previous studies we have found evidence of impaired flow mediated dilatation, thought to indicate endothelial dysfunction, in the brachial and femoral arteries of young subjects with a variety of vascular risk factors including cigarette smoking,⁵ insulin dependent diabetes mellitus,¹¹ hypercholesterolaemia,¹² and homocystinuria.¹³ This method contrasts the changes in brachial artery diameter in response to increased flow and to sublingual glyceryl trinitrate. Flow mediated arterial dilatation has been shown in both experimental and clinical studies to depend on intact endothelial function, whereas arterial dilatation in response to glyceryl trinitrate does not.

The relation between non-invasive measurement of flow mediated dilatation and endothelial function is supported by a correlation with coronary endothelial function tests using acetylcholine in the same subjects¹⁴ and by the finding that brachial artery dilatation to flow using our technique can be blocked by N^G-monomethyl-L-arginine (LNMMA), a specific antagonist to EDRF/NO production by the endothelium.¹⁵ In the present study, we have assessed the precision of measurement of small diameter changes using this technique, and investigated 40 adults on four occasions each, to determine the natural variability in arterial responses over time. This quantitative

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Accepted for publication
28 February 1995

information provides the basis for the use of this method in prospective clinical studies of reversibility of abnormal endothelial pathophysiology.

Methods

PHANTOM

A phantom with 10 cylinders ("arteries") was constructed by moulding agar around metal cylinders of known diameter (Danish Phantom Design, Jyllinge, Denmark). The "arteries" measured 2.8, 3.0, 3.2, 3.4, 3.6, 4.0, 4.1, 4.2, 4.3, and 4.4 mm (similar to the range of brachial artery diameters analysed in the *in vivo* studies) and were arranged in random order. The anterior aspect of each "artery" was approximately 15 mm from the surface of the phantom. This provided seven pairs of "arteries" with a diameter difference of 0.2 mm and four pairs with a difference of 0.1 mm.

SUBJECTS

Forty adults (20 males, 20 females) aged 22–51 years were recruited from among hospital staff. All were asymptomatic, normotensive, and non-diabetic. Eight were current smokers throughout the study, eight were former smokers, and three had a total plasma cholesterol concentration > 250 mg/dl. None was taking regular medications. Each patient had four studies, incorporating intervals between visits of one to two days, one to two weeks, and two to four months. The subjects were not necessarily scanned at the same time of each day, and were not instructed to avoid food, drink, or cigarette smoking before any study. In each case the operator was unaware of the previous study results in that subject. All subjects gave informed consent, and the study protocol was approved by the local committee on ethical practice.

STUDY DESIGN

The method used to test arterial physiology non-invasively has been described in detail elsewhere.¹⁰ High resolution ultrasound was used to measure changes in arterial diameter in response to increased flow (flow mediated dilatation is dependent on intact endothelial function) and to glyceryl trinitrate (which causes vasodilatation by an endothelium independent mechanism). The brachial artery above the elbow was scanned in longitudinal section, and scans were taken at rest, during

reactive hyperaemia, again at rest, and after glyceryl trinitrate. Changes in diameter were assessed by external B mode ultrasound imaging (Acuson 128XP/10 equipped with a 7.0 MHz linear array transducer; Acuson, Mountain View, California, USA). All studies were performed in a temperature controlled room (20–25°C). The subject rested in the supine position for 10 minutes before the first scan and remained supine until the final recording was acquired. The centre of the vessel was identified when the clearest images of the anterior and posterior walls of the artery were obtained, and the transmit zone was set to the level of the anterior vessel wall. Depth and gain settings were optimised to identify the lumen to vessel wall interface, and were kept constant during each study (transmit power –9 dB, log compression 40 dB, pre-processing curve 2 ("crisp borders"), persistence A, postprocessing curve 5 ("high contrast"), and overall gain 3 dB). Images were magnified with the resolution box function leading to a television line width of approximately 0.065 mm.

Flow increase was induced by inflation of a pneumatic tourniquet placed around the forearm to 300 mm Hg, followed by cuff deflation four to five minutes later (reactive hyperaemia). The artery was scanned continuously for 90 seconds after cuff deflation. A second resting scan was recorded 10 minutes later, and was followed by administration of sublingual glyceryl trinitrate (400 µg spray). The final scan was acquired three to four minutes later.

DATA ANALYSIS

Phantom

Two experienced vascular ultrasound operators scanned the 10 phantom "arteries" on separate occasions each, with all scans recorded on super-VHS videotape. Ultrasound settings and processing curves were identical to those used for the *in vivo* studies. "Artery" diameters were then measured in random order by each of four observers, all of whom were unaware of the operator identity and the "artery" sizes. Measurements were taken from the trailing edge of the near wall interface to the leading edge of the far wall interface (fig 1). For each scan of each phantom "artery", three measurements were taken by each observer. Thus a total of 720 readings was taken (10 "arteries", two operators, three occasions, four observers, three measurements each).

Figure 1 Typical B mode ultrasound images obtained from a phantom "artery", and the right brachial artery of an adult subject.

Table 1 Sources of variation in measuring phantom "artery" diameter

Sources of variation	Variance	Percent of total
Between observer	0.00005	0
Between operator	0.00009	0
Within operator	0.00004	0
Within observer	0.00157	1
Interaction	0.00047	0
Between "arteries"	0.30673	99

In vivo studies

Arterial diameters were measured directly from super-VHS videotape recordings by two independent observers who were unaware of the scan sequence and the identity of the subject. Vessel diameters were measured from the anterior to the posterior interface between media and adventitia (the "m line") at a fixed distance from an anatomical marker, such as a vein or a fascial plane. The mean diameter was calculated from four cardiac cycles incident with the R wave on the ECG. For the hyperaemia scan, vessel diameter was measured 45 to 60 seconds after cuff release.

STATISTICAL ANALYSIS

Descriptive statistics are expressed as the mean (standard deviation).

Phantom

An analysis of variance estimated components attributable to between "arteries", between observers, between operators, within observers, within operators, and residual interaction. The three measurements made by each observer on each scan were averaged and rounded to the nearest 0.1 mm to give 240 observations. Recorded differences between "arteries" known to be 0.1 mm and 0.2 mm different were tabulated. The average bias between observed and true diameter was calculated.

In vivo studies

For each study of each patient (total of 160 studies), flow mediated dilatation was calculated for each of the two observers as the arterial diameter during the hyperaemia scan

divided by the average diameter of the two resting scans. Diameter changes were expressed as the percentage change relative to the average baseline scan (100%). Inter-observer variation was then summarised by calculating the mean and standard deviation of the absolute difference between observers for flow mediated dilatation at each of the 160 studies. For each study, flow mediated dilatation was calculated as the average of the values obtained by the two observers. A four level nested analysis of variance was then carried out to determine the relative magnitude of the different sources of variation: between patients, between days (within weeks), between weeks (within months), and between months (within patients). These sources of variability were combined into a between scan (within patient) standard deviation. Coefficients of variation were obtained from this standard deviation divided by the overall mean flow mediated dilatation, expressed as a percentage of the baseline scan. As a further indication of within patient variability, the mean or "true" flow mediated dilatation was calculated for each subject and subtracted from each of the four values obtained from the four visits for each individual.

The between scan (within patient) variability obtained allows an estimate of the percentage improvement in flow mediated dilatation that would have to be observed in an individual patient in order to be confident that the change was not simply due to spontaneous variation. It was then assumed that investigators would wish to design studies with 80% statistical power and a confidence level where $P = 0.05$ (two sided test). The number of individuals required to detect a range of hypothesised benefits could then be calculated for various study designs using the results from the analysis of variance (technical details are provided in the appendix). Results were calculated for crossover and parallel group trial designs, for different numbers of studies performed in each subject before and after intervention.

Since the primary target of interventional studies would be individuals with low or absent flow mediated dilatation, this estimate was made for the whole group and for the subset of 17 subjects in whom the mean flow mediated dilatation was less than 5%.

Results

PHANTOM

In an analysis of variance, 99% of the total measurement variation was explained by the variation in "artery" size and only 1% was due to the variability within and between operators and observers (table 1). Differences between pairs of "arteries" with diameters 0.2 mm apart were correctly estimated as 0.2 mm in 112 of 168 cases (67%). No measurement of this difference by any observer was > 0.1 mm in error. Differences in vessel diameters of 0.1 mm were correctly identified in 50 of 96 cases (52%). Similarly, no measurements differed by > 0.1 mm from the true value (fig

Figure 2 Plot of the accuracy of measurement of "artery" diameter differences of 0.1 mm and 0.2 mm in the phantom experiment.

Table 2 Sources of variation in measuring flow mediated dilatation in the brachial artery of 40 adults, studied on four occasions each

Sources of variation	Variance	Percent of total
Between patients	11.1	72
Between months	1.3	8
Between weeks	0.1	1
Between days	2.8	1.9

Figure 3 Plot of the difference of each study from the mean flow mediated dilatation (FMD) for each subject, showing that the observed FMD was within 4% of the mean FMD in 95% of subjects.

2). Overall, the mean error in estimation of diameter differences was 0.04 mm.

SUBJECTS

All 160 ultrasound studies were of sufficient quality to be analysed. The flow mediated dilatation at the first study was 7 (SD 1)% (range 0–17%), whereas the dilatation to glyceryl trinitrate was 16 (2)% (range 6–33%). The interobserver variability for measurement of flow mediated dilatation was 1.2 (0.4)%.

SOURCES OF VARIATION

The sources of variation in flow mediated dilatation (between subjects, within subjects between months, between weeks, and between days) are shown in table 2. The largest sources of variation were between patients and between days within patients. No significant additional variability was found between studies separated by weeks or up to four months. The overall between-scan variance was 3.45.

Table 3 Percentage improvement in flow mediated dilatation (FMD) required for statistical confidence ($P < 0.05$, two sided) that the change in FMD for an individual studied before and after an intervention is real rather than due to spontaneous variation

	Number of scans*		
	1	2	4
For any subject†	5.2	3.6	2.6
For a non-responder‡	4.5	3.2	2.3

*Number of studies before and after the intervention.

†Calculated using data from all subjects ($n = 40$).

‡A “non-responder” was any subject in whom the mean FMD over four studies was $< 5\%$ ($n = 17$).

WITHIN PATIENT VARIABILITY

The overall coefficient of variation for flow mediated dilatation for all subjects was 1.8% (1.6% for women, 1.9% for men, $P = 0.18$). The difference between flow mediated dilatation in each of the 160 studies and the mean or “true” flow mediated dilatation for each of the 40 subjects is shown in fig 3. In 34 of the 40 subjects (85%), all measurements of flow mediated dilatation were within 2.5% of the mean flow mediated dilatation for that subject. Only 5% of measurements in any subjects were more than 4% from the mean for that individual. The two most “consistent” subjects had flow mediated dilatation measured as 10%, 10%, 10%, and 11% and 4%, 4%, and 4%. The two most variable subjects had flow mediated dilatation measured as 9%, 10%, 11%, and 0% and 1%, 1%, 7%, and 8%; both of these were men aged between 30 and 40 years. The overall coefficient of variation for glyceryl trinitrate induced dilatation was 2.8%.

INDIVIDUAL VARIATION

Table 3 shows the minimum percent increase in flow mediated dilatation required to show a significant improvement in endothelium dependent arterial physiology at the 95% confidence level. For example, a subject who is studied once before and once after an intervention would need to show a greater than 5% improvement in flow mediated dilatation to be confident that the change is due to a true beneficial effect rather than to natural variability. By increasing the number of studies before and after intervention, the amount of improvement required decreases (if a subject is studied four times before and after intervention, an improvement of only approximately 3% would be required). In subjects who are “non-responders” (flow mediated dilatation $< 5\%$ over four studies), less improvement is needed to detect a significant change.

CLINICAL TRIAL DESIGN

Figure 4 shows the relationship between hypothesised percentage improvement in flow mediated dilatation and the number of subjects required for a trial with 80% power at

Figure 4 Relation between hypothesised improvement in flow mediated dilatation (FMD) and number of subjects required in a crossover trial design (80% power, 95% confidence level). The three curves represent three different monitoring strategies, as shown.

Figure 5 Relationship between hypothesised improvement in flow mediated dilatation (FMD) and number of subjects required in a "parallel group" trial design (80% power, 95% confidence level). The three curves represent three different monitoring strategies, as shown.

the 5% level. Curves are drawn to represent this relationship for 1, 2, and 4 studies being performed in each subject before and after intervention, assuming that the between scan coefficient of variation is as low as that found in our study. For example, if a new cholesterol lowering therapy improved flow mediated dilatation by 2.5% in subjects with hypercholesterolaemia, and these subjects were studied once before and after each treatment period, a sample size of 10 subjects would have an 80% power of demonstrating a significant benefit.

PARALLEL TRIAL

Figure 5 shows the same relationship for a parallel-group trial design. Much larger numbers of subjects are required; in the example above, a sample size of approximately 90 subjects (45 in each group) would be needed to have the same statistical power.

Discussion

There is considerable interest in measuring endothelial function in man, because endothelial dysfunction occurs in patients with established atherosclerosis,^{3,4,9} is an early event in animal models of atherogenesis,^{1,13} and has recently been demonstrated in asymptomatic children and young adults with risk factors for atherosclerosis.¹⁰ Clinical evaluation of endothelial function in large arteries relies on the measurement of small changes in vessel diameter in response to pharmacological stimuli such as infusions of acetylcholine,¹⁶ papaverine,⁹ and substance P.⁸ Invasive studies of endothelial function have been performed in the coronary,^{17,18} pulmonary,¹⁹ and femoral²⁰ arteries using quantitative angiography, which has been validated in phantom experiments.²¹ Our data show that changes in the diameter of systemic arteries of as little as 0.1 mm can be measured accurately and reliably using B mode external ultrasound imaging, and that the variability in flow mediated dilatation in healthy adults is small over periods of one day to four months.

In experimental and clinical studies in peripheral and coronary arteries arterial dilatation in response to flow increase has been shown to depend on endothelial function.^{9,22-24} The relationship between arterial diameter changes and endothelial function as

measured by our technique is supported by two recent studies. In the first, a correlation within subjects was observed between coronary artery responses to acetylcholine infusions and non-invasive brachial artery responses to reactive hyperaemia¹⁴ using our technique. In the second, flow mediated dilatation in the brachial artery was substantially reduced by intra-arterial infusion of LNMMA, a specific antagonist for endothelial production of nitric oxide, using a similar protocol to our own.¹⁵

PHANTOM STUDY

The diameter changes of 0.1–0.2 mm which could be detected accurately in our phantom study are consistent with the theoretical limit of resolution of 7.0 MHz ultrasound (0.11 mm). Furthermore the variability within and between observers and operators was negligible. The depth of the "arteries" in the phantom was equivalent to that of the brachial artery in typical adult subjects, and the density of the background matrix was similar to that of soft tissue. The machine settings (depth, gain, persistence, preprocessing, and postprocessing curves) used for the phantom experiments were similar to those for the in vivo study, and no modifications to the commercially available machine were made. Phantom studies show the "best case" for measurement accuracy, as the "arteries" are smooth and non-pulsatile. In the clinical setting, measurement of arterial diameter may be more difficult because of minor irregularities of the vessel wall/lumen interface. This difficulty can be overcome by careful selection of the arterial segment to be scanned and measured, so that in this study there were no scans that were unsuitable for analysis.

REPRODUCIBILITY STUDY

In our laboratory, there was a very low coefficient of variation for in vivo measurement of flow mediated dilatation. We studied 40 young adult subjects with smooth arteries, and selected ultrasound indices to maximise the contrast between lumen and vessel wall ("crisp borders"). The experiment was carried out under highly controlled conditions, by an experienced vascular technician and experienced ultrasound observers. Each measurement of arterial diameter represented the average of eight values (four measurements by each of two observers). These factors might act to reduce variability significantly. Therefore under optimum conditions designed to minimise measurement error, we have found that within-patient variability of flow mediated dilatation is small, over a period of up to four months.

These findings are consistent with our previous report on the effect of cigarette smoking and hypercholesterolaemia on endothelial function, in which 21 different subjects were studied two or three times, and the coefficient of variation was 2.3%.¹⁰ Data are still lacking to show whether endothelial responses to pharmacological stimuli (such as acetylcholine) are similarly reproducible when

measured by quantitative angiography, or whether endothelial function tests in subjects with established atherosclerosis will give consistent results on different occasions.

In only two of our subjects was there large variation over the four scans (7–10% between the lowest and highest values for flow mediated dilatation); this could either have been due to biological variability or to technical factors. The results in the remaining subjects were highly reproducible, despite the fact that no attempt was made to standardise such potentially important biological influences as time of day or relationship to meals. It is noteworthy that variability in females was no greater than in males, suggesting that the oestrus cycle has little influence on this measure of endothelial function. Variability within patients was mainly from day to day, with little additional variation introduced by longer intervals between studies. This suggests that the gap between studies before and after interventions is not critical as long as the day to day variability is taken into account (up to a period of months).

IMPLICATIONS FOR REVERSIBILITY STUDIES

Changes in arterial reactivity which reflect endothelial function can now be measured non-invasively in vivo, and are likely to represent an early stage in the atherogenic process. Risk factors that have a proven relationship with late atherosclerosis and its complications have been shown to be associated with impaired flow mediated dilatation in asymptomatic individuals who smoke,⁵ have hypercholesterolaemia,¹² have insulin dependent diabetes mellitus,¹¹ or who are hypertensive.⁶ The recent demonstration that L-arginine, the substrate for the production of endothelium derived relaxing factor, not only protects endothelium dependent relaxation but may also inhibit atheroma formation²⁵ is provocative. It suggests that loss of normal endothelial function is not simply a marker of early disease, but may be intimately involved in the pathogenesis of atherosclerosis. It is therefore timely and appropriate to test whether the abnormal vascular responses that we¹⁰ and others^{7,18} have found in clinical studies can be modified or reversed in high risk asymptomatic subjects by appropriate interventions. Our non-invasive method is ideally suited to the performance of prospective studies of this kind. The effect of interventions can be assessed in individual patients, and performing several studies before and after treatment will maximise the chances of showing a real benefit. The power function analyses presented allow rational design of trials, as the number of patients required is dependent on the hypothesised improvement in flow mediated dilatation and the type of study (crossover or parallel). Improvements of at least 1–2% in flow mediated dilatation would be required in any trial for a significant benefit to be found, no matter how many patients were enrolled. The more studies performed pre- and postintervention on each patient, the fewer patients are required, particularly in a

crossover study design. In parallel trials, the numbers required are much greater, as a result of between-patient variability.

CONCLUSION

Vascular responses of systemic arteries to endothelium dependent and independent stimuli can be studied accurately and reproducibly in vivo using a non-invasive technique. The low natural variation in measured responses suggests that it is feasible to test whether early manifestations of arterial damage can be modified by appropriate intervention.

Appendix

Let T be the statistic that will be used to estimate the mean difference in flow mediated dilatation between control and treatment periods, and V_T/I be the variance of T , where I is the total number of patients in the study. Let V_p and V_w denote the between and within patient variance respectively. When a single patient is measured on K days, their mean response will have variance $W = I V_w/K$. In a crossover trial, assuming no carryover and no treatment period interaction, $V_T/I = 2W/I$. In a parallel study with $I/2$ patients per group measured on K days, $V_T/I = 4(W + V_p)/I$. Note the parallel study both introduces between-patient variability and halves the number of patients per treatment.

Suppose we hypothesise a reduction due to treatment of $d\%$. Under this alternative hypothesis T has a distribution with mean d and variance V_T/I , and the number of patients in the study to have 80% power to correctly reject the hypothesis of no effect with a two sided test at the 5% level is given by

$$I = (0.842 + 1.96)^2 V_T/d^2$$

Plotting I against d provides the figs 4 and 5.

For a single patient the mean response has a standard deviation of $\sqrt{(2W)}$, and hence from random variation alone there is a 5% chance the response will show a change of at least $\pm d = 1.96 \sqrt{(2W)}$. This provides the entries in table 3 for the reduction that might be expected by chance.

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