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Original Article

# *In vivo* assessment of endothelial function in small animals using an infrared pulse detector

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## Abstract

**Objective:** Endothelial dysfunction is the earliest change in atherosclerosis. Flow-mediated dilatation (FMD) is used to assess endothelial function in humans. However, this assessment is not easy in small animals. This study demonstrated the reliability and reproducibility of a proposed instrument for *in vivo* assessment of FMD in a rodent model using infrared pulse sensors. **Materials and Methods:** We used 24 adult male Wistar Kyoto rats randomly divided into three groups. FMD was measured under continuous infusion of normal saline followed by intra-arterial infusion of acetylcholine (Ach; $n$ = 8), sodium nitroprusside (SNP; $n$ = 8), or $N^{\omega}$-nitro-$_L$-arginine methyl ester ($_L$-NAME; $n$ = 8). **Results:** The dilatation indices (DIs) of all three groups were similar before application of the vasoactive agents (1.82 ± 0.46, 1.81 ± 0.44, and 1.93 ± 0.40, $P$ = 0.877, by one-way analysis of variance). The DI was significantly increased during infusion of Ach (2.97 ± 1.03 vs. 1.82 ± 0.46, $P$ = 0.015), unchanged during infusion of SNP (1.81 ± 0.44 vs. 1.98 ± 0.40, $P$ = 0.574), and attenuated during infusion of $_L$-NAME (1.91 ± 0.40 vs. 1.42 ± 0.35; $P$ = 0.028). **Conclusion:** The results of this study correlated well with those of human studies, suggesting that this method can be used for *in vivo* evaluation of endothelial function in small animals.

**Keywords:** *Endothelial function, Flow-mediated dilatation, Nitric oxide*

## Introduction

The vascular endothelium is a single layer of cells lining the inner surface of vasculature, which also plays an important role in cardiovascular homeostasis. In addition to maintaining vascular tone, the endothelial cell has anti-thrombotic, fibrinolytic, and anti-inflammatory effects. It also inhibits proliferation of underlying smooth muscle cells [1]. *Ex vivo* assessment of endothelial function has been used since early 1980 by measuring the constriction and relaxation of isolated thoracic aortic ring with the application of acetylcholine (Ach) [2]. Initially, endothelial function was assessed invasively by measuring vasomotor changes on endothelium during local administration of drugs, such as Ach, substance P, or bradykinin [3,4].

Several noninvasive methods were developed over the past three decades. One commonly used measurement, flow-mediated dilatation (FMD), also called reactive hyperemia, assesses increases in the regional blood flow or vessel diameter after transient occlusion of upstream or downstream arteries. The increase in blood flow and also shear stress on the wall after reperfusion stimulate production and release of endothelium-derived nitric oxide, thereby causing vasodilatation [5]. FMD is a simple and reliable method to assess endothelial function and nitric oxide bioavailability, with the use of ultrasonography or tonometry to record changes in vessel diameter or blood flow [6]. From clinical observations, endothelial dysfunction occurs in the early stage of atherosclerosis and attenuation of FMD is associated with many atherosclerosis-related diseases [7,8]. Since endothelial dysfunction predicts poor clinical outcomes or occurrence of atherosclerosis-related diseases [9-14], closely monitoring endothelial function may provide an early warning and an opportunity for early intervention.

A growing number of therapeutic interventions (e.g., exercise, weight reduction, smoking cessation, and treatment with statins and angiotensin-converting enzyme inhibitors) are known to decrease atherosclerosis-related risks and also improve endothelial function in human subjects [15-17]. However, it is not easy to assess endothelial function in small animals, which is an important issue for drug

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development and exploration of disease pathophysiology. In rodents, most reported methods are invasive and require sacrificing the animals at each time point during the study [18-20]. The only method available for repeated assessment of endothelial function in living rats uses a 35-MHz high-resolution ultrasound imaging system to measure changes in the diameter of the femoral artery during FMD [21]. It is time-consuming and must be performed by experienced technicians using expensive equipment. This system has been used in only a few studies [22]. Here, we propose an instrument of novel design that uses infrared photoplethysmographic (PPG) sensors to provide easy and reliable measurements of endothelial function (i.e., FMD) in rats without the necessity to sacrifice animals at each time point and furthermore allows repeated measurements over a long period. We also proved that changes in volume flow are mostly mediated by endothelium-releasing nitric oxide.

## Materials and methods

### Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of Tzu Chi General Hospital (IACUC Approval No. 98-43-1). Male Wistar Kyoto (WKY) rats (6–9 weeks old) were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. These animals were bred in a specific pathogen-free environment at 25°C with 12:12 h light-dark cycles and allowed free access to food and water in the Animal Center of Tzu Chi University. The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and placed in a supine position on a heating pad maintained at 37°C [23].

### Measurement of flow-mediated dilatation

An infrared sensor was fixed on the footpad of the right hind limb [Figure 1]. The common iliac artery and its branch, the internal iliac artery, were isolated carefully under a dissecting microscope [24] [Figure 2]. A P10 polyethylene tube was inserted into this artery and attached to a 3-mL syringe. The syringe was fixed to an infusion pump (NE-1600 New Era Pump Systems Inc., NY, USA). An infrared sensor was attached to the footpad of the right hind limb [Figure 1]. The volume pulses were recorded by PPG, transmitted to a signal processing module containing a USB-6009 DAQ (National Instruments, Austin, TX, USA), and converted to digital signals as previously described [23]. The signals were filtered with a band-pass of 0.3–3 Hz, amplified, and then processed to a personal computer for storage and analysis.

### Experimental protocol for flow-mediated dilatation

It took about 30 min for cannulation and acclimatization. After cannulation, the animals received a continuous intra-arterial infusion of normal saline at a rate of 10 μL/min. After pulse stabilization (about 5 min), the baseline volume pulses were recorded for 1 min. Then, the external iliac artery was occluded using a hemostatic clip at the proximal site for 5 min [Figure 3a]. The dilatation index (DI) was calculated as the ratio of the mean pulse amplitude recorded during a 1-min period 1 min after release of the clip to the 1-min mean pulse amplitude before occlusion of the artery [Figure 3b] [25].

After recording the baseline DIs, the animals received an intra-arterial infusion of Ach (Sigma-Aldrich, St. Louis, MO, USA), sodium nitroprusside (SNP, Mayne Pharma Pty Ltd, Melbourne, Victoria, Australia), or a nitric oxide synthase inhibitor, $N^{\omega}$-nitro-$_L$-arginine methyl ester ($_L$-NAME, Sigma-Aldrich, St. Louis, MO, USA). The infusion rate was 10 μl/min at a dosage of 1 μg/min for Ach, 1 μg/min for SNP, and 1.7 μg/min for $_L$-NAME [26]. The same procedures were repeated to check DIs during infusion of each drug.

To test reliability of the measurements, two consecutive measurements of the DI were performed in the right and left hind limbs of five rats at an interval of 1 week. The agreement of the measurement was demonstrated by Bland–Altman plotting.

### Statistical analysis

All data are expressed as mean ± standard deviation. To check the reliability of the measurement, we performed linear regression between the first and second measurements. We used Student's *t*-test to check the difference in the DIs between the left and right side. The effects of drugs on FMD

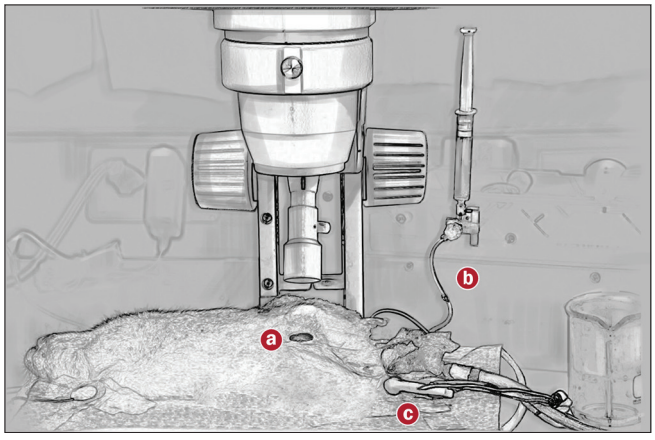


**Figure 1:** Setup of the experimental platform. The deep femoral artery was explored at the inguinal area (a) and then connected to an infusion machine with a P10 polyethylene tube (b). An infrared sensor (c) was attached to the footpad of the hind limb and then connected to a signal processing module containing a USB-6009 DAQ card for filtration and digitalization

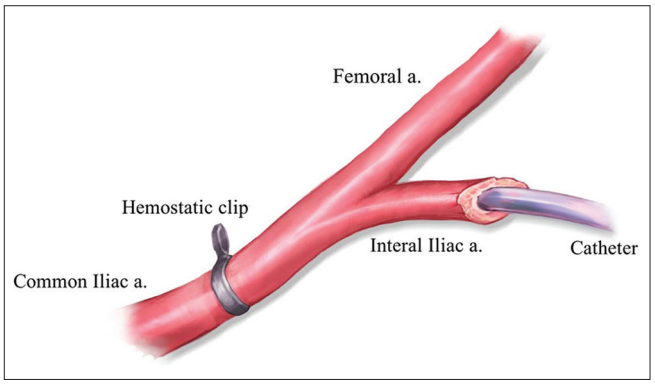


**Figure 2:** Anatomic relationship of the arteries used for the hyperemic test at the dissection site. The internal iliac artery was catheterized with a P10 polyethylene tube and connected to an infusion pump for continuous infusion of pharmacological agents. For the hyperemic test, a hemostatic clip was placed at a proximal site on the common iliac artery for transient occlusion of blood flow

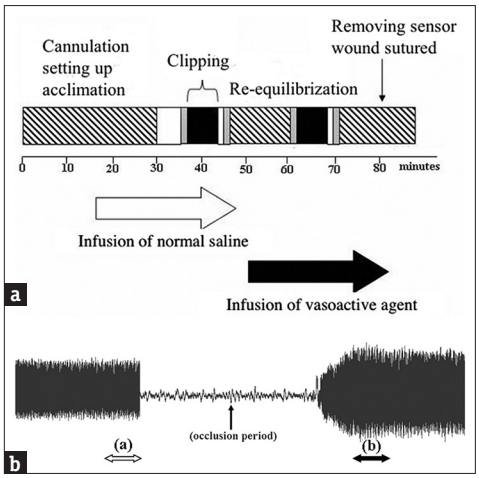


**Figure 3:** (a) The experimental protocol. About 30 min were required for cannulation and acclimatization. Then the animals received a continuous intra-arterial infusion of normal saline until stabilization of the pulse (about 5 min). The baseline pulse amplitude was recorded for 1 min (gray bar), and the common iliac artery was occluded by a hemostatic clip for 5 min (black bar). One minute after releasing the clip, the pulse amplitude was recorded for 1 min (gray bar), and the normal saline infusion was changed to acetylcholine, sodium nitroprusside, or $N^{\omega}$-nitro-$_L$-arginine methyl ester. (b) The dilatation index was defined as the mean 1-min pulse amplitude (period b) 1 min after release of the clip divided by the mean 1-min pulse amplitude (period a) 1 min before occlusion of the left femoral artery

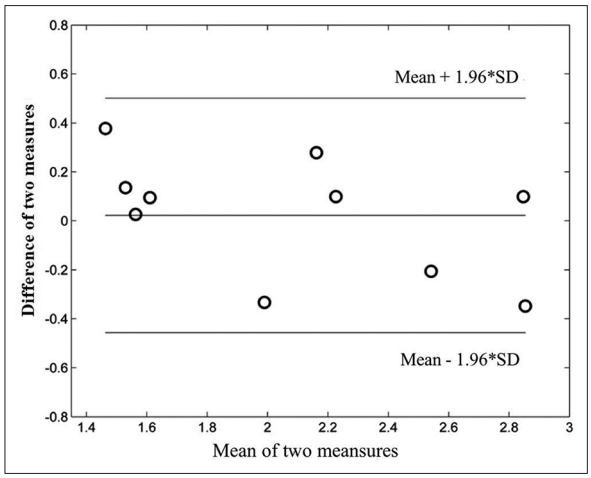


**Figure 4:** Reproducibility of the measurements was demonstrated by Bland–Altman plotting between the first and second assessments of both hind limbs of five animals at an interval of 1 week

were assessed by comparing the FMD before and during infusion of the agents, and changes in FMD were analyzed with the nonparametric Mann–Whitney U-test. The homogeneity of baseline DIs was examined by one-way analysis of variance (ANOVA). All analyses were performed using SPSS (version 10.0, SPSS, Inc., Chicago, IL, USA). Statistical significance was accepted at $P < 0.05$.

## Results

### Reproducibility of measurements

Two consecutive measurements of FMD were carried out on both sides of five rats, and therefore, 10 DIs were calculated the first time. One week later, FMDs were measured again. Ten more measurements were recorded in the 2nd week. There was very good agreement between the first and second measurements as assessed by Bland–Altman plotting [Figure 4]. There was no significant difference in measurements between sides (2.03 ± 0.69 vs. 1.83 ± 0.56; $P = 0.94$).

### Effects of drugs on reactive hyperemic vasodilatation

Twenty-four WKY rats were divided into three groups of eight. DIs did not differ before infusion of the drugs ($P = 0.877$, by one-way ANOVA). DIs were increased from 1.82 ± 0.46 (at baseline) to 2.97 ± 1.03 ($P = 0.015$) during infusion of Ach, were unchanged (1.81 ± 0.44 vs. 1.98 ± 0.40; $P = 0.574$) after infusion of SNP, and were decreased (1.91 ± 0.40 vs. 1.42 ± 0.35; $P = 0.028$) after infusion of $_L$-NAME. Although the baseline DI was higher in animals infused with $_L$-NAME than in animals infused with Ach or SNP, the difference was not statistically significant by one-way ANOVA [Table 1 and Figure 5].

**Table 1: The effects of drugs on flow-mediated dilatation**

| Drug | DI at baseline | DI during infusion of drug | *P* |
|---|---|---|---|
| Acetylcholine (*n*=8) | 1.82±0.46 | 2.97±1.03 | 0.015§ |
| Sodium nitroprusside (*n*=8) | 1.81±0.44 | 1.98±0.40 | 0.574§ |
| $_L$-NAME (*n*=8) | 1.91±0.40 | 1.42±0.35 | 0.028§ |
| *P* | 0.877* | | |

The DI of the animals before and during intra-arterial infusion of vasoactive agents. Values are presented as mean±SD. *There was no individual difference in DI before infusion of these drugs by one-way ANOVA, §Because of individual differences in response to vasoactive agents, the changes during and before infusion of drugs were evaluated by the nonparametric Mann–Whitney U-test. Statistical significance was accepted at $P<0.05$. SD: Standard deviation, DI: Dilatation index, ANOVA: Analysis of variance

## Discussion

PPG is a simple optical technique that has been used to detect changes in microcirculatory perfusion in many tissues, including the fingertips and earlobes. With the advantages of low cost, simple manipulation, and portability, PPG instruments with red or infrared sensors have been used to measure oxygen saturation, blood pressure, cardiac output, and autonomic function [27]. We have used a dual-channel PPG to measure the pulse-wave velocity in humans. These acquisitions and measurements of PPG have been shown to be reliable and reproducible [28,29].

In this study, we used a modified instrument to measure endothelial function in anesthetized adult WKY rats using infrared PPG to detect blood flow volume changes in the footpad. There are several definitions of DI, using either the ratio of the peak increase of blood flow after a hyperemic test to the baseline blood flow or the average of the pulse amplitude in a time interval to the mean amplitude at baseline [25,30]. In human study, DIs recorded during the 90–120-s postdeflation period had the strongest correlation with cardiovascular risk factors [31]. However, there is no established definition of DI for rats. The only study in which the DI in rats was measured with ultrasonography used the ratio of the maximal diameter to the baseline diameter after the hyperemic test to the baseline diameter [21]. We followed the protocol published by Nohria *et al*. to estimate the DI and used the ratio of the average amplitude of the pulse wave volume over a 1-min period

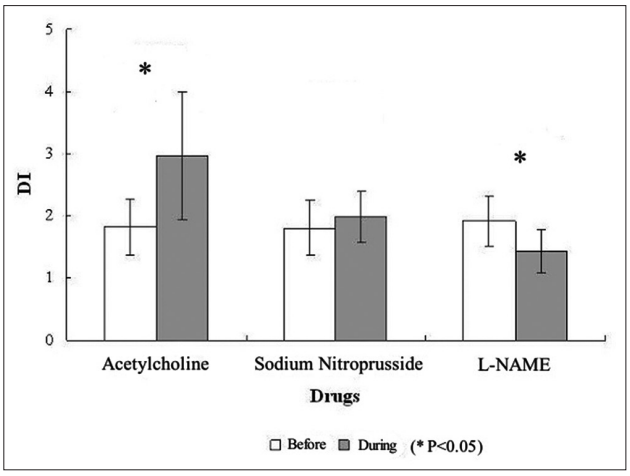


**Figure 5:** The effects of infusion of different vasoactive agents on the dilatation index ($n$ = 8 in each group). Acetylcholine enhanced dilatation indices significantly ($P$ = 0.015), $N^{\omega}$-nitro-$_L$-arginine methyl ester attenuated dilatation indices ($P$ = 0.028) and sodium nitroprusside did not have a significant effect on the FMD ($P$ = 0.574)

starting 1 min after release of the hemostatic clip divided by the average of the pulse amplitude during a 1-min interval before occlusion of the artery [32]. Many factors affect the reproducibility of PPG, such as environmental temperature, proper sensor attachment, motion artifacts, and the subject's posture, breathing, and wakefulness [27]. In this study, we showed that excellent reproducibility could be achieved under general anesthesia and controlled room temperature conditions. Moreover, this instrument can be used for long-term and dynamic *in vivo* studies of endothelial function in small animals.

FMD is mainly mediated by the release of endothelial nitric oxide. In this study, we used three vasoactive agents to confirm that the volume pulse signal changes that we recorded by infrared sensors were also mediated by nitric oxide. Figure 5 shows their effects on the DI. The endothelium-dependent vasodilator Ach increased DI significantly. In contrast, intra-arterial infusion of $_L$-NAME, a nitric oxide synthase inhibitor, attenuated flow-mediated dilatation. Another vasodilator, SNP, acting on vascular smooth muscle cells directly, did not affect the DI. These findings proved that the FMD data obtained by measurement with infrared PPG were similar to those obtained using other devices such as tonometry or high-resolution ultrasound in humans and rats [21,32].

Because the worldwide mortality and morbidity of atherosclerosis-related diseases is increasing, the development of noninvasive monitoring of progression of atherosclerosis has become a very important issue for prevention and early treatment of these diseases. Dysfunction of the vascular endothelium has been shown to be the earliest change in atherosclerosis from clinical observations over three decades [4,33]. It has been proved that FMD is a reliable assessment of the bioavailability of endothelial nitric oxide. Impaired FMD in asymptomatic individuals correlates with cardiovascular risk factors such as hypertension, insulin resistance, diabetes mellitus, elevated serum low-density lipoprotein, smoking, and even sedentary lifestyles. Therefore, FMD was recently recommended as a valid parameter in the noninvasive assessment of subclinical atherosclerosis in children and adolescents [34]. Although therapeutic targeting on vascular endothelial dysfunction has been proposed as a useful strategy in the treatment of atherosclerosis and related diseases [35-37], long-term evaluation of the therapeutic impact on endothelial function in clinical studies is not easy. In addition, the causes of atherosclerosis such as genetic polymorphism or environmental toxins, diet, and oxidation stress are difficult to assess in human subjects. The use of a physiologically and genetically similar animal model, therefore, can serve this purpose. Several animal models of atherosclerosis are available for intervention studies [38,39], but few reliable, noninvasive methods can be used to assess endothelial function. To overcome this limitation, we developed the aforementioned instrument for reliable and easy assessment in long-term monitoring of endothelial function in animal models. This can offer a deeper understanding of the disease process and guide further pharmacological and interventional treatment strategies against atherosclerosis.

## Conclusion

We designed a reliable instrument to assess endothelial function in small animals using an infrared pulse detector. Similar to that in human beings, the FMD acquired with our system was shown to be mainly nitric oxide mediated. This instrument can be used for the study of the pathogenesis of atherosclerosis through assessing changes in endothelial function and evaluating the efficacy of therapeutic regimens against atherosclerosis in animal models.

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### Conflicts of interest

There are no conflicts of interest.

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