Applanation Tonometry in Mice

A Novel Noninvasive Technique to Assess Pulse Wave Velocity and Arterial Stiffness

Arthur J.A. Leloup, Paul Fransen, Cor E. Van Hove, Marc Demolder, Gilles W. De Keulenaer, Dorien M. Schrijvers

Abstract—Arterial stiffening is the root cause of a range of cardiovascular complications, including myocardial infarction, left ventricular hypertrophy, stroke, renal failure, dementia, and death, and a hallmark of the aging process. The most important in vivo parameter of arterial stiffness is pulse wave velocity (PWV). Clinically, PWV is determined noninvasively using applanation tonometry. Unlike the clinical value of arterial stiffness and PWV, techniques to determine PWV in mice are scarce. The only way to determine aortic PWV noninvasively in the mouse is by using ultrasound echo Doppler velocimetry. It is a fast, efficient, and accurate technique, but the required tools are expensive and technically complex. Here, we describe the development and validation of a novel technique to assess carotid–femoral PWV noninvasively in mice. This technique is based on applanation tonometry as used clinically. We were able to establish a reproducible reference value in wild-type mice (3.96±0.05 m/s) and to detect altered carotid–femoral PWV values in endothelial nitric oxide synthase knockout mice (4.66±0.05 m/s; P<0.001 compared with control), and in mice sedated with sodium pentobarbital (2.89±0.17 m/s; P<0.001 compared with control). Also, carotid–femoral PWV was pharmacologically modulated and measured in a longitudinal experiment with endothelial nitric oxide synthase knockout mice to demonstrate the applicability of this technique. In general, applanation tonometry can be used to measure carotid–femoral PWV noninvasively in mice. The experimental setup is simple, and the technical requirements are basic, making this technique readily implementable in any mouse model–based research facility interested in arterial stiffness.

Key Words: aging ▪ mice ▪ pulse wave analysis ▪ vascular stiffness

Progressive large artery stiffening is the predominant cause of increased pulse pressure, a marker of cardiovascular risk in the general population, and a predictor of cardiovascular events. Furthermore, it reduces myocardial perfusion efficiency and increases left ventricular afterload and mechanical stress on smaller vessels and capillaries, potentially damaging the capillary wall of strongly perfused organs such as the heart, brain, and kidneys.

The most important in vivo parameter of arterial stiffness is pulse wave velocity (PWV) or the propagation speed of the arterial pressure wave in the arterial wall. Clinically, carotid–femoral PWV (cfPWV) is considered the gold standard for assessment of central arterial stiffness and a surrogate marker for cardiovascular morbidity and mortality, independent of atherosclerosis or brachial blood pressure. Using applanation tonometry with high-fidelity pressure sensors, cfPWV can be determined noninvasively from the delay of pressure waves at the carotid and femoral artery and from the distance traveled by the pulse, which is usually a measure of the surface distance between the 2 recording sites. It is a fast, efficient, and accurate technique and therefore frequently used to assess large artery stiffening in humans. However, PWV assessment in mice, which are regularly used in preclinical research of cardiovascular disease, is less straightforward because of the small size and high heart rate of 400 to 600 bpm. Here, invasive intravascular tonometry can be used to measure aortic PWV (aPWV) or cfPWV, but it cannot be applied in longitudinal studies. To determine aPWV noninvasively in mice, most frequently, ultrasonic echo Doppler velocimetry is used. Hartley et al. showed that the velocity profile of the blood is more or less synchronized with the pressure profile (±1 ms). Hence, the echo Doppler–based velocity profile of the blood in the aortic arch and abdominal aorta can be used to assess aPWV as a measure of aortic stiffness in mice. Echo Doppler velocimetry is accurate, sensitive, and the current gold standard for noninvasive aPWV determination in mice. However, the experimental setup is complex and cost consuming, thereby limiting the accessibility of the technique.

The main goal of the present study was to develop a novel noninvasive technique based on applanation tonometry for...
the assessment of cfPWV as a measure of large artery stiffness in mice. This technique had to be easy to use, cost effective, accurate, sensitive, and efficient. Therefore, we tested whether applanation tonometry as used in clinic can be adapted for the use with mice. The capacity of this adapted technique to generate reproducible results was evaluated, and reference values to compare with echo Doppler velocimetry data in mice, as displayed in the literature, were collected. Finally, the sensitivity of this new technique to pick up alterations in cfPWV was tested using mouse models known to have elevated or decreased aPWV.

Materials and Methods

Animals
All animals were housed in the animal facility of the University of Antwerp in standard cages with 12–12-hour light–dark cycles with free access to regular chow and tap water, unless stated otherwise. A total of 33 wild-type (WT) mice (C57Bl/6J strain; age, 5.6±0.2 months; Charles River Laboratories, Belgium) and 22 endothelial nitric oxide synthase knockout (eNOS−/−) mice (C57Bl/6J background; age, 12.3±0.8 months; Jackson Laboratories) were used. Five eNOS−/− mice were treated during 12 days with 4.5 mg/kg per day perindopril erbumine (TOCRIS Bioscience, United Kingdom), assuming a daily water intake of 6 mL/mouse. The vehicle for perindopril erbumine was 0.1% ethanol, and thus vehicle-treated eNOS−/− mice (n=6) served as controls. Depending on the setup, sevoflurane anesthesia (8% and 4%–5% in oxygen for induction and maintenance, respectively, 1.5 L/min) or sodium pentobarbital (Nembutal, 75 mg/kg IP) anesthesia was used to immobilize the animals during tonometry measurements. All experiments were conducted following the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and were approved by the Ethical Committee for Animal Experiments of the University of Antwerp.

After induction of anesthesia, the mouse was placed supine on a heating pad. To stimulate vasodilatation of the femoral artery, an additional heating lamp was placed above the mouse. The heating lamp was placed in a way that both tonometers and connecting wires were equally heated to avoid variation in the signal propagation time. The fur on the right carotid and femoral artery was removed, and 2 pulse tonometers (SPT-301, Millar Instruments) were stably applanated on the skin using a micromanipulator (Figure S1 in the online-only Data Supplement). Both measuring sites were marked to allow precise determination of the external carotid–femoral distance.

Determination of Carotid–Femoral Transit Time
Carotid–femoral transit time (Δt) was determined using the time difference between the foot of carotid and femoral artery pulses (foot-to-foot method). Factors such as blood pressure and age can significantly influence the waveform, therefore the foot rather than other points of the pressure wave was used to calculate Δt because of the limited interference with reflected waves. The foot of the pulse pressure wave was defined as the second derivative maximum. This is a straightforward method, and reproducibility was shown to be superior as compared with other methods (eg, pressure minimum or first derivative maximum). Fifty consecutive pulses with sufficient amplitude and a reproducible waveform were analyzed; pulses that interfered with respiratory movement peaks were excluded. For a trained person, the procedure took between 5 and 20 minutes per mouse, and the actual signal acquisition of the arterial pulse took ≈2 minutes.

Determination of Carotid–Femoral Distance and cfPWV
Similarly to the clinical setting, the distances between the sternal notch and both measurement sites (carotid and femoral artery) were accurately measured using a scientific sliding caliper. The difference between both distances was defined as the external carotid–femoral distance (d; Figure S1). Finally, cfPWV was calculated as the external carotid–femoral distance divided by transit time (cfPWV=d/Δt).

Figure 1. Carotid artery tonometer signal tracings of different wild-type mice. A, High-quality tonometer signal with a reproducible waveform allows the accurate determination of the pressure pulse foot (•). Despite the presence of interfering respiratory movement peaks (dashed lines), there are sufficient discrete arterial pressure pulses to determine transit time. B, Inverted waveforms have been reported in the clinical setting also, making determination of the foot of the pulse impossible. C, Low-amplitude (<5 μV) arterial pressure peaks (•) limiting the accurate determination of the pulse foot. D, Low-amplitude peaks (<5 μV) with variable waveform, the transit time is impossible to determine. E, The respiration rate is high causing no discrete arterial pressure peaks to be present between 2 respiration peaks (dashed lines).
amplitude could be detected to determine $\Delta t$ accurately. The signal was considered reliable when the waveform was characterized by a fast systolic upstroke followed by a slower decline with interfering reflected waves, when the amplitude of the amplified tonometer output as acquired in LabChart was $\geq 5 \mu V$, and when interference by respiratory movement peaks was limited (Figure 1).

To assess $\Delta t$, high-quality pulse waves were acquired with both tonometers in position (1 on the carotid artery and 1 on the femoral artery; Figure 2). The transit time ($\Delta t$) was then calculated as the mean foot-to-foot delay of 50 simultaneously recorded carotid and femoral pulse waves (Table).

### Reproducibility of the Method

All relevant parameters ($\Delta t$, d, and cfPWV) were determined in 4 different groups of 6 WT mice (Table). As illustrated in Figure 3, cfPWV was determined with high reproducibility ($P<0.93$).

### Sensitivity of the Technique

Sensitivity of the technique was tested by measuring cfPWV in conditions where alterations of aPWV have been described. With echo Doppler–based velocimetry, it was described that sodium pentobarbital anesthesia lowered aPWV significantly compared with other anesthetic agents.$^{10}$ In addition, the absence of endothelial nitric oxide release in eNOS $^{-/-}$ mice resulted in elevated aPWV. $^{12,22}$ Here, we applied applanation tonometry to test whether the technique was sensitive enough to reproduce these echo Doppler–based observations. Applanation tonometry results were indeed similar to the observations with echo Doppler velocimetry (Figure 4). Nembutal-anesthetized animals displayed significantly slower (2.89±0.17 m/s; $P<0.001$ [n=9]) and sevoflurane-anesthetized eNOS $^{-/-}$ mice displayed significantly faster cfPWV (4.66±0.16 m/s; $P<0.001$ [n=11]) as compared with sevoflurane-anesthetized WT mice (3.96±0.05 m/s [n=24]).

### Application of the Technique: A Longitudinal Experimental Setup

Because the noninvasive approach of our technique allows to follow cfPWV variation with time, a longitudinal study was performed in which cfPWV was pharmacologically modulated. CfPWV remained unchanged after 12 days in vehicle-treated (0.1% ethanol) eNOS $^{-/-}$ mice. Chronic treatment of eNOS $^{-/-}$ mice with perindopril (4.5 mg/kg per day) resulted in a significant $\approx 15\%$ decrease of cfPWV (Figure 5) to values nonsignificantly different from WT mice.

### Discussion

In 1997, Hartley et al$^{10}$ presented a novel method to determine aPWV noninvasively in mice using the blood velocity profile.
rather than the pressure signal because the latter could only be obtained using (invasive) intravascular pressure catheters. In the present study, we were able to show that high-fidelity pressure tonometers can be used to acquire the carotid and femoral artery pressure signal noninvasively and that this technique is applicable to study arterial stiffness in mice by measuring cfPWV. The technique allowed to collect reproducible reference values of cfPWV in WT mice and was sensitive enough to confirm changes in PWV described earlier in eNOS−/− mice, a genetic mouse model with arterial stiffness, and in WT mice anesthetized with sodium pentobarbital. Furthermore and most advantageous, the technique could be used to measure cfPWV in a longitudinal study in which cfPWV was pharmacologically modulated.

Accurate cfPWV determination is limited by the temporal resolution of the tonometer signal and the accuracy of the carotid–femoral distance measurement. With a sampling rate of 2 kHz and hence 0.5-ms sampling intervals, the temporal resolution was sufficient to measure carotid–femoral foot-to-foot transit time. Improving the temporal resolution even further is technically possible. However, a major source of variation is the determination of carotid–femoral distance. External measurements (eg, sternal notch–femoral artery minus sternal notch–carotid artery distance) instead of measurements of the actual carotid–femoral path length are used to calculate cfPWV in the clinical setting. Exactly the same surface measurements were used for the calculation of cfPWV in the experiments presented here. The use of external rather than the effective carotid–femoral distance results in an overestimation of the actual cfPWV. This, however, is inherently linked to the noninvasive approach and, when anatomic dissimilarities are absent, will not limit the ability to compare cfPWV values between subjects, groups, or in time.

Other than the determination of carotid–femoral distance and temporal resolution of the tonometer signal, the timing algorithm that is used to determine Δt can strongly influence the calculated PWV value. In the present study, the maximum second derivative method was used. It gives a more reproducible measure of transit time compared with other methods such as the pressure wave minimum or the maximum first derivative method. Nevertheless, the use of other algorithms can result in equally reproducible but different absolute values of PWV. In the present study, the maximum second derivative method was used. It gives a more reproducible measure of transit time compared with other methods such as the pressure wave minimum or the maximum first derivative method. Nevertheless, the use of other algorithms can result in equally reproducible but different absolute values of PWV as is illustrated by the differences between echo Doppler–based aPWV values from literature that range from $3 \pm 0.35$ to $3.8$ m/s (Figure 6). The highly reproducible reference values of $3.96 \pm 0.05$ m/s for C57Bl/6J mice obtained in the present study by measuring 4 groups of mice from different cages and measured at different timings are high compared with PWV values obtained...
and sensitive, and the technical requirements are basic, making this technique readily implementable in any standard laboratory environment investigating cardiac and vascular biology in mice.

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Disclosures
None.

References


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### Novelty and Significance

**What Is New?**

- The development of a novel technique for the noninvasive assessment of carotid–femoral pulse wave velocity (PWV) as a measure of arterial stiffness in mice.

**What Is Relevant?**

- There is accumulating evidence that arterial stiffness is strongly correlated with cardiovascular complications, thus increasing the need for fundamental and preclinical research in animal models.
- The noninvasive assessment of PWV in mice was, until now, limited to laboratories with access to echo Doppler velocimetry, a cost-consuming and technically challenging technique.

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### Summary

Appplanation tonometry can be used to assess carotid–femoral PWV noninvasively in mice. This technique is reproducible, capable of detecting known alterations in PWV, and can be applied in a longitudinal study in which carotid–femoral PWV is pharmacologically modulated over time.
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Figure S1. Experimental set-up of carotid-femoral transit time determination. Carotid-femoral distance (d) is defined distance A minus distance B. sn: suprasternal notch.