A Simple Method for Normalization of Aortic Contractility

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Keywords

Aorta · Blood vessel · Isometric contraction · Myography · Phenotypic switch · Vascular smooth muscle

Abstract

Vascular contractile function changes in proliferative vascular diseases, e.g. atherosclerosis, and is documented using isolated blood vessels; yet, many laboratories differ in their approach to quantification. Some use raw values (e.g., mg, mN); others use a “percentage of control agonist” approach; and others normalize by blood vessel characteristic, e.g. length, mass, etc. A lack of uniformity limits direct comparison of contractility outcomes. To address this limitation, we developed a simple 2-step normalization method: (1) measure blood vessel segment length (mm), area (mm²) and calculate volume (mm³); then, (2) normalize isometric contraction (mN) by segment length and volume. Normalized aortic contractions but not raw values were statistically different between normal chow and high-fat diet-fed mice, supporting the practical utility and general applicability of normalization. It is recommended that aortic contractions be normal-

ized to segment length and/or volume to reduce variability, enhance efficiency, and to foster universal comparisons across isometric myography platforms, laboratories, and experimental settings.

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Introduction

Cardiovascular disease is the leading cause of death worldwide. Atherosclerosis is the primary form of cardiovascular disease that increases the risk of myocardial infarction, stroke, and peripheral arterial disease. An underlying mechanism of atherosclerosis involves a “phenotypic switch” in vascular smooth muscle cells (VSMC) that transcriptionally moves VSMC from a contractile to a proliferative or “synthetic phenotype” [1, 2]. Thus, measuring contractility in isolated blood vessels provides functional evidence of phenotypic change reflective of the

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underlying local cellular and biochemical alterations such as in atherosclerosis. Thus, laboratories around the world measure vascular contractile function *in vitro*, wherein an isolated blood vessel, e.g. aorta, is contracted with an agonist, e.g. phenylephrine (PE), endothelin-1, U46,619 – a thromboxane A₂ analog, etc... and data reported [3–5]. The widespread use of *in vitro* vascular contractility assays is revealed in PubMed search using the terms “vascular” and “dysfunction,” which netted 214,358 hits, indicating a sizeable research investment.

Although raw isometric data reflect vessel contractility, raw values (e.g., mg or mN tension) [6] cannot be compared across different laboratories due to obvious differences in the size of the blood vessel segment (e.g., length, diameter), species, animal size, age, diet, and disease state as well as the specific blood vessel or blood vessel location (e.g., thoracic vs. abdominal aorta). Additionally, different platforms or myography systems (e.g., DMT) are used to assess isometric force production. So, even if two laboratories use the same system, the reporting of data can make it difficult to compare contractility due to dissimilar units (mg vs. mN) or a different data normalization procedure, e.g. percent of control agonist such as percent contraction of high-potassium solution) [7, 8] or differential normalization, e.g. mg tension/cross-sectional area; mg tension/mg wet weight; mN/mm length [3, 4, 9]. To address this obvious limitation and to foster more across-study and laboratory comparisons, aortic contractions were normalized by several easily measured parameters.

Several criteria need to be met to establish a universal normalization method. For example, the method needs to be accessible, and thus, it should not require costly equipment that may exclude users. It should be simple enough that all users can implement the method. Moreover, the method should be cost effective in that the time spent performing should be worth the effort. Because current laboratories already use some form of normalization (e.g., as “% of control agonist”) [8], a new approach must be cost effective and enhance data quality over an existing practice. Finally, the method should foster data comparisons for both cross-laboratory fertilization and greater biological understanding. To this end, we offer a simple and cost-effective method for normalization of vascular contractility that requires minimal equipment and effort on the user’s behalf, yet refines the measurement of aortic contraction to a degree wherein even modest changes in contractility (e.g., due to a treatment, aging, diet, etc...) are more readily discerned. Contractility data that are universally normalized can be shared and compared between laboratories around the globe. Adoption of this ap-

proach may improve assessment of the biological actions of drugs, diets or toxicants, for example, and thus, minimize overall use of resources.

Materials and Methods

Rodents

Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) or raised in house. Mice were fed normal chow (NC; 13% kcal fat, Rodent Diet 5010; LabDiet, St. Louis, MO, USA) or high-fat (HFD; 42% kcal fat, TD.88137; Harlan Teklad, Madison, WI, USA) diet from 8 to 20 weeks of age. Male Sprague-Dawley rats (wild type [WT] and low-density lipoprotein receptor deficient [LDLR-KO]) were obtained from SAGE (Sigma-Aldrich) and fed NC diet up to 1 year of age [10]. Rodents were treated according to APS’s Guiding Principles in the Care and Use of Animals, and all protocols were approved by University of Louisville IACUC. All rodents were anesthetized with sodium pentobarbital (i.p., 150 mg/kg) and exsanguinated via right ventricle blood draw.

Equipment

The following equipment was used: digital handheld microscope (zORB, Carson, New York, NY, USA); scientific ruler (1 mm increments; Fine Science Tools); 10% neutral buffered formalin solution (NBF; Sigma); Falcon® 12-well, clear, flat bottom plate (Corning Costar); ImageJ (NIH free imaging software); razor blades; dental wax (Z Pink); general surgical tools (scissors, forceps); microfuge tubes; organ bath myography system; and, microbalance. All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Aorta Isolation and Organ Bath Systems

Aortas were isolated via ventral thoracotomy and placed into cold phosphate-buffered solution (PSS) containing (in mM): NaCl, 130; KCl, 4.7; MgSO₄·7H₂O, 1.17; KH₂PO₄, 1.18; NaHCO₃, 14.9; CaCl₂, 2.0; glucose, 5.0; pH 7.4. High-potassium (HI K⁺) PSS contained the following (in mM): NaCl, 34.7; KCl, 100; MgSO₄·7H₂O, 1.17; KH₂PO₄, 1.18; NaHCO₃, 14.9; CaCl₂, 2.0; glucose, 5.0; pH 7.4. Distal thoracic aorta (located 0.8–1 cm from aortic arch apex) segments were cut from each aorta (2–4 mm), cleaned of perivascular adipose tissue, and mounted in one of two systems: (1) horizontal pin (DMT, Ann Arbor, MI, USA); or, (2) vertical wire system. In the horizontal pin system, a short, aortic segment (≈2 mm) was mounted on stainless steel pins in a 5-mL heated (37°C) organ bath. One pin was connected to an isometric strain gauge transducer, and the other was attached to a micrometer. Transducer signals were fed directly into a PC with LabChart software (v. 7 or 8; ADI). In the vertical wire system, an aortic segment (3–5 mm) was placed on wire hooks (100-μm diameter tungsten wire) with a hook connected to an isometric strain gauge transducer (Kent Scientific, Litchfield, CT) and the other attached to a fixed support glass rod in a 15-mL heated (37°C) organ bath. Transducer signals were fed into an 8-channel PowerLab A/D converter and recorded on a PC using LabChart software (v. 4.3.2; ADI). Prior to each use, each system was calibrated using standardized weights. In both systems, after 10 min without tension, aortic rings were equilibrated to ≈9.81 mN (≈1 g) loading tension over 30 min before agonist stimulation [3].

Fig. 1. Images of method for aortic parameter data acquisition. **a** Digital images of blood vessel segment length (mm) acquired with digital mini-microscope camera. **b** Digital image of blood vessel cross-section overlaid with digital outlines of outer and inner circumferences (NIH ImageJ). The outer area (OA) minus the inner area (IA; lumen) is the segment cross-sectional area (mm^2). **c** Calculation of blood vessel segment volume where, volume = length (mm) $\times$ cross-section area (mm^2) in mm^3 .

Statistics

Values are expressed as mean $\pm$ SD or $\pm$ SE (where appropriate). Comparisons between 2 groups (e.g., horizontal vs. vertical system; user 1 vs. user 2; etc...) were done with a paired or unpaired *t* test as appropriate. $p < 0.05$ was considered significant.

Results and Discussion

Acquisition of Aortic Segment Parameters

Normalization of contractions was done using a simple, sequential, multistep process that is described pictorially in Figure 1, and described here as a general protocol: (1) immediately formalin-fix a blood vessel segment; (2) obtain wet weight (to 0.1 mg; dab extra fluid on tissue paper); (3) image segment lengthwise with scientific ruler (Fig. 1a); (4) cut (with razor blade) and image (with handheld digital microscope) a thin (0.5 mm) blood vessel cross-section (x-sec) with a ruler in the image (Fig. 1b); (5) using ImageJ, quantify segment length (mm) and x-sec area (mm^2) (Fig. 1c); and, (6) calculate segment vol-

ume (mm^3) as the product of length (mm) and x-sec area (mm^2), and then normalize isometric contractions by segment length (mN/mm), x-sec area (mm^2), and volume (mN/ mm^3).

Aortic Function

Aortic vascular contractile function was assessed using both a horizontal and a vertical isometric tension organ bath system as reported previously [3]. To illustrate the utility of the normalization protocol, the distal thoracic aorta of a mouse was sectioned into 2 obvious, unequal lengths. A shorter piece (1.5–2.5 mm) was mounted in the horizontal pin system and a longer piece (3–4 mm) was mounted in the vertical system. Both segments were equilibrated similarly (≈ 9.81 mN tension). As intended in this “proof of principle” example, the absolute (raw) tension of aortic contractions (mN) induced either by cumulative concentrations of PE (1 nM to 10 μM ; Fig. 2a) or by high-potassium solution (100 mM; Fig. 2b) was significantly different between the two due to the intentional difference in blood vessel segment size ($\approx 2\times$).

Fig. 2. Normalization of vascular contractility data. Contraction data (raw, mN) and data normalized by aorta cross-sectional area (mN/mm²), length (mN/mm), and volume (mN/mm³) were plotted. To generate tension data, two aortic segments were exposed to contractile agents: phenylephrine (PE; 1 nM to 10 μ M) (**a**) and high-

potassium (100 mM) buffer (**b**); in a horizontal pin (DMT; 1, filled symbols), or a vertical wire (2, open symbols) organ bath system. After experimentation, length and cross-sectional area of formalin-fixed blood vessel segments were measured and the segment volume calculated (volume = length $\times$ cross-section area; see Fig. 1).

Table 1. Thoracic aorta segment parameters from C57BL/6 mice fed normal chow ($n = 9$) or high-fat diet ($n = 10$) for 12 weeks

Parameter	Diet	
	normal chow	high-fat diet
Wall area, mm ²	0.246 $\pm$ 0.024	0.225 $\pm$ 0.020
Length, mm	3.96 $\pm$ 0.56	4.37 $\pm$ 0.41
Volume, mm ³	0.976 $\pm$ 0.178	0.990 $\pm$ 0.171

Values = means $\pm$ SE; $n = 9, 10$ mice, respectively.

Table 2. Normalization of high potassium-induced (100 mM) isometric contractions in thoracic aortas isolated from male mice fed either normal chow (NC) or high fat diet (HFD) for 12 weeks

Group	mN	mN/mm	mN/mm ²	mN/mm ³
NC	8.26 $\pm$ 1.03	2.08 $\pm$ 0.22	33.16 $\pm$ 3.32	8.37 $\pm$ 0.71
HFD	5.54 $\pm$ 0.61	1.26 $\pm$ 0.13	24.61 $\pm$ 2.60	5.66 $\pm$ 0.57
p value	0.0330	0.0047	0.0562	0.0079

Values = mean $\pm$ SE; $n = 9, 10$ mice, respectively; p values shown are results of unpaired t tests between NC and HFD groups.

Normalization of Aortic Contraction

After acquisition of aortic parameters, cumulative PE and high potassium-induced contractions were normalized to x-sec area, length, and volume (Fig. 2). Normalization of raw tension by segment volume (mN/mm³) resulted in the most similar PE-induced tension curves (see curves of “volume 1” and “volume 2”; Fig. 2a). Whereas normalization of raw values to either x-sec area or length was beneficial in reducing the difference between “raw 1” and “raw 2” values, but neither was superior to normalization by volume. Notably, in a separate study, a group used a standardized mouse aorta segment length (3 mm) to normalize contractility and arrived at values similar to ours, thus, supporting the idea that normalized contractions even to one

dimension (e.g., length; mN/mm) likely are better than no normalization (Fig. 2a) [11]. Normalization efficacy was independent of the contractile agonist because a similar effect was observed when HI K⁺-induced maximal tension was normalized to x-sec area, length and volume (Fig. 2b). As for PE, normalization to volume appeared to perform better than using either length or area alone.

In a second example, real-world contractility was measured in aortas isolated from C57BL/6 mice fed either an NC (13% kcal fat) or HFD (42% kcal fat) for 12 weeks. As all aorta segments had similar lengths, x-sec areas, and thus, volumes (Table 1), direct comparison of raw contractility values revealed that 12 weeks of HFD appeared

Fig. 3. Normalization of vascular contractility of aortas from C57BL/6 mice fed normal chow (NC; *n* = 9 mice) or high-fat diet (HFD; *n* = 10 mice) for 12 weeks. Phenylephrine (PE)-induced raw contraction data (**a**; mN) and raw data normalized by aorta length (**b**; mN/mm), x-sect area (**c**; mN/mm²), and volume (**d**; mN/mm³). Values = mean ± SE. *n* = 9 or 10 aortas (1 aorta per mouse). * *p* < 0.05 between NC and HFD.

to decrease PE-induced contractions relative to aortas of NC-fed mice, yet there were no statistically significant differences (Fig. 3a). To assess whether normalization could unmask differences in contractility, raw values were normalized by length (Fig. 3b), area (Fig. 3c), and volume (Fig. 3d). When normalized to aortic segment length or volume, contractions were statistically different between NC and HFD mice (Fig. 3b, d). Overall, these data indicate that normalization of aortic contractility enhanced detection of statistically significant changes induced by HFD (Fig. 3b, d). Similarly, aortic contractions induced by 100 mM HI K⁺ buffer were also significantly

Table 3. Thoracic aorta segment parameters from wild-type (WT) and LDLR-null (LDLR-KO) 1-year-old Sprague-Dawley rats

Parameter	Strain	
	WT	LDLR-KO
Wall area, mm ²	1.251±0.213	1.168±0.224
Length, mm	3.07±0.49	2.74±0.48
Volume, mm ³	3.90±1.055	3.269±1.138

Values = means ± SE. *n* = 5, 6 rats, respectively.

Fig. 4. Normalization of vascular contractility of aortas from Sprague-Dawley wild type (WT; $n = 5$ rats) and LDLR-KO rats ($n = 6$ rats) fed normal chow for 1 year. Phenylephrine (PE)-induced raw contraction data (**a**; mN) and raw data normalized by aorta length (**b**; mN/mm), x-sec area (**c**; mN/mm²), and volume (**d**; mN/mm³). Values = mean $\pm$ SE. $n = 5$ or 6 aortas (1 aorta per rat). * $p < 0.05$ between WT and LDLR-KO rats.

between NC and HFD-fed groups both as raw values and when normalized by length and volume (Table 2).

Another real-world example was performed using aortas isolated from 1-year old Sprague-Dawley rats that were WT or LDLR-KO. As all aorta segments had similar lengths, x-sec areas, and thus, volumes (Table 3), direct comparison of raw contractility values revealed that genetic deletion of LDLR increased PE-induced contractions relative to aortas of WT rats (Fig. 4a). To assess whether normalization could alter the statistical differences observed, raw values were normalized by length (Fig. 4b), area (Fig. 4c), and volume (Fig. 4d). Normaliza-

tion of raw contractility data to aortic segment length or volume yet not area enhanced the statistically significant differences (lower p values; Table 4) between WT and LDLR-KO rats (Fig. 4b-d). Overall, these data showed that normalization of aortic contractility to length or volume further improved detection of statistically significant differences and, importantly, had no detrimental effect on statistical outcomes.

Method Validation

The “proof of principle” tests (i.e., platforms; NC vs. HFD; WT vs. LDLR-KO) support the general applica-

Fig. 5. Reproducibility of aorta parameter data acquisition: intra-assay variation. The same images of a blood vessel lengthwise and in cross-section (ring) were repeatedly examined for length and cross-sectional area (x-sec area), respectively, as described above using ImageJ a total of 10 times by two different observers. Blood vessel volume (mm^3) was calculated by multiplying length (mm) $\times$ x-sec area (mm^2). Values = mean $\pm$ SD.

bility of our normalization approach, so we also performed four validation tests. These were: (1) two different “users” quantified the same ring images using ImageJ; (2) two different users quantified the same image multiple times (e.g., 10 times); (3) two different users imaged and then quantified multiple sections from a single aorta; and (4) normalization was performed using segment volume calculated by 2 different methods: (a) our image analysis approach and (b) volume estimated from segment weight (in mg) and density ($\text{mg}/\mu\text{L}$; mg/mm^3).

Reproducibility

Image analysis for length and x-sec area proved highly reproducible (Fig. 5). It appeared that users vary in their first attempt to quantify length, but users rapidly adjusted and subsequently made consistent measurements of the same image. These data support the simple and cost-effective nature of our approach. Images were made with an inexpensive microscope camera (USD < 50) and were analyzed with free imaging software (NIH ImageJ). The approach was independent of the user because measurements made by 2 users of both area

Table 4. Normalization of PE-induced ($10 \mu\text{M}$) isometric contractions in thoracic aortas isolated from wild-type (WT) and LDLR-null (LDLR-KO) 1-year-old Sprague-Dawley rats

Group	mN	mN/mm	mN/ mm^2	mN/ mm^3
WT	15.10 $\pm$ 2.55	4.85 $\pm$ 0.66	12.09 $\pm$ 1.59	3.98 $\pm$ 0.52
LDLR-KO	22.29 $\pm$ 2.34	8.36 $\pm$ 1.09	19.57 $\pm$ 2.35	7.50 $\pm$ 1.27
<i>p</i> value	0.0680	0.0283	0.0327	0.0413

Values = means $\pm$ SE. $n = 5, 6$ rats, respectively. *p* values shown are results of unpaired *t* tests between WT and LDLR-KO groups.

(Fig. 6a) and length (Fig. 6b) using the same 5 blood vessel segments were similar. Although noticeable divergence in volumes between users was present (Fig. 6c), both variability and directionality of values were preserved – indicating both robustness and consistency. Our approach also compared favorably to using blood vessel volume estimated as the quotient of blood vessel mass and using a reported aortic density, i.e. $0.86 \text{ mg}/\text{L}$ [12] (Fig. 6d). We think that there is inherently more difficulty in accurately measuring the mass of a wet blood vessel than in using our image-based approach. First, it should be recognized that a short, cleaned segment of mouse aorta weighs approximately $0.1\text{--}0.2 \text{ mg}$, and a high-quality balance capable of such a measurement is costly. Second, much smaller blood vessels, e.g. mesenteric, may be difficult to weigh accurately because of lumen fluid retention that is difficult to remove without rapidly “drying out” the blood vessel segment [13]. Thus, our low-cost approach is more practical and avoids these pitfalls.

We also tested whether a single blood vessel segment would yield different x-sec areas along its length and whether this was dependent on user evaluation. Although variation in x-sec area was apparent, it appeared less dependent on user and more on image quality and segment location (Fig. 7). These data further emphasize that specific user-based analysis of an image is a less important determinant of x-sec area than is the image quality and blood vessel location (e.g., thoracic aorta tapers proximal to distal). Of course, quality control of image analysis needs to be established in each laboratory adopting this approach. We purposefully used a low cost, highly portable “hobby” or classroom-style digital camera/microscope to emphasize that even rudimentary optics are sufficient to perform blood vessel analysis, yet practice is required.

Fig. 6. Reproducibility of aorta parameter data acquisition: inter-assay variation. **a** Aorta cross-sectional area (mm²) measured in five separate aortas independently by two users (user 1; user 2). **b** Aorta segment length (mm) measured independently by two different users in five separate aortas. **c** Volume (mm³) calculated five times by two users. **d** Aortic volume calculated from image analysis compared with volume derived from segment weight and aorta density (0.86 mg/L). Values = mean $\pm$ SD.

Resiliency to Prolonged Formalin Fixation

Formalin fixation for 1 week (Fig. 8a), 1 month (Fig. 8b), and up to 2 months (Fig. 8c) had little effect on quantified aortic segment volume, so it is feasible to formalin fix a blood vessel and still obtain reliable and robust measurements for use in normalization long after contractility experiments were performed. Additionally, fixed blood vessel segments can be processed, embedded, sectioned, and stained for more refined morphometry and histological staining of the blood vessel wall [14].

Major Advantages and Limitations

The current study describes a novel method for normalization of vascular contractility in vitro, and highlights 4 major advantages: (1) the method is simple and low cost, and thus, accessible worldwide; (2) the method is validated and is reproducible across 2 different species, platforms and users; (3) the method is resilient to chronic storage of blood vessels in formalin; and (4) if adopted, normalized vascular contraction data with a “universal unit” (either mN/mm or mN/mm³) will allow for more across study and across laboratory comparisons, and thus, more efficient use of research resources.

Fig. 7. Intra-aortic variation in the cross-sectional area. A single thoracic aorta segment was cut into five cross-sections (“donut”) and each cross-section was imaged and the area measured by two independent users (user 1; user 2). Values = mean $\pm$ SD.

There are several caveats to this method. This method did not use an optimal stretch (i.e., we used ≈ 1 g [9.81 mN] isometric tension for baseline stretch [3–5, 15]; optimal tension depends on stretch [16]) or identify the mechanisms of change in contractility (e.g., endothelium dysfunction would still need to be tested). Moreover, it is expected that laboratory conditions likely will vary in many ways that meaningfully contribute to real differences in contractility (e.g., buffer composition, loading tension, temperature, inhibitors, denuding, etc...), but “all things being equal,” this approach provides a common denominator, which currently is not used by the vast majority of laboratories. As indicated above, the method reduces variability in raw values by correcting for differences that arise as a function of blood vessel segment size. Blood vessel segments may differ in size simply due to randomness or because two different laboratories use different platforms. An obvious limitation is that we only

Fig. 8. Resiliency of aorta parameter data acquisition over time. Comparison of aorta volumes quantified after formalin fixation of the blood vessel. **a** Aorta volume measured 1 week after formalin fixation. **b** Aorta volume measured 1 month after formalin fixation. **c** Aorta volume measured 2 months after formalin fixation. Values = mean $\pm$ SD.

tested the protocol using aortas (albeit both from mice and rats); however, the method will likely work in other blood vessels because there is an inherent reliance of contraction on vascular smooth muscle that makes up the bulk of the media in aorta and all other arteries as well [14]. In any case, we predict that normalization (with segment length and/or volume) is superior to using only raw contraction values. In one of our real-world examples, we detected significant changes in aortic contractility with HFD feeding (vs. NC) only when we normalized contractions (Fig. 3). This outcome has value because importantly it reduces the overall cost of a study by reducing the number of animals needed in a study. Finally, our approach was found to be robust, consistent, reproducible, and resilient. It is recommended that isometric contractions of isolated aorta be normalized to either blood vessel segment length (mN/mm) and/or volume (mN/mm³) to

both improve quantitative assessment of contractility and foster across study comparisons. In future studies, this normalization method can be applied in other blood vessels as and when validated for each vascular bed.

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Disclosure Statement

No potential conflicts of interest relevant to this article were reported.

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