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Research Article

Quantitative Analysis of the Relationship between Blood Vessel Wall Constituents and Viscoelastic Properties: Dynamic Biomechanical and Structural *In Vitro* Studies in Aorta and Carotid Arteries

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The purposes of this work were to perform in sheep a quantification of the elastic, viscous, and inertial moduli obtained in carotid and aortic artery segments during *in vitro* dynamic studies that mimic the normal circulatory function; a quantitative determination of collagen, elastin, and vascular smooth muscle of the carotid and aortic segments analyzed *in vitro*; the correlation between the amounts of each arterial wall constituent and the viscoelastic properties. To this end, nine healthy sheep were included. One artery was selected from each animal to evaluate its biomechanical properties: (a) in three sheep the ascending aorta, (b) in three the thoracic descending aorta, and (c) in the remaining three the proximal segments of the carotid artery. Each selected artery was instrumented with pressure and diameter sensors. After excision, a small ring-shaped sample was set apart from each segment for histological analysis. In conclusion, (a) the arterial compliance showed a positive association with the absolute and relative amount of the parietal elastin, and (b) arterial viscosity was positively associated with the relative amount of smooth muscle, and this association was increased when the correlation was calculated considering the amount of collagen as well as the amount of smooth muscle.

1. Introduction

The relationship between the structure and the function of the tissues of the arterial wall has been analyzed for more than 50 years, being the work of Alan Burton one of the most important among those that focused the attention on this basic subject [1]. Indeed, the structural bases for the static and dynamic mechanical properties of the vascular wall have been extensively studied both in *in vitro* experiments [2] and in *in vivo* experimental animals [3]. Furthermore, the influence of the specific constituents of the arterial wall on the mechanical properties of the vessel has been investigated, focusing the analyses on determining their role in the elasticity of arteries [4].

The aforementioned structure/function vascular studies evaluated both the parietal constituents and the physiology of the vessel, using the standardized methods and techniques available, to estimate (a) the amount of elastin, smooth muscle, and collagen and (b) the viscoelasticity of the vascular wall. Since the technology and methodologies that ensure accurate measurements are continuously in evolution, it is very important to focus on this important fact. For instance, at the time in which Wolinsky and Glagov described the structural basis for the static mechanical properties of the aortic tunica media, nonaccurate measurements were performed to evaluate the amount of elastin, collagen and smooth muscle [2]. The study described the arterial wall constituents

mentioning that *interlamellar fine fibrils showed no consistent pattern of orientation*, pointing out that the collagen fibers *tended to be parallel to one another* and that *in general, orientation of medial smooth muscle followed that of interlamellar elastin* [2]. As can be observed, there is no real quantification of elastin, smooth muscle, or collagen, but instead only qualitative observations about their appearance are described. At present, there are reliable techniques that allow the accurate quantification of the amount of each arterial wall constituent, as described by Kawasaki et al. [5].

The improvement of the techniques that allowed obtaining reliable measurements of the amount of arterial wall constituents has been accompanied by a similar evolution in the methods to calculate arterial wall functional indexes and in the data acquisition and storage techniques. In this sense, our group has developed several methodologies that allow estimating the elastic, viscous, and inertial moduli [6, 7].

At present, hypertension, atherosclerosis, and cardiac failure are public health issues that affect a great number of patients, as at the time when the mentioned authors performed their studies [2, 3]. Furthermore, there are therapeutic interventions where the relevance of the role of vascular smooth muscle has been identified as a determinant of the arterial wall response to treatment. More specifically, intraaortic counterpulsation, a technique developed to treat acute heart failure, produces smooth muscle-dependent changes in the aortic wall [8, 9]. This is a demonstration that further studies would be necessary to understand the interrelationship between functional parameters and accurate estimations of the arterial wall constituents.

The purposes of this work were to perform, in sheep, (1) a quantification of the elastic (E), viscous (η), and inertial (M) moduli obtained in carotid and aortic artery segments during *in vitro* dynamic studies that mimic the normal circulatory function, (2) a quantitative determination of the amounts of collagen, elastin, and vascular smooth muscle of the carotid and aortic segments analyzed *in vitro*, and (3) the correlation between the amounts of each constituent of the arterial wall and their viscoelastic properties.

2. Methods

In this study, nine healthy male Corriedale sheep, weighing 25–35 kg and aged between 12 and 16 months, were included. The protocol was approved by the Research and Development Council of the participant institutions and was conducted in accordance with the National Institutes of Health Guidelines for the care and use of laboratory animals (U.S.N.R. Council, Guide for the Care and Use of Laboratory Animals, Washington, DC: National Academy Press, 1996).

All animals were vaccinated and treated for parasites by a specialized veterinary team. During the 20 days prior to the experimental surgeries, the sheep were appropriately fed and their healthy clinical status controlled. Each animal fasted the night before surgery. A general anaesthesia was induced using 20 $mg \cdot kg^{-1}$ of intravenous sodium thiopental and maintained with 1% halothane, administrated through a Bain tube connected to a ventilator (Neumovent 910; Tecme S.A., Cordoba, Argentina). A pulse oximeter was used in all animals (Pulse Oximeter 515A, Novametrix Medical Systems Inc., Wallingford, USA) in order to monitor respiratory parameters. Respiratory rate and tidal volume were checked and maintained among physiological ranges: arterial pCO_2 at 35–45 mmHg, pH at 7.35–7.4, and pO_2 above 80 mmHg.

During anesthesia, in each animal, an artery was selected to evaluate its biomechanical properties: (a) in three sheep the thoracic ascending aorta, (b) in three sheep the thoracic descending aorta, and (c) in three sheep proximal segments of the carotid artery. Segments from straight parts of the selected arteries were obtained, allowing to evaluate segments with cylindrical shape. Two suture stitches were used to delimitate *in vivo* a 6 cm length arterial segment, accurately measured with a caliper. All procedures were identical to those used in previous works [10].

Each segment was instrumented with pressure and diameter sensors, positioned before the excision. A solid-state pressure microtransducer (Model P2.5, 1200 Hz frequency response; Konigsberg Instruments, Inc., Pasadena, CA, USA) was inserted in each arterial segment; additionally, two miniature piezoelectric crystal transducers (5 MHz, 2 mm in diameter) were positioned on opposite sides of each selected vessel and sutured to the adventitia in order to measure the external artery diameter. The optimal location of the microcrystals was assessed by an oscilloscope (model 465B, Tektronix, Beaverton, OR, USA). The transit time of the ultrasonic signal was converted to distance using a sonomicrometer (1000 Hz frequency response, Triton Technology Inc., San Diego, CA, USA). Both pressure and diameter signals were digitalized using an AD converter, acquired using a Data Acquisition Board (PCI 1200, National Instruments, Austin, TX, USA) and stored in a PC (Pentium 4, 2.6 GHz) using a program developed in our laboratory in LabView 5.1.1 language (National Instruments, Austin, TX, USA). This allowed obtaining 20 to 30 beats in steady-state conditions, which were sampled at a frequency of 200 Hz. This instrumentation allowed *in vivo* instantaneous measuring of the external diameter of the vessels and their corresponding intraluminal pressure, which were monitored on the screen of the computer. The pressure transducer was calibrated using a mercury manometer. The diameter measurement instruments were calibrated using the internal calibration system of the sonomicrometer.

After *in vivo* confirmation of the adequate quality of the diameter and pressure signals, each animal was sacrificed with an intravenous overdose of sodium thiopental, followed by potassium chloride. The instrumented segments were excised and nontraumatically mounted at the same *in vivo* length, in an *in vitro* setup previously used and reported by our group [11, 12]. A small ring-shaped sample was set apart from each segment for posterior histological analysis.

The *in vitro* setup consisted of a polyethylene perfusion line, variable flow resistances and a Windkessel chamber, powered by a pneumatic pump (Jarvik Model 5, Kolff Medical Inc.). Each segment was placed in the chamber and left to attain equilibrium during 10 minutes under steady flow (approximately 450 $mL \cdot min^{-1}$), stretching rate (108

beats·min^{-1} = 1.8 Hz), and mean pressure (approximately 85 mmHg) conditions. Flow stability was monitored using an ultrasonic flowmeter (Model T206, Transonic Systems Inc., Ithaca, NY). During the *in vitro* experiments, each arterial segment was kept immersed and perfused with thermally regulated (37°C) and oxygenated Tyrode's solution (pH = 7.4) [11, 12].

In vitro pressure and diameter measurements were obtained in the instrumented segments as described earlier for *in vivo* conditions. The pneumatic pump, Windkessel chamber, and flow resistances were finely adjusted to reproduce the *in vivo* wave morphology, enabling adequate isobaric, isoflow, and isofrequency analysis.

2.1. Data Analysis. The pressure-diameter or stress-strain relationship of the arterial wall is nonlinear; this is due to the different mechanical behaviors of each constituent of the vessel. The continuous loading and unloading of the arterial wall presents a characteristic pathway or pressure-diameter loop. When physiological adjustments are triggered, variations of the arterial loading determine a dynamic response of the arterial wall, which at small loads is linear, and whose slope increases at higher load levels [6].

A model developed by our group allows assessing the elastic response (i.e., developed pressure (P) or stress (σ) and consequent strain (ε)) of *elastin* (σ_E), *collagen* (σ_C), and *smooth muscle* (σ_{SM}) fibers and the *viscous* (σ_η) and *inertial* (σ_M) behavior of the aortic wall and has been extensively used [6]. The mentioned model allows characterizing the mechanical behavior of the different wall constituents and can be represented by the following equation:

$$\sigma = \sigma_E + \sigma_C + \sigma_{SM} + \sigma_\eta + \sigma_M. \quad (1)$$

The pressure-diameter (P-D) or stress-strain (σ-ε) relationship can be described more specifically by the following equation:

$$\sigma = E_E \cdot (\varepsilon - \varepsilon_{0E}) + E_C \cdot f_C \cdot \varepsilon + E_{SM} \cdot f_A \cdot \varepsilon + \eta \cdot \frac{d\varepsilon}{dt} + M \cdot \frac{d^2\varepsilon}{dt^2}, \quad (2)$$

where ε_{0E} is strain at zero stress; E_E, E_C, and E_{SM} are the elastic moduli of elastin, collagen, and the maximally contracted vascular smooth muscle, respectively, f_C is the *recruitment function* of collagen fibers, f_A is an *activation function* for the smooth muscle fibers, η is the viscous modulus, and M is the inertial modulus [6]. The stress was calculated considering the arterial wall thickness, according to the previously described procedure [6].

The P-D or σ-ε relationship encloses an area. This represents the existence of viscous and inertial component. To assess the purely elastic stress-strain relationship (i.e., considering only the elastic modulus E), the inertial modulus is initially considered equal to zero (see (1)), and then, by increasing the values of the viscous modulus, the hysteresis area can be reduced to a minimum maintaining the clockwise rotation of the loop. This allows obtaining the viscous modulus (i.e., the proposed viscous value that minimizes the hysteresis loop); a similar procedure is used to quantify the inertial modulus [6]. In this work, arterial compliance was calculated as $1/E$.

2.2. Histological Studies. The ring-shaped samples (n = 9) previously set apart from each artery segment (ascending aorta, thoracic descending aorta, and proximal carotid artery) were fixed by immersion in buffered 10% formaldehyde and embedded in paraffin, which later allowed obtaining 7 microns-thick sections, cut perpendicularly to the longitudinal axis of the artery. Five 7 microns-thick sections were analyzed for each artery. The samples were then deparaffinized and hydrated and stained following the Cajal-Gallego staining method. This method allows differential staining, in the same section, of the muscular component (yellow-green), elastin (deep red), and collagen (light blue). The images obtained from each sample were digitized on 630 × 1024 pixel frames using an optical microscope (eyepiece lens (10x) × objective lens (40x) = total magnification (400x)). To quantify the relative amount of each arterial wall component, the images were analyzed using the procedure described by Kawasaki et al. [5]. See Figures 1, 2, and 3. Using digital filters, the pixels not belonging to vascular tissues were removed from the images. Later, the amount of pixels for each individual component (elastin, collagen, and muscle) was determined, as well as the total amount of pixels. The relative amounts of the components are calculated as the percent proportion between the amount of pixels representing each coloring and the total amount of pixels. For a particular arterial segment, its properties values were the average of five 7 microns-thick sections.

The absolute amounts of each component can be determined from the relative indexes. The sectional area is calculated as the difference between the total vascular area (π·external radius^2) and the luminal area (π·internal radius^2) [6]. The total amount of each observed component (elastin, collagen and smooth muscle), in mm^2, is derived from the relative amount (%) and the total sectional area.

2.3. Statistical Analysis. Values reported are expressed as mean ± standard deviation (MV ± SD). To compare dynamic *in vitro* and structural data, a one-way analysis of variance (ANOVA) followed by Bonferroni test was used. Regression analysis was performed in order to detect association between biomechanical and histological data. A $P < 0.05$ value was adopted as statistically significant. Statistical analyses were performed with SPSS software (version 18.0, Statistical Package for the Social Sciences).

3. Results

Table 1 shows the hemodynamic parameters obtained during *in vitro* studies of the nine vessels analyzed. The isobaric condition can be verified and diameter values, as expected, showed significative differences among them ($P < 0.05$). The frequency and the intraluminal flow were the same as mentioned in Section 2.

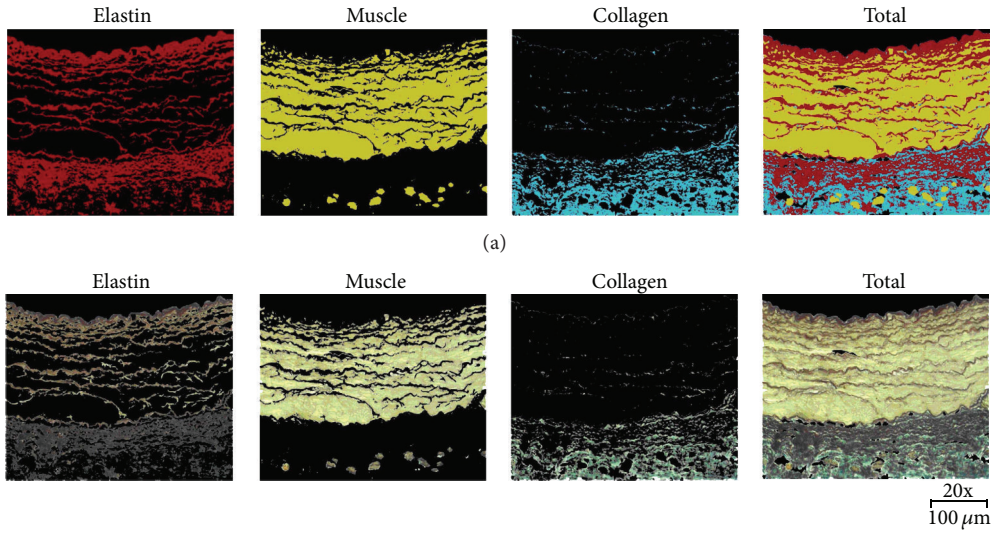


Figure 1: Illustration of the histological analysis processing stages in an ovine carotid artery. (b) Characteristic colors of the histological preparations, from left to right: elastin, smooth muscle, collagen, and the original image, where the three integrated constituents can be visualized throughout the arterial wall. (a) Similar discrimination with assigned fictional colors that allow a clearer visualization of the location of each wall constituent. Note the thick layer of smooth muscle in the tunica media and the disposition of collagen in the tunica adventitia.

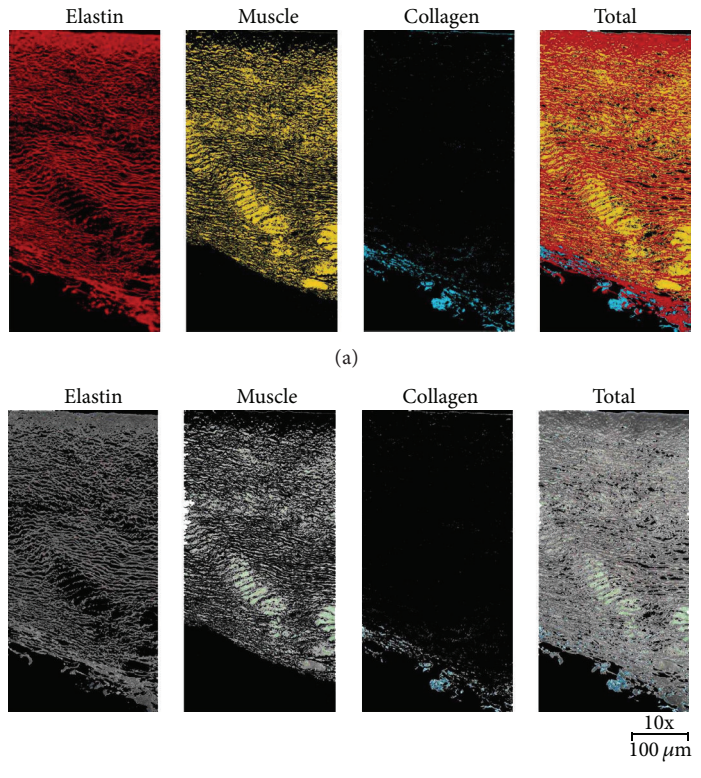


Figure 2: Illustration of the histological analysis processing stages in an ovine ascending aorta. (b) Characteristic colors of the histological preparations, from left to right: elastin, smooth muscle, collagen, and the original image, where the three integrated constituents can be visualized throughout the arterial wall. (a) Similar discrimination with assigned fictional colors that allow a clearer visualization of the location of each wall constituent. Note the high amount of elastin in the tunica media.

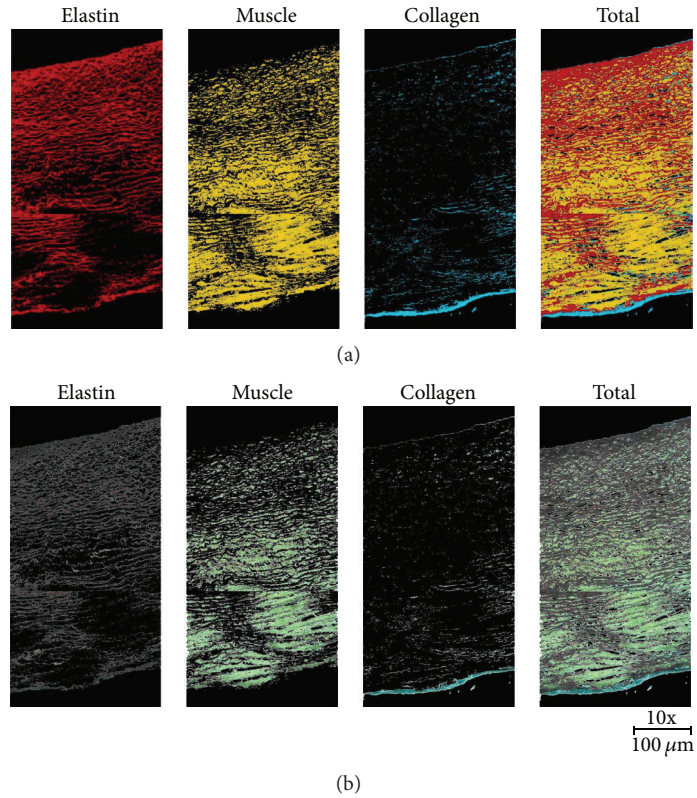


Figure 3: Illustration of the histological analysis processing stages in an ovine descending aorta. (b) Characteristic colors of the histological preparations, from left to right: elastin, smooth muscle, collagen, and the original image, where the three integrated constituents can be visualized throughout the arterial wall. (a) Similar discrimination with assigned fictional colors that allow a clearer visualization of the location of each wall constituent. Note the increase in the amount of smooth muscle and collagen with respect to the ascending aorta (Figure 3).

Table 1: Hemodynamic parameters.

	Systolic pressure (mmHg)	Diastolic pressure (mmHg)	Pulse pressure (mmHg)	Mean pressure (mmHg)	Mean diameter (mm)
Arteries					
Carotid	134.3 ± 5.6	61.1 ± 4.3	73.2 ± 5.0	85.5 ± 5.0	6.38 ± 0.32
Ascending aorta	135.8 ± 4.6	67.1 ± 4.3	68.7 ± 5.0	90.0 ± 4.0	22.40 ± 1.15[a]
Descending aorta	138.1 ± 4.6	63.9 ± 5.4	74.2 ± 4.5	88.7 ± 4.7	15.81 ± 0.97[a,b]

[a] $P < 0.05$ with respect to carotid artery.
[b] $P < 0.05$ with respect to ascending aorta.

The *mechanical characterization* of the arterial wall, performed using three indexes, shows that compliance was higher in ascending than in descending aorta ($P < 0.05$) and the lowest value was observed in carotid artery ($P < 0.05$). The viscous index at the level of the ascending aorta was lower than that observed in the descending aorta ($P < 0.05$), while the maximum value of the arterial wall viscosity was found in the carotid artery ($P < 0.05$). Finally, inertial values calculated for the carotid artery were the highest ($P < 0.05$) (see Table 2).

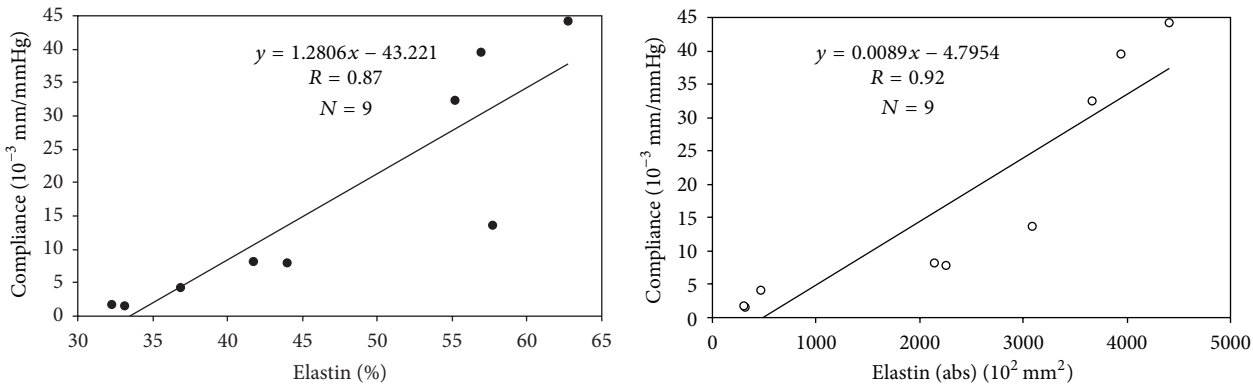
The *histological analyses* show that the relative values of smooth muscle and collagen measured in the descending aorta were higher than those observed in the ascending aortic artery ($P < 0.05$), while the relative amounts of elastin and elastic tissue were lower ($P < 0.05$). Carotid arteries show the highest relative values of collagen with respect to the ascending and descending aorta, while the relative elastin values were the lowest with respect to the both mentioned aortic segments ($P < 0.05$). Finally, the relative amount of smooth muscle of the carotid artery was higher than that observed in the ascending aorta ($P < 0.05$), and the relative amount of elastic tissue was higher in the ascending aorta than in the carotid artery ($P < 0.05$).

The absolute value of elastin observed in the descending aorta was lower than that measured in the ascending aorta ($P < 0.05$). The absolute values of smooth muscle, elastin, and collagen measured in carotid artery were lower than those observed in the descending aorta ($P < 0.05$), while only the

Table 2: Biomechanical parameters.

	Compliance (10^{-3} mm/mmHg)	Viscosity (mmHg·s/mm)	Inertia (10^{-2} mmHg·s^2/mm)
Carotid artery	1.79 ± 0.05	26.84 ± 1.17	20.77 ± 1.86
Ascending aorta	38.20 ± 4.37[a]	1.30 ± 0.40[a]	1.25 ± 0.57[a]
Descending aorta	7.54 ± 0.49[a,b]	5.71 ± 0.89[a,b]	6.57 ± 1.64[a]

[a] $P < 0.05$ with respect to carotid artery.
[b] $P < 0.05$ with respect to ascending aorta.



(a) (b)

Figure 4: Association between the relative amounts of elastin and the arterial wall compliance (a) and between the absolute amount of elastin and the parietal compliance (b).

smooth muscle and elastin absolute values were lower than those obtained in ascending aorta ($P < 0.05$).

The *correlation analysis* shows that the arterial compliance exhibits a positive association with the absolute and relative amount of the arterial wall elastin ($P < 0.05$). See Figure 4. However, arterial wall viscosity was correlated with the relative amount of arterial smooth muscle ($P < 0.05$). Furthermore, this association was increased when the mentioned correlation was performed including collagen to the amount of smooth muscle ($P < 0.05$). See Figure 5.

4. Discussion

To the best of our knowledge, this is the first work that specifically characterizes the relationship between arterial wall viscosity and the arterial wall constituents; furthermore, the analyses performed utilized a reliable index previously validated [6] that was correlated with accurate measurements of the amounts of smooth muscle and collagen in the arterial wall [5]. Previous reports, such as the one authored by Gow and Taylor [13], reported data of arterial wall viscosity derived from a meticulous study of harmonics but did not discriminate the isolated contribution of elastin, collagen, and vascular smooth muscle. Consequently, no correlations such as those reported in our study were obtained.

Arterial wall viscosity was evaluated by the first time by Hardung in 1953; however, until present, it is not considered a standardized index as useful as the well-known vascular elasticity index [14]. In the mentioned study, in coincidence with our experiments, the evaluated vessel was the aortic artery. From thereon, several studies were published, calculating, in different vessels, the characteristic value of the arterial wall viscosity [6, 15]. In a work of Armentano et al., it was clearly demonstrated that parietal viscosity of human carotid and femoral arteries was altered in a uniform manner in hypertensive patients [15].

Experimental studies in spontaneous hypertensive rats constitute a traditional way of performing analyses of the effects of the sustained increase of systemic arterial blood pressure. However, at present, the etiology of the most frequent cause of systemic hypertension is unknown. Arribas et al., in 2010, demonstrated that the enhanced survival of vascular smooth muscle cells was responsible for the accumulation of elastin in the arterial wall, and that this abnormal growth determines the subsequent development of systemic hypertension [16]. If we take into account the high correlation between the relative amount of smooth muscle and the arterial wall viscosity found in systemic arteries included in our study, future research could be focused in the analysis of arterial wall dynamics (including viscosity).

As was mentioned in our study, the calculated values of arterial wall viscosity increase from the aortic root towards the descending aorta. This is probably due to the increases of the relative amounts of collagen and smooth muscle; it is important to highlight that the latter represents only 15% of the dry weight of the proximal aorta. Furthermore, the elastin/collagen relationship is always larger than 1 in the thoracic aorta, and the inverse at abdominal level [17]. On the

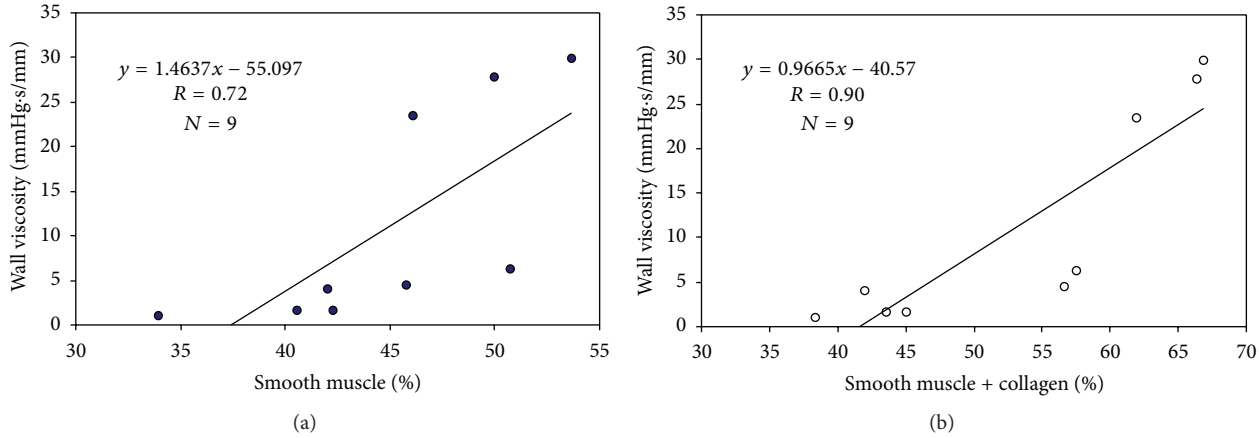


Figure 5: Association between the relative amounts of smooth muscle and the parietal viscosity (a) and between smooth muscle and collagen and arterial wall viscosity (b).

Table 3: Structural parameters obtained from histological analyses.

	Carotid artery	Ascending aorta	Descending aorta
	MV ± SD	MV ± SD	MV ± SD
Smooth muscle (%)	49.69 ± 3.56	39.34 ± 4.62[a]	46.43 ± 4.05[b]
Elastin (%)	34.46 ± 2.50	57.84 ± 3.52[a]	47.52 ± 8.76[ab]
Collagen (%)	15.82 ± 1.63	3.16 ± 2.17[a]	5.61 ± 5.10[ab]
Elastic tissues (%)	50.28 ± 3.52	61.00 ± 5.19[a]	53.13 ± 4.72[b]
Smooth muscle (abs) (10^2 mm^2)	537.03 ± 38.43	2891.67 ± 339.87[a]	2452.20 ± 213.83[a]
Elastin (abs) (10^2 mm^2)	372.38 ± 27.00	4252.04 ± 258.92[a]	2509.79 ± 462.79[ab]
Collagen (abs) (10^2 mm^2)	171.00 ± 17.64	232.19 ± 59.53	296.27 ± 69.33[a]

Relative (%) and absolute (abs) values of the arterial wall constituents evaluated through histological techniques. Elastic tissue values are the sum of the elastin and collagen amounts.

[a] $P < 0.05$ with respect to carotid artery.

[b] $P < 0.05$ with respect to ascending aorta.

other hand, arterial compliance values decrease distally from ascending to descending aorta. Furthermore, in our work, the evaluation of the arterial wall elasticity showed a significative correlation with the amount of elastin contained in the arterial wall, confirming previous reports that have characterized the contribution of elastin and collagen to the abdominal aorta stiffness in *in vivo* studies [18, 19].

The proportional analysis of the amounts of arterial wall constituents allows minimizing the effects of the vessel diameter differences observed mainly between aortic segments and carotid artery. Curiously, a large standard deviation was observed between ascending and descending aortic segments (Table 3).

The mock-circulation loop utilized in this work has been largely validated by our group [11, 12] and ensures isofrequency, isobaric, and isoflow analyses of vascular segments; perhaps the main limitations in this sense are the impossibility to perform isodiameter experiments when the segments are obtained from different territories of the arterial tree. Data analysis takes into account direct measurements of instantaneous pressure and diameter values, obtaining an instantaneous loop that enables to consider all x-y points of each cardiac cycle. This analysis allows obtaining a complete characterization of the arterial wall mechanical behavior, which involves quantifying the three moduli: elastic, viscous, and intertial [6]. About the animal model, in the evaluation of cardiac and vascular biomechanical properties and/or in anatomical/structural investigations, the use of large domestic animals (i.e., pig, cows, and sheep) instead of small ones (i.e., rats, and mouse) has shown to be more appropriate, as large animals incorporate anatomical and physiological characteristics more closely resembling those observed in humans. For instance, the similarities allow for the development of models where devices (i.e., left ventricle mechanical assistance devices and intraaortic balloon pumping) are implanted in a site intended for clinical use (i.e., site-specific) and allow for a more accurate analysis of safety and clinical efficacy [6–10]. In particular, the sheep is considered a suitable model for cardiovascular research due to anatomical and physiological characteristics [20]. About this, cardiopulmonary anatomy is similar to that of humans. Additionally, physiological parameters (i.e., heart rate, blood pressure, cardiac index, and intracardiac pressures) have been assessed in both anesthetized and conscious sheep, and many

hemodynamic and metabolic variables have shown to be similar to those of other large mammals. In particular, it has been shown that sheep physiological parameters often approximate those of humans, especially parameters related with thrombogenicity [20]. In contrast, swine tend to be exquisitely sensitive to exogenous anticoagulation therapy in an unpredictable fashion. On the other hand, sheep grow at a rate comparable to human growth, which allows for an adequate comparative analysis. In contrast, larger ruminants such as cattle and large variety of swine experience rapid and prolonged growth until reaching a large adult size. Such rapid growth can cause difficulties in experimental protocols (i.e., size matching between prosthetic valves and native valve annulus, resulting in paravalvular leaks and functional valve stenosis) [20]. In addition to the anatomical and functional advantages described above, earlier are docile animals, another factor that makes them suitable for acute and chronic cardiovascular research studies.

We conclude that (a) arterial wall compliance exhibited a positive association with the absolute and relative amounts of parietal elastin, and (b) arterial viscosity was positively associated with the relative amount of arterial smooth muscle, and this association was increased when the mentioned correlation was performed including collagen to the amount of smooth muscle.

Conflict of Interests

The authors have no conflict of interests.

Acknowledgments

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