Thoracic aorta thickness and histological changes with aging: an experimental rat model

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The three main components of the media layer of the aorta are elastic fibers, collagen fibers and smooth muscle cells (SMC). This layer’s elastic properties are major determinants of its biomechanics in health and disease states. Age-related changes in such elastic properties are associated with altered hemodynamic parameters, such as systolic hypertension and end-organ damage, and are thought to result from changes of its main components.

Most elastic fibers in the media layer are incorporated into concentric layers called lamellae, while collagen, proteoglycans, SMC and other elastic fibers are contained in the interlamellar spaces. Since elastin synthesis is negligible after the neonatal period in mammals, elastic fibers are lifelong exposed to the factors associated with aging process. Media layer collagen, on the other side, is synthesized by fibroblasts, and its content increases with age. Parallel to such changes in extracellular matrix, age-related media layer smooth muscle cell senescence has been recognized along with a reduction in media layer SMC count.

This animal-based study has been conceived as a rat biological model of normal aortic arterial wall aging. It has been focused in the histologic phenomena of aging, to allow for a simultaneous morphoquantitative evaluation of all aforementioned major components of the arterial media layer thought to be involved in age-related changes in vascular structure and function, that is, elastic and collagen fibers and SMC, along with a morphometric evaluation of arterial wall thickness, throughout the aging process.

Our research was approved by Ethics Committee for Research with Animals of Federal University of Paraná (protocol number 23075.031142/2013–73, certificate number 732). Study animals were 60 male Wistar rats (Rattus norvegicus albinus), maintained under a light-dark cycle of 12 h and controlled temperature (22°C) and receiving water and food (standard pellet diet; Nuvilab-Nuvital®, Curitiba, Brazil) ad libitum until they were sacrificed. Rats were divided in six even groups, kept in even conditions until the age each group was ascribed for the time of the sacrifice for the study (Table 1).

Animals underwent euthanasia by intraperitoneal injection of a solution of Ketamine and Xylazine. Thereafter, a 1 cm long segment was dissected from the descending part of the thoracic aorta immediately below the aortic arch. Segments’ dimensions were assessed using a digital caliper rule (Digimess®).

Next, aortic segments were fixated in Bouin’s solution (picric acid, 40% formaldehyde and acetic acid) for 18 h, and then dehydrated in a decrescendo sequence of xylene and ethanol. Such material was then included in Paraplast®, and each rat provided material for three histological laminae.

Table 1. Number of rats per group and life time.

| Group | n  | Age   |
|-------|----|-------|
| 1     | 12 | 3 months |
| 2     | 12 | 6 months |
| 3     | 12 | 9 months |
| 4     | 12 | 18 months |
| 5     | 12 | 24 months |

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each with 10 slices 5 μm thick. The three laminae were stained, respectively, with Hematoxin & Eosin (HE), Masson’s trichrome, and Weigert’s elastin stain.

For histological analysis, an Olympus® BX50 microscope with acquisition camen DP71 3CCD pro series was used. Images were exported to a Sony Trinitron® colored screen and scanned by an Oculus TCX (Coreco®) scanner. The software for image acquisition was MetaSystems® VSViewer 2.1.102.

The same software was employed for morphometric analysis. Intima, media and adventitia layer thicknesses were measured in laminae stained by HE, five measurements per layer per rat on alternate slices, and the average thickness of each layer of each rat was registered in micrometers.

For morphoquantitative analysis, media layer snapshots were taken as follows: for smooth muscle cell count, five snapshots with 0.0080 mm² each, in the same relative position of alternate slices, in each HE stained lamina; for collagen quantification, five snapshots with 0.0100 mm² each, in the same relative positions of alternate slices, in each Masson’s trichrome stained lamina; for elastic fiber quantification, 10 snapshots with 0.0042 mm² each, in 10 standard positions around media layer’s circumference, in each Weigert stained lamina.

Morphoquantitative data were obtained from each snapshot as follows. For SMC, visual count of nuclei was performed, and result registered as number of nuclei per 0.0080 mm² area. For elastic and collagen fiber quantification, one parted from the principle that elastin is stained purple by Weigert, while collagen is stained blue by Masson’s trichrome, therefore the proportion of the area of each snapshot occupied by these colors was determined using the chrome, therefore the proportion of the area of each snapshot occupied by these colors was determined using the software Media Cybernetics® Image-Pro Plus 6.0 for Windows®. Such color area quantification was performed using HSI histogram based color segmentation, after color equalization by the same software. Elastin’s purple color on Weigert stained laminae was empirically determined to be in the HSI range (0–255, 255–255, 0–255), while collagen’s blue color on Masson’s trichrome stained laminae was empirically defined to be in the HSI range (60–180, 70–255, 0–255), and empty spaces in the HSI range (10–255, 0–40, 220–255). Colors falling out of these ranges corresponded to the remaining components of the vessel’s media layer.

The areas occupied by each of these color ranges were measured by the software, and the quantification of collagen and elastic fibers was expressed by the percentage of the field area stained for each of them: elastic fibers = 100 × elastic fibers area/(elastic fibers area + collagen area + remaining components area) and collagen = 100 × collagen area/(collagen area + remaining components area). This procedure was performed for each snapshot, and the average of each animal was considered for statistical analysis.

Images with artifacts were excluded from the analysis. If the entire lamina contained artifacts, the rat was not evaluated for the variable correspondent to that lamina, but could be evaluated for other variables if images exempt from artifacts were present in other laminae.

Data were analyzed with the software IBM® SPSS Statistics v.20. Results of the studied variables were described by mean, median, minimum and maximum values and standard deviations. For comparisons between all study groups, ANOVA model with one factor was used, and for multiple post-hoc comparisons the LSD test was used. Normal distribution of the variables was determined using the Kolmogorov-Smirnov test. Statistical significance was indicated by $P$-values $< 0.05$.

All study animals remained alive for the lifetime ascribed for each of them. Two animals from group 18 months were found to have tumors by the time of the tissue harvest, not affecting any aspects of the research’s methodology. Number of subjects ($n$) per group differed from 12 in individual analyses due to the presence of artifacts compromising the analysis of histological specimens. Notably, the entire 18 months group was excluded from elastic fiber analysis, and the entire 24 months group from collagen analysis for this reason.

Media layer thickness had a statistically significant increase with age (Table 2), and comparisons between individual groups are presented on a separate table (Table 3).

SMC count and elastic fiber concentration decreased, whereas collagen concentration had a tendency to increase that did not reach statistical significance (Tables 4 and 5).

Our study has built a model of aortic arterial wall aging in rats that allowed us to both describe the increment in media layer thickness that occurs during normal aging, and quantitatively assess the changes in elastic and collagen fibers and SMC, namely a decrease in elastic fibers and SMC content. As relative elastic fiber content decreased while media layer thickness increased, such thickening is supposed to have been at the expense of increased collagen

| Group  | $n$ | Media layer thickness, μm | Mean  | SD  | $P$-value |
|--------|-----|---------------------------|-------|-----|-----------|
| 3 mo   | 11  | 89.67                     | 12.09 |     |           |
| 6 mo   | 12  | 101.70                    | 13.42 |     |           |
| 9 mo   | 12  | 106.38                    | 14.02 |     |           |
| 18 mo  | 10  | 121.42                    | 18.81 |     |           |
| 24 mo  | 12  | 114.36                    | 17.47 |     | < 0.001   |
fiber content (i.e., fibrosis), a trend that was observed in our results, though without reaching statistical significance.

From the quantitative point of view, although the total content of elastic fibers in elastic lamellae remain constant throughout life,[14] its concentration reduces, as a result of increased collagen synthesis between lamellae,[14] and almost complete substitution of elastic fibers by collagen and proteoglycans in the interlamellar space.[15] From a qualitative standpoint, phenomena as altered aminoacid crosslink,[16] glyco-oxidative reaction,[17,18] fragmentation,[19–21] increase matrix metalloproteinases (MMP) activity,[15,19] and calcification have been observed.[22]

Media layer elastic module has two components: elastic fibers account for the first component, physiological, with a flatter stress-strain relationship, while collagen fibers account for the second component, whose stress-strain relationship is steeper.[23] Age-related elastic fiber changes[17,18] are thought to account for loss of its elastic properties[16] and, as a consequence, transmitting of hemodynamic forces to underlying, stiffer,[24] media layer collagen fibers,[25] resulting in increased blood vessel stiffness.[11,26,27]

Collagen fiber staining involved more steps than other employed staining, thus increasing the possibility of artifact insertion, such that more laminae had to be excluded from the analysis due to presence of artifacts, thus possibly lowering the sample power to detect a significant change in collagen fiber content between groups. Moreover, Masson’s trichrome, the stain we utilized in our analysis, is less specific, for quantitative purposes, than Picrosirius’ red stain,[28–30] which has been successfully employed in other study[31] to quantitatively assess collagen content in aortic media layer and make comparisons between groups.

Recent data propose a possible link between loss of elastin concentration and integrity to smooth cell growth signaling.[32,33] This link has been suggested based on findings that normal elastin interacts with growth factors in the paracellular level in the aortic media layer.[34–36] Others researchers have found that aortic media layer SMC themselves become stiffer with aging, raising the possibility of a direct contribution of the cellular component of the media layer to arterial stiffening besides stiffening of the extracellular matrix.[12] Media layer cellularity, which is made up predominantly by SMC, was found to decrease with age[13] though the mechanism for this remains to be elucidated. Importantly, SMC dysfunction has a potential pathogenic role in atherosclerosis and aneurysm formation.[37]

In the present study, rats have been raised up to the age of 24 months, which has been shown to parallel human 60-year age.[38] Therefore, although our data can be regarded as reflecting the aging process through adulthood to old age, but not necessarily its continuation from that point on. On the other side, since we found statistical significance, our results may signal to possible future studies that that age is enough time to wait for aging changes in rats.

The present study described age-related histological alterations in the thoracic aorta in an experimental model with rats. Extracellular matrix increases were observed, leading to layer thickening. This increase seems to be driven by the collagen content, resulting in decreased elastic fiber concentration and smooth muscle cell count.

Table 3. Comparison of media layer thickness between individual groups.

| Compared groups       | Media layer thickness | P-value |
|-----------------------|-----------------------|---------|
| 3 mo × 6 mo           | 0.065                 |         |
| 3 mo × 9 mo           | 0.012                 |         |
| 3 mo × 18 mo          | < 0.001               |         |
| 3 mo × 24 mo          | < 0.001               |         |
| 6 mo × 9 mo           | 0.457                 |         |
| 6 mo × 18 mo          | 0.004                 |         |
| 6 mo × 24 mo          | 0.048                 |         |
| 9 mo × 18 mo          | 0.026                 |         |
| 9 mo × 24 mo          | 0.206                 |         |
| 18 mo × 24 mo         | 0.286                 |         |

SMC count is presented as number of cells per 0.0080 mm² field. Elastic fiber and collagen quantification is given as the proportion of the sample field occupied by each component. SMC: smooth muscle cell.

Table 4. Results of SMC count, elastic fiber and collagen quantitative analysis.

| Group | n  | Mean | SD | P-value | n  | Mean | SD | P-value | n  | Mean | SD | P-value |
|-------|----|------|----|---------|----|------|----|---------|----|------|----|---------|
| 3 mo  | 12 | 32.1 | 3.4|         | 10 | 62.03| 6.04|         | 12 | 19.06| 7.63|         |
| 6 mo  | 12 | 28.5 | 4.1|         | 9  | 57.89| 9.04|         | 12 | 27.35| 10.55|         |
| 9 mo  | 12 | 27.6 | 3.7|         | 10 | 53.26| 4.48|         | 12 | 21.63| 8.70|         |
| 18 mo | 12 | 31.3 | 6.0|         | 11 | 46.28| 6.67|         | 0  | -    | -  |         |
| 24 mo | 12 | 20.9 | 4.4| < 0.001 | 0  | -    | -  | < 0.001 | 12 | 26.57| 6.91| 0.068   |

SMC count is presented as number of cells per 0.0080 mm² field. Elastic fiber and collagen quantification is given as the proportion of the sample field occupied by each component. SMC: smooth muscle cell.
Table 5. Comparisons of SMC count and elastic fiber proportion between individual groups.

| Compared groups | SMC P-value | Elastic fibers P-value |
|-----------------|-------------|------------------------|
| 3 mo × 6 mo     | 0.057       | 0.185                  |
| 3 mo × 9 mo     | 0.017       | 0.006                  |
| 3 mo × 18 mo    | 0.672       | < 0.001                |
| 3 mo × 24 mo    | < 0.001     |                        |
| 6 mo × 9 mo     | 0.612       | 0.140                  |
| 6 mo × 18 mo    | 0.134       | < 0.001                |
| 6 mo × 24 mo    | < 0.001     |                        |
| 9 mo × 18 mo    | 0.047       | 0.022                  |
| 9 mo × 24 mo    | < 0.001     |                        |
| 18 mo × 24 mo   | < 0.001     |                        |

SMC: smooth muscle cell.

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References

1. Mescher AL. The circulatory system. In Junqueira’s Basic Histology; Mescher, AL, Ed, 13 edition; McGraw Hill: New York, USA, 2013.
2. Baldwin AK, Simpson A, Steer R, et al. Elastic fibres in health and disease. Expert Rev Mol Med 2013; 15: e8.
3. Chung J, Lachapelle K, Cartier R, et al. Loss of mechanical directional dependency of the ascending aorta with severe medial degeneration. Cardiovasc Pathol 2017; 26: 45–50.
4. Kaess BM, Rong J, Larson MG, et al. Aortic stiffness, blood pressure progression, and incident hypertension. J Am Med Assoc 2012; 308: 875–881.
5. Weisbrod RM, Shiang T, Al Sayah L, et al. Arterial stiffening precedes systolic hypertension in diet-induced obesity. Hypertension 2013; 62: 1105–1110.
6. Maroules CD, Khera A, Ayers C, et al. Cardiovascular outcome associations among cardiovascular magnetic resonance measures of arterial stiffness: the Dallas heart study. J Cardiovasc Magn Reson 2014; 16: 33.
7. Laurent S, Boutourlie P, Asmar R, et al. Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. Hypertension 2001; 37: 1236–1241.
8. Lyle AN, Raaz Y. Killing me unsoftly: causes and mechanisms of arterial stiffness. Arterioscler Thromb Vasc Biol 2017; 37: c1–c11.
9. O’Connell MK, Murthy S, Phan S, et al. The three-dimensional micro- and nanostructure of the aortic medial lamellar unit measured using 3D confocal and electron microscopy imaging. Matrix Biol 2008; 27: 171–181.
10. Godfrey M, Nejezchleb PA, Schaefer GB, et al. Elastin and fibrillin mRNA and protein levels in the ontology of normal human aorta. Connect Tissue Res 1993; 29: 61–69.
11. Tsamis A, Krawiec JT, Vorp DA. Elastin and collagen fibre microstructure of the human aorta in ageing and disease: a review. J R Soc Interface 2013; 10: 20121004.
12. Qiu H, Zhu Y, Sun Z, et al. Short communication: vascular smooth muscle cell stiffness as a mechanism for increased aortic stiffness with aging. Circ Res 2010; 107: 615–619.
13. Collins JA, Munoz JV, Patel TR, et al. The anatomy of the aging aorta. Clin Anat 2014; 27: 463–466.
14. Spina M, Garbisa S, Himrie J, et al. Age-related changes in composition and mechanical properties of the tunica media of the upper thoracic human aorta. Arterioscler 1983; 3: 64–76.
15. Fritze O, Romero B, Schleicher M, et al. Age-related changes in the elastic tissue of the human aorta. J Vasc Res 2012; 49: 77–86.
16. Watanabe M, Sawai T, Nagura H, Suyama K. Age-related alteration of cross-linking amino acids of elastin in human aorta. Tohoku J Exp Med 1996; 180: 115–130.
17. Sawabe M. Vascular aging: from molecular mechanism to clinical significance. Geriatr Gerontol Int 2010; 10 (Suppl 1): S213–S220.
18. Konova E, Baydani S, Atanasova M, Velkova A. Age-related changes in the glycation of human aortic elastin. Exp Gerontol 2004; 39: 249–254.
19. Greenwald SE. Ageing of the conduit arteries. J Pathol 2007; 211: 157–172.
20. Toda T, Tsuda N, Nishimori I, et al. Morphometrical analysis of the aging process in human arteries and aorta. Acta Anat (Basel) 1980; 106: 35–44.
21. Avolio A, Jones D, Tafazzoli-Shadpour M. Quantification of alterations in structure and function of elastin in the arterial media. Hypertension 1998; 32:170–175.
22. Bielak LF, Turner ST, Franklin SS, et al. Age-dependent associations between blood pressure and coronary artery calcification in asymptomatic adults. J Hypertens 2004; 22: 719–725.
23. Kohn JC, Lampi MC, Reinhart-King CA. Age-related vascular stiffening: causes and consequences. Front Genet 2015; 6: 112.
24. Kobielarz M, Chwilikowska A, Turek A, et al. Influence of selective digestion of elastin and collagen on mechanical properties of human aortas. Acta Bioeng Biomech 2015; 17: 55–62.
25. Chow MJ, Turcotte R, Lin CP, Zhang Y. Arterial extracellular matrix: a mechanobiological study of the contributions and interactions of elastin and collagen. Biophys J 2014; 106: 2684–2692.
26. Ninomiya OH, Tavares Monteiro JA, Higuchi Mde L, et al. Biomechanical properties and microstructural analysis of the human nonaneurysmal aorta as a function of age, gender and location: an autopsy study. J Vasc Res 2015; 52: 257–264.
Aronson D. Cross-linking of glycated collagen in the pathogenesis of arterial and myocardial stiffening of aging and diabetes. *J Hypertens* 2003; 21: 3–12.

Drifka CR, Loeffler AG, Mathewson K, et al. Comparison of Picrosirius red staining with second harmonic generation imaging for the quantification of clinically relevant collagen fiber features in histopathology Samples. *J Histochem Cytochem* 2016; 64: 519–529.

Street JM, Souza AC, Alvarez-Prats A, et al. Automated quantification of renal fibrosis with Sirius Red and polarization contrast microscopy. *Physiol Rep* 2014; 2: e12088.

Wegner KA, Keikhosravi A, Eliceiri KW, Vezina CM. Fluorescence of picrosirius red multiplexed with immunohistochemistry for the quantitative assessment of collagen in tissue sections. *J Histochem Cytochem* 2017; 65: 479–490.

Wheeler JB, Mukherjee R, Stroud RE, et al. Relation of murine thoracic aortic structural and cellular changes with aging to passive and active mechanical properties. *J Am Heart Assoc* 2015; 4: e001744.

Li DY, Brooke B, Davis EC, et al. Elastin is an essential determinant of arterial morphogenesis. *Nature* 1998; 393: 276–280.

Wagenseil JE, Ciliberto CH, Knutsen RH, et al. The importance of elastin to aortic development in mice. *Am J Physiol Heart Circ Physiol* 2010; 299: H257–H264.

Lannoy M, Slove S, Jacob MP. The function of elastic fibers in the arteries: Beyond elasticity. *Pathologie Biologie* 2014; 62: 79–83.

Karnik SK, Brooke BS, Bayes-Genis A, et al. A critical role for elastin signaling in vascular morphogenesis and disease. *Development and Disease* 2003; 130: 411–423.

Horiguchi M, Ota M, Rifkin DB. Matrix control of transforming growth factor-b function. *J Biochem* 2012; 152: 321–329.

Albinsson S, Sward K. Targeting smooth muscle micrornas for therapeutic benefit in vascular disease. *Pharmacol Res* 2013; 75: 28–36.

Andreollo NA, Santos EF, Araujo MR, Lopes LR. Rat’s age versus human’s age: what is the relationship? *Arq Bras Cir Dig* 2012; 25: 49–51.