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Orientation, anisotropy, clustering, and volume fraction of smooth muscle cells within the wall of porcine abdominal aorta

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Abstract

We analyzed tissue samples of the normal porcine abdominal aorta using stereological assessment of histological sections through the tunica media layer. The results demonstrated that the local volume fraction of smooth muscle cells within tunica media does not differ among samples taken round the circumference of the artery, and that volume fraction can be assessed in sections stained with green trichrome as well as with immunohistochemistry against actin. The distribution of angles between the long axes of nuclei of the smooth muscle cells and the radial direction was different from normal. The profiles of smooth muscle cells were distributed in an isotropic, but an inhomogeneous manner.

Keywords: Aorta, arterial wall, microscopy, stereology

1. Introduction

Constitutive models of the arterial wall are either based on phenomenological approach, or take structural information of the underlying histology into account (see e.g. [6] for a thorough review of modelling of arterial layers). The leading idea of this paper was to support the latter strategy with use of modern stereological tools.

1.1. Microscopic design of the tunica media of arterial wall

An idealized healthy elastic artery is composed of three layers: tunica intima, media, and adventitia. From the histomechanical point of view, the most important layer is the tunica media. The basic architecture of the fibrous and cellular components of tunica media of large arteries consists of repeating concentric and spiral layers of smooth muscle cells (SMC) separated by complex network of collagen and elastic fibres, the latter forming interconnected fenestrated sheets, or lamellae (fig. 1). The number of lamellar units in a vascular segment is related linearly to tensional forces within the wall, with the greatest number of elastic layers occurring in the larger, more proximal vessels that experience the highest wall stress [5]. When the vessel wall is forming, SMC differentiation, lamellar number, and elastin content increase coordinately with the gradual rise in blood pressure until the proper number of lamellar units are organized. It has been suggested that the artery wall is designed to distribute uniformly the tensile stresses

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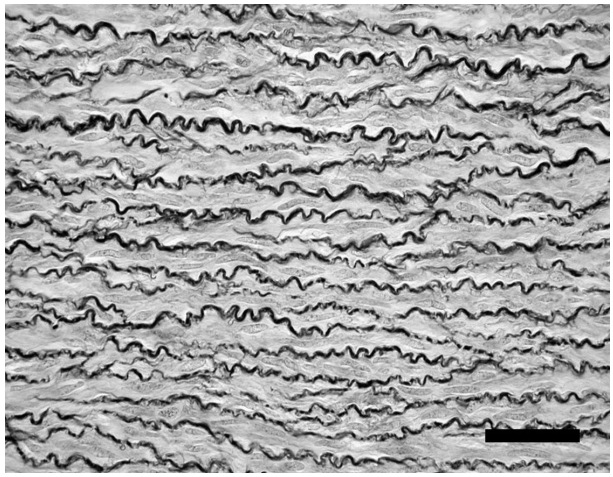

Fig. 1. Lamellar units within the wall of porcine aorta consist of repeating concentric layers of smooth muscle cells, elastin and collagen fibres. Bar 50 μm.

to which it is subjected. The basic morphological plan occurs in various modifications. Larger mammals have larger arteries with proportionately more elastin layers, as proved by morphometric studies discussed by Shadwick [11]. Diameter and wall thickness increase in nearly constant proportion and the lamellar thickness remains constant at approximately 15 μm. The number of lamellar units increases in direct proportion to the radius and wall thickness. This led to the conclusion that the elastin-muscle-collagen lamella is the basic structural and functional unit of the aorta. Further refinement of this model based on scanning electron microscopy showed that the elastic tissue between concentric layers of circumferentially oriented smooth muscle cells actually consists of two layers of elastin fibres, each associated with adjacent muscle layers and containing interposed bundles of wavy collagen fibres. While there are no apparent connections between fibres of elastin and collagen, both appear to be linked to the membranes of adjacent muscle cells, from which they are synthesized [11].

In computational modelling of the tunica media, the collagen fibres [4] as well as the smooth muscle cells [8] often assumed to be arranged in symmetrical helices. For description of elastin, which is probably the most spatially complex constituent of the media, several methods have been suggested (e.g., [12, 13, 14, 15]). However, quantitative data published on the real microscopic morphology of the tunica media are really scarce.

1.2. Quantitative light microscopic detection of smooth muscle cells

The first step in quantitative assessment of smooth muscle cells is the visualization of these cells. Smooth muscle cells can be detected in histological sections with use of either overall staining methods, or with use of more specific affinity-histochemical labelling methods (mainly immunohistochemistry or lectin histochemistry).

As the vascular SMC occurs together with connective tissue elements, it can be easily distinguished from the surrounding connective tissue with use of trichrome stains. These widely used methods apply two or more acid dyes of contrasting colours to selectively colour different basic tissue components, i.e. SMC in contrast to collagen. In green trichrome method [9], the plasma of SMC stains reddish with a acid fuchsin, which is of intermediate molecular weight.

At first, acid fuchsin is applied for long enough to stain all the tissue, but after differentiation with a polyacid (phospho-wolfram acid), only the cytoplasm of SMC and other cells remains red, as the large molecule of the polyacid removes the plasma stain from the collagen. The collagen fibres are then stained with a light or naphtol green with a molecular weight larger than the plasma stain. Although this routine stain is cheap, efficient and offers a good contrast between SMC and collagen, it is not specific for the SMC.

A more specific, but also a more expensive visualization of vascular SMC is based on detection of either cytoplasmic intermediate muscle filaments (desmin) or cytoplasmic microfilaments (α-smooth muscle actin). These multi-step immunohistochemical methods localize proteins (e.g., desmin, or α-smooth muscle actin) in cells of a tissue section, using the principle of primary antibodies binding specifically to antigens. The localization of the primary antibody in the tissue section is commonly detected e.g. with an biotinylated secondary antibody conjugated to a reporter enzyme (e.g., a peroxidase), which reacts with another solution to produce a brown staining wherever primary and secondary antibodies are attached. The intensity of staining correlates with the amount of the protein of interest (for details, see [1]).

However, it remains unclear, whether the possible difference between the cheap and fast trichrome methods and the expensive immunohistochemical method is significant.

1.3. Aim of the study was to test the following hypotheses:

H_A — The volume fraction of smooth muscle cells within tunica media is the same in four segments systematically sampled in a circumferential direction round the tunica media.

H_B — The volume fraction of smooth muscle cells within tunica media in sections stained with green trichrome is the same as in sections labelled immunohistochemically against α-smooth muscle actin.

H_C — The distribution of angles between the long axes of nuclei profiles of smooth muscle cells visible in transversal section through the tunica media, and the radial direction, is a normal distribution.

H_D — The profiles of smooth muscle cells in longitudinal sections through the tunica media are distributed in an isotropic manner in the plane of the two-dimensional section and can be modelled as isotropic rather than anisotropic network.

H_E — The centres of profiles of smooth muscle cells in longitudinal sections through the tunica media are not arranged in non-random clusters.

2. Material and methods

2.1. Histological processing

The formalin-fixed sample of aorta was processed with a common paraffin technique. The sample was divided into two halves, one of them with the cutting plane perpendicular to the long axis of the aorta, whereas the second one was sectioned as parallel to this axis. Some of the 5 μm-thick sections were stained with Verhoeff's hematoxylin and green trichrome [9] or with the overall hematoxylin and eosin staining. Other sections were processed immunohistochemically for α-smooth muscle actin detection. The sections were incubated with a monoclonal mouse anti-human antibody (clone 1A4, dilution $1 : 150$; Dako, CA, USA) for 12 hours at 4 °C. As stated in the manufacturer's declaration, the antibody cross-reacts with the alpha-smooth

muscle actin-equivalent protein. The secondary antibody (45 min, 37 °C) and avidin-biotin peroxidase complex (45 min, 37 °C) were applied, using the Novostain Super ABC Universal Kit (Novocastra Laboratories Ltd., GB). Following immunohistochemistry, the background tissue was stained with Gill's haematoxylin (30 s, Bio-Optica, Italy).

2.2. Volume fraction of smooth muscle cells

We used the stereological point-grid method allowing a reliable estimation of volume fraction of smooth muscle cells V_V(SMC, media) within the tunica media, see Equation 1:

$$V_V(SMC, media) = \frac{estV(SMC)}{estV(media)} \cdot 100\ (\%), \tag{1}$$

where $estV(SMC)$ was the volume of smooth muscle cells within the reference volume of corresponding segment of the tunica media $estV(media)$. As the quantification was based on a series of micrographs taken with medium-power magnification, it was performed repeatedly in four stacks of micrographs sampled in a systematic uniform random manner round the circumference of the aortic media (fig. 2).

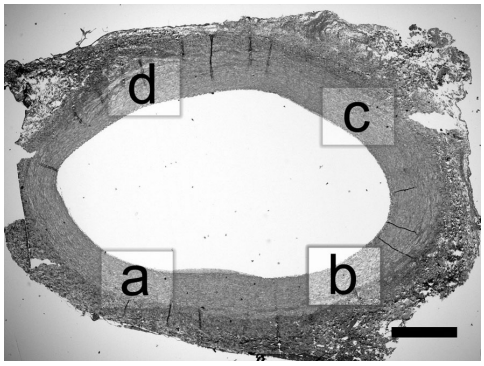

Fig. 2. From each section, four micrographs were analyzed. Modified green trichrome, scale bar 1 mm

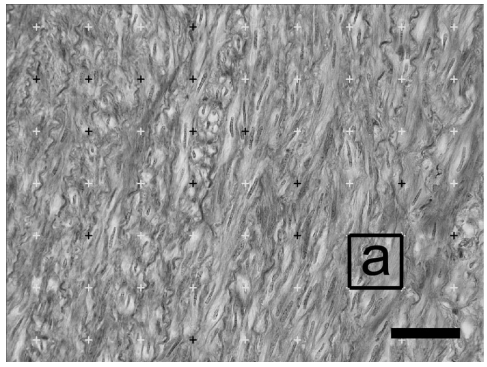

Fig. 3. Intersections of the testing point grid with smooth muscle cell profiles. The probability to hit the cells was proportional to their area fraction. Modified green trichrome, scale bar 50 μm

The area of profiles of smooth muscle cells was estimated in each section, see Equation 2:

$$estA = a \cdot P, \tag{2}$$

where $estA$ was the estimated area, grid parameter a was the area corresponding to one test point and P was the number of test points hitting the SMC profiles (fig. 3). The Cavalieri principle was used for the estimation of the volume V of the SMC within the reference segment of the tunica media, see Equation 3:

$$estV = T \cdot (A_1 + A_2 + \ldots + A_m), \tag{3}$$

where $estV$ was the Cavalieri volume estimator, $T = 0.045$ mm was the distance between the two following selected sections, and A_i was the area of the SMC in the i-th section of the relevant stack. In accordance with a previously published pilot study performed in porcine

abdominal aorta [12, 13], we evaluated twelve sections as representatives of each of the four aortic segments (stacks), i.e. $m = 12$ [7]. Stereological analysis was done with the PointGrid module of the Ellipse3D software (ViDiTo, Košice, Slovakia). The total number of micrographs analyzed was 96, one half of them being stained with the trichrome method, the other half being processed with immunohistochemistry. The total number of points counted was at least 200 in all sections of each stack.

2.3. Two-dimensional orientation of profiles of nuclei of smooth muscle cells

Smooth muscle cells are spindle shaped and the long axes of their oval nuclei run parallel to the long axis of the muscle cells. Unlike the contours of individual muscle cells, the nuclei are easy to be visualized, e.g. with the hematoxylin stain. Using the Orientation module of the Ellipse3D software, the profiles of nuclei ($n = 528$) were marked in seven micrographs and the angle between the long axes of these nuclei and the radial direction, i.e., across the profile of tunica media (fig. 4) was assessed (fig. 5–6). The microscopic stage was rotated to guarantee the orientation of the micrograph in compliance with the coordinate system described in fig. 5. According to our experience in testing the orientation of the linear grid of an objective micrometer, the total measuring error of both procedures involved (i.e. precise orientation of the micrographs and drawing the lines over the nuclear profiles) did not exceed $\pm\ 1.5\,^{\circ}$. Due to the lamellar structure, the orientation of smooth muscle cells and their nuclei was a fair estimate of the orientation of the extracellular fibres of connective tissue.

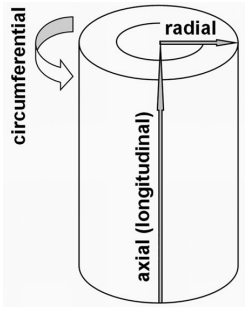

Fig. 4. Circumferential, radial, and axial directions of an artery

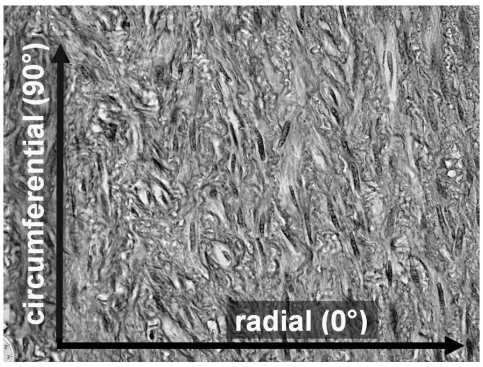

Fig. 5. Transversal section through tunica media with the orientation of the micrograph of tunica media with respect to the blood vessel

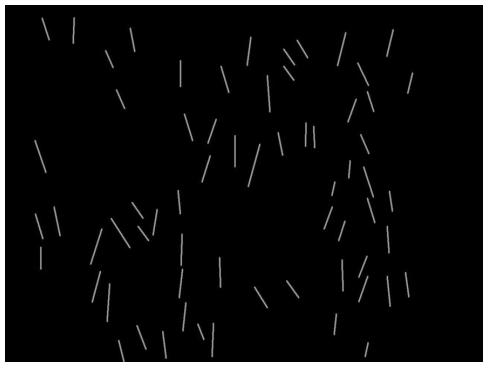

Fig. 6. Lines marking the orientation of nuclei of smooth muscle cells in a micrograph of tunica media oriented according to the fig. 5

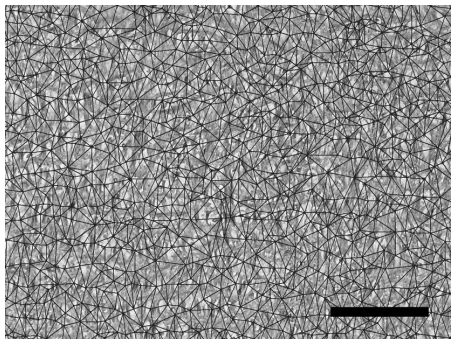

Fig. 7. The centres of smooth muscle cell profiles were marked and the resulting points were interconnected (triangulated). Modified green trichrome, scale bar 50 μm

2.4. Two-dimensional anisotropy of smooth muscle cell profiles

The anisotropy of two-dimensional distribution of centres of SMC profiles ($n = 15\,257$) visible in six longitudinal sections through the tunica media was assessed with the Delaunay triangulation (DT) [3] calculated by Qhull software [2]. DT for a set $\boldsymbol{P}$ of points in the plane is a triangulation DT($\boldsymbol{P}$) such that no point in $\boldsymbol{P}$ is inside the circumcircle of any triangle (i.e., the circle which passes through all three vertices of a triangle) in DT($\boldsymbol{P}$). DT($\boldsymbol{P}$) maximizes the minimum angle of all the angles of the triangles in the triangulation (fig. 7). For this purpose the positions of SMC profiles were identified with the centres of the SMC profiles visible in the micrographs. The points were interconnected (triangulated), and the parameters of model grids (square grid, isosceles and equilateral triangles) were computed in order to reveal any possible differences between the interpoint distances of the SMC profiles in the radial and circumferential direction. Such a difference would have revealed the presence of layers of SMC within the tunica media.

The number of SMC profiles per area unit was estimated with the LocalizeInWall plugin of the Ellipse3D software. We estimated the number of SMC profiles ($n = 9\,751$) per area unit in three virtual "layers" delineated arbitrarily over three longitudinal sections through the tunica media. We sampled 9 micrographs from each layer in a systematic uniform random manner, i.e. 27 micrographs in total (total area of 10.044 mm^2). A difference among these three layers would have suggested that parts of the tunica media closer to the arterial lumen would have had different density of SMC profiles than those adjacent to the tunica adventitia.

2.5. Two-dimensional clustering of smooth muscle cell profiles

For assessment of the distribution of smooth muscle cells, we identified the clusters of the SMC profiles (local increase of density) and we tested their randomness. A suitable method has been already published [10], based on the analysis of paired correlation (clustering) function implemented in the module Gold of the Ellipse3D software. In order to prove that the observed deviations from total randomness were significant, values of confidence intervals of the paired correlation function were calculated by method based on the Monte Carlo simulations. We tested the randomness of spatial patterns of smooth muscle cell profiles (Makro Makro $n = 20\,068$) in six longitudinal histological sections, describing the distribution of interpoint distances among the centres of the profiles. The testing procedure was performed for intervals $\langle 0; 300\rangle$, $\langle 50; 540\rangle$, $\langle 100; 540\rangle$, $\langle 200; 540\rangle$, $\langle 250; 540\rangle$, and $\langle 300; 540\rangle$ μm including increments

of the lower endpoint of 50 μm within the first interval of $\langle 0; 300\rangle$ μm (i.e., $\langle 0; 50\rangle$, $\langle 0; 100\rangle$, $\langle 0; 150\rangle$, $\langle 0; 200\rangle$, $\langle 0; 250\rangle$, and $\langle 0; 300\rangle$). The presence of non-random clusters would have been useful for assessment of inhomogeneity of the tunica media consisting of SMC embedded in fibrillar and amorphous extracellular matrix.

2.6. Statistics

The data were processed with the Statistica Base 7.1 (StatSoft, Inc., Tulsa, OK, USA). The Shapiro-Wilk W test was used in testing for normality. The Levene test and the Brown-Forsythe test were used to assess the homogeneity of the variances in the repeated observations, which was an assumption for the parametric T-test as well as for ANOVA. In the normal probability plot used for assessment of the normality of orientation of nuclei of SMC, standardized values of the normal distribution (plotted on the y-axis) were based on the assumption that the data come from a normal distribution. The test results were considered significant at $p < 0.05$

3. Results

3.1. Volume fraction of smooth muscle cells

The quantification was based on transversal sections perpendicular to the longitudinal axis (fig. 8). In trichrome-stained samples, the V_V(SMC, media) was 64.5 ± 8.76 % (mean$\pm$standard deviation). In α-smooth muscle-stained sections, the value was 69.0 ± 5.32 %. Statistical evaluation of the difference between these two groups is presented in fig. 12. Analysis of variance among the four stacks of micrographs captured within the four segments of tunica media is given in fig. 14 for the trichrome stain, and in fig. 15 for the actin immunohistochemistry. We rejected neither the H_A nor the H_B hypothesis.

3.2. Two-dimensional orientation of profiles of nuclei of smooth muscle cells

The orientation was assessed in 528 profiles of nuclei of smooth muscle cells found in 7 transversal sections. The angle between the long axes of the profiles of the nuclei and the radial direction ranged from $45\,^\circ$ to $135\,^\circ$ (fig. 9). In more than in 99% of the nuclei, the angle ranged

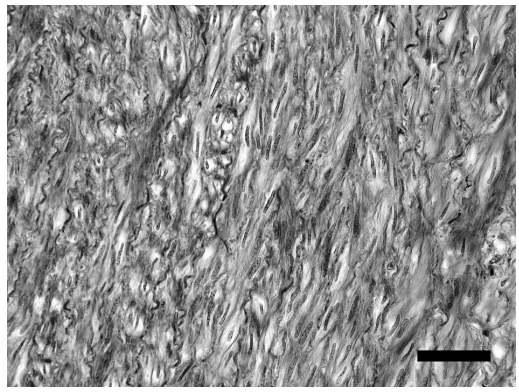

Fig. 8. Smooth muscle cells stained with modified green trichrome method, transversal section through tunica media. Scale bar 50 μm

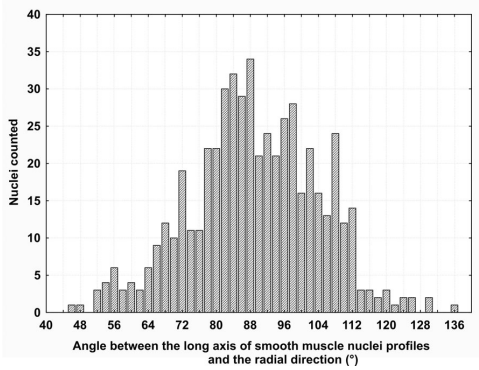

Fig. 9. Distribution of the angle between the long axes of smooth muscle cells and the radial direction

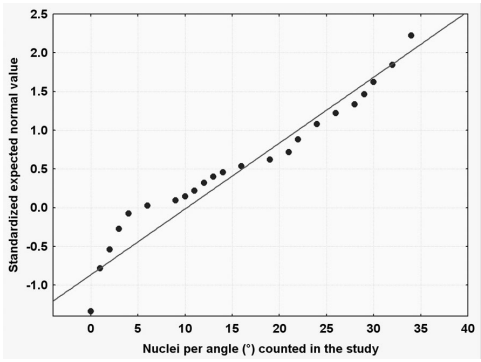

Fig. 10. Normal probability plot with standardized values of the rank ordered deviations from the mean (residuals, on the y-axis) plotted against observed values (on the x-axis). The Shapiro-Wilk $p < 0.001$.

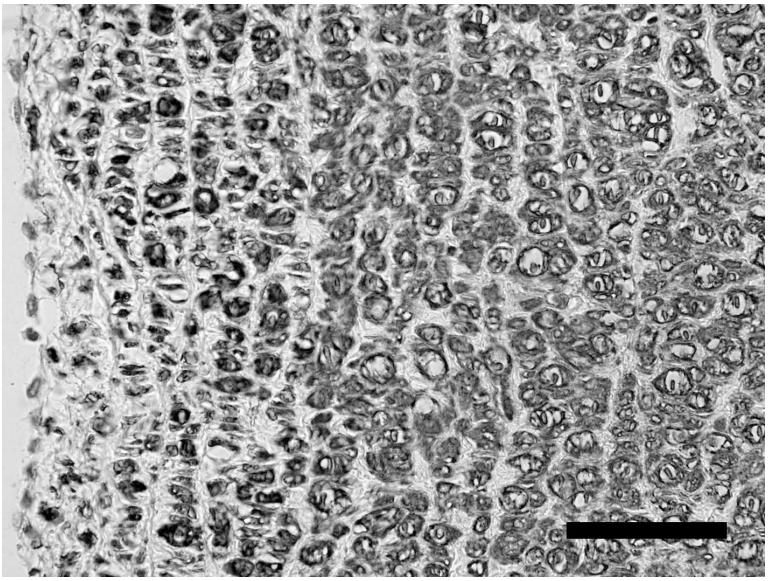

Fig. 11. α-smooth muscle actin stained with immunohistochemistry, longitudinal section through tunica media. Scale bar 50 μm

from $52\,^\circ$ to $125\,^\circ$, i.e. within the interval $\langle -38; 35\rangle\,^\circ$ with regard to the circumferential direction. As demonstrated in fig. 9–10, the distribution of the values was not normal (Shapiro-Wilk W test, $p < 0.001$). We rejected the H_C hypothesis.

3.3. Two-dimensional anisotropy of smooth muscle cell profiles

Assessment of anisotropy and clustering was based on longitudinal sections (fig. 11). Delaunay triangulation of centres of smooth muscle cell profiles ($n = 15\,257$) did not reveal any anisotropy in arrangement of SMC profiles. The assessment of the orientation and length of

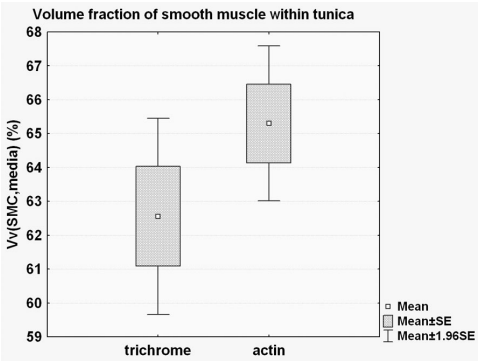

Fig. 12. Comparison of two histological methods used for identification of smooth muscle cells. The T-test did not prove significant difference ($p = 0.15$) between the trichrome stain and α-smooth muscle immunohistochemistry. In both sets of micrographs ($n = 48$), the values had a normal distribution (Shapiro-Wilk W $p = 0.12$ and $p = 0.23$, respectively) and their variances were homogeneous (Levene $p = 0.10$, Brown-Forsythe $p = 0.10$)

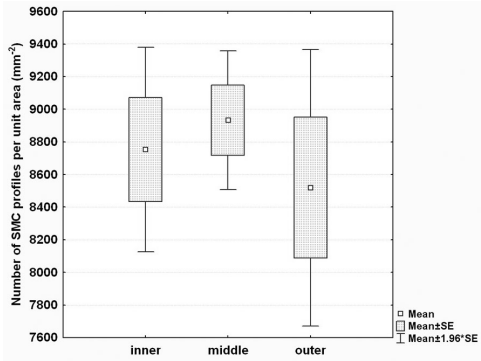

Fig. 13. Comparison of the number of smooth muscle cell profiles per unit area observed in micrographs ($n = 27$) of the inner, the middle, and the outer third of the thickness of arterial wall (longitudinal sections). The ANOVA did not prove significant difference ($p = 0.68$) among these virtual layers. The values in all sets of micrographs had a normal distribution (Shapiro-Wilk W $p = 0.28 - 0.91$) and their variances were homogeneous (Levene $p = 0.29$, Brown-Forsythe $p = 0.35$)

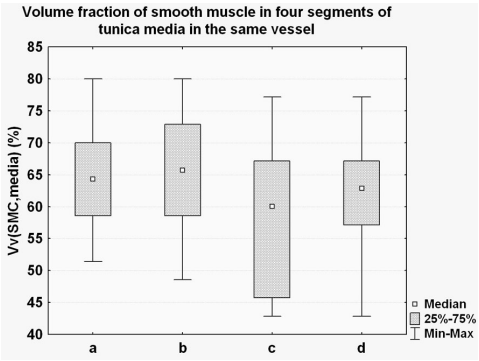

Fig. 14. Analysis of variance among the four segments of trichrome-stained stacks labeled a, b, c, d did not prove significant difference ($p = 0.28$). Values in all four stacks ($n = 12$) had a normal distribution, and their variances were homogeneous (Levene $p = 0.42$, Brown-Forsythe $p = 0.53$)

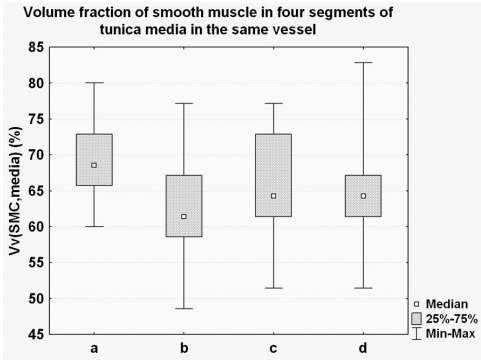

Fig. 15. Analysis of variance among the four segments of α-smooth muscle-stained stacks labeled a, b, c, d did not prove significant difference ($p = 0.20$). Values in all four stacks ($n = 12$) had a normal distribution, and their variances were homogeneous (Levene $p = 0.70$, Brown-Forsythe $p = 0.69$)

the edges was closer to an idealized isotropic network of equilateral triangles rather than to anisotropic network of isosceles triangles. The mean distance of centres of profiles of neighbouring SMC was $19.15 \pm 0.76\,\mu$m, providing that the cells were modelled in vertices of a network formed by equilateral triangles.

The mean number (±standard deviation) of profiles of SMC per unit area of the tunica media was $8\,737.5 \pm 312.8\,\mathrm{mm}^{-2}$ and the differences among the three layers of the tunica media were not significant (fig 13). We did not reject the H_D hypothesis.

3.4. Two-dimensional clustering of smooth muscle cell profiles

We rejected the H_E hypothesis. We proved that the dimensions of non-random clusters ($p < 0.01$) of SMC profiles ($n = 20\,068$) were 50–300 μm.

4. Discussion

Both the overall green trichrome stain and the immunohistochemistry of smooth muscle actin left a small fraction of the SMC cytoplasm unstained. The distribution of the unstained fraction of the cytoplasm was comparable in both stains. The slightly higher value of volume fraction of SMC in tunica media found in sections stained against actin most probably reflects the variability between the adjacent sections stained with the two techniques, as well as the variability among the image fields sampled for the analysis. We proved, that it was not necessary to perform expensive immunohistochemical staining of the smooth muscle cells to estimate their volume fraction. However, this was true in normal specimen only. Quantification of smooth muscle in pathological samples would require more sophisticated histological processing, as the vascular smooth muscle cells often change the expression of contractile microfilaments (e.g., actin), and intermediate filaments (e.g., desmin and vimentin). When modelling tissues under pathological conditions, we should characterize the phenotype and staining properties of the modified smooth muscle cells. Moreover, the rotational symmetry of the microstructure of the tunica media found in this study in samples of healthy aorta would be also probably altered in pathological samples.

As we did not assess the two-dimensional orientation of the profiles of the SMC nuclei separately for the right and for the left side of the artery, it was difficult to interpret the rejection of the H_C hypothesis. The micrographs used for this task represented the whole thickness of the tunica media in a systematic uniform manner. Therefore we suggest that the dominant representation of lower angles between the nuclei and the radial direction was caused by the lower radius of curvature of the nuclei profiles representing the SMC closer to the tunica intima. In the future work, the distribution of angles will be tested against the von Mises (i.e., the circular normal) distribution.

As we can consider the H_D hypothesis to be valid, we claim that an isotropic network of equilateral triangles is a more suitable description for the arrangement of the SMC profiles than anisotropic network of isosceles triangles or rectangles. In other words, the SMC profiles do not form any detectable layers and the average distance of the neighbouring profiles in the radial direction does not differ from their average distance in the circumferential direction. The mean distance of the centres of an idealized isotropic network of SMC profiles (i.e., $19.15 \pm 0.76\,\mu$m) is fairly close to the idealized thickness of the lamellar unit published by [11] (i.e., 15 μm). Whether the SMC profiles are arranged in spirals or in concentric layers, is an interesting question that deserves further study.

Although we did not prove the anisotropy of two-dimensional distribution of centres of individual SMC visible in histological sections, this finding is neither directly related to the possible anisotropy of the arrangement of SMC in the whole three-dimensional wall of aorta, nor to the arrangement of fibres of extracellular matrix (e.g., collagen). It remains unproven,

whether there is a simple relation between the structural anisotropy and anisotropy of mechanical properties of the tissue (which has not been tested in this study). This would have required a three-dimensional study involving all the cardinal tissue constituents (including the elastin and collagen fibres, their associated fibrillar glycoproteins, and the surrounding amorphous matrix), and their contributions to the tissue mechanics.

As we rejected the H_E hypothesis, we suggest that the microscopic structure of tunica media should be considered as statistically inhomogeneous, at least within the intervals listed in the results. However, the H_B, H_C, H_D, and H_E hypotheses were formulated and tested in two-dimensional sections only. As the real constituents of the tunica media are three-dimensional structures, we suggest to test these hypotheses in the three-dimensional space in the future as well.

5. Conclusion

The green trichrome stain is a sufficient method usable for quantification of smooth muscle within the wall of normal porcine aorta. The quantitative results of this study, i.e. the volume fraction of smooth muscle cells, the stochastic description of two-dimensional orientation, anisotropy, and clustering of the smooth muscle cells are suggested to be used for modelling of biomechanical properties of the tunica media of aorta based on the real morphology.

Acknowledgements

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References

[1] J. D. Bancroft, A. Stevens, (Eds), Theory and practice of histological techniques, Churchill Livingstone, New York, 1996.

[2] C. B. Barber, D. P. Dobkin, H. T. Huhdanpaa, The Quickhull Algorithm for Convex Hulls. ACM Transactions on Mathematical Software, (22) (1996) 469–483.

[3] B. Delaunay, Sur la sphere vide, Izvestia Akademii Nauk SSSR, (7) (1934) 793–800.

[4] N. J. Driessen, W. Wilson, C. V. Bouten, F. P. Baaijens, A computational model for collagen fibre remodelling in the arterial wall, Journal of Theoretical Biology (226) (2004) 53–64.

[5] G. Faury, M. Pezet, R. H. Knutsen, W. A. Boyle, S. P. Heximer, S. E. McLean, R. K. Minkes, K. J. Blumer, A. Kovacs, D. P. Kelly, D. Y. Li, B. Starcher, R. P. Mecham, Developmental adaptation of the mouse cardiovascular system to elastin haploinsufficiency, Journal of Clinical Investigation (112) (2003) 1 419–1 428.

[6] T. C. Gasser, R. W. Ogden, G. A. Holzapfel GA, Hyperelastic modelling of arterial layers with distributed collagen fibre orientations, Journal of the Royal Society Interface (3) (2006) 15–35.

[7] H. J. G. Gundersen, E. B. Jensen EB, The efficiency of systematic sampling in stereology and its prediction. Journal of Microscopy (147) (1987) 229–263.

[8] G. A. Holzapfel, Determination of material models for arterial walls from uniaxial extension tests and histological structure, Journal of Theoretical Biology (238) (2006) 290–302.

[9] J. Kočová, Overall staining of connective tissue and the muscular layer of vessels, Folia Morphologica (18) (1970) 293–295.

[10] A. A. Philimonenko, J. Janacek, P. Hozak, Statistical evaluation of colocalization patterns in immunogold labelling experiments, Journal of Structural Biology (132) (2000) 201–210.

[11] R. E. Shadwick, Mechanical design in arteries, Journal of Experimental Biology (202) (1999) 3 305–3 313.

[12] Z. Tonar, S. Nemecek, T. Muzik, Morphology of elastin network in porcine aorta. In: J. Chraponski, J. Cwajna, L. Wojnar (Eds): Proceedings of 9th European Congress on Stereology and Image Analysis and 7th STERMAT International Conference on Stereology and Image Analysis in Materials Science, Vol. II (2005) 368–375.

[13] Z. Tonar, S. Němeček, R. Holota, Microscopic image analysis of elastin and morphometry of wall of thoracic and abdominal porcine aorta, IFMBE proceedings (11) (2005) 1 474–1 479.

[14] Z. Tonar, J. Janáček, R. Poledne, Stereological methods for quantitative description of atherosclerotic lesions in optical microscopy. Cor et Vasa (49) (2007) 95–101.

[15] Z. Tonar, P. Kochová, M. Holeček, J. Janáček, Stereological assessment, mechanical measurement and computer modelling of smooth muscle, Materials Science Forum, (567–568) 353–356.