Acta of Bioengineering and Biomechanics
Vol. 17, No. 2, 2015

Original paper
DOI: 10.5277/ABB-00184-2014-02

Influence of selective digestion of elastin and collagen on mechanical properties of human aortas

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Purpose: There are two families of fibres taking part in the process of mechanical loads transfer, i.e. elastin and collagen fibres. Their number, spatial arrangement and specific properties determine the capacity of a blood vessels to resist mechanical loads resulting from the impact of blood on vessel walls. The purpose of the present paper is to define the load-bearing capacities of elastin and collagen scaffolds equivalent to natural fibre arrangements of human aorta and produced by selective digestion. Methods: Samples of thoracic human aortas were digested by using phosphate buffer of trypsin at pH 8.0 for 22 hours in order to degrade elastin and by autoclaving followed by incubation in 90% formic acid for 22 hours. The efficacy of digestion was assessed immunohistochemically. Mechanical properties of pre-stretched native and digested samples were determined by uniaxial tensile test. Results: Samples subjected to autoclaving have been successfully deprived of both types of collagen and elastin has been intact. Treatment with trypsin caused a removal of elastin and the presence of type I and IV collagen was demonstrated. Digestion of aortic samples either by formic acid or trypsin has resulted significantly decreasing mechanical properties in comparison with native samples. Conclusions: Collagen and elastin scaffold-like stuctures have been effectively produced by selective digestion of thoracic human aorta and their contribution to the load-bearing process was evaluated. Isolated collagen network are more durable and stiffer and less deformable than elastin network, hence are responsible for load-bearing process at higher strain since the range of working of elastin is at lower strain values.

Key words: *collagen, elastin, selective digestion, mechanical properties, human aorta*

1. Introduction

The basic load-bearing structural components of a multilayer aortic wall are elastin and collagen fibres as well as smooth muscle cells. Thanks to their ability to contract and relax, smooth muscle cells are responsible for active mechanical properties of the vessel wall. Elastin and collagen fibres, on the other hand, are responsible for the passive mechanical properties of tissue. The amount, natural spatial arrangement and interaction of both families of load-bearing fibres determine the ability of the blood vessel wall to transfer mechanical loads under condi-

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Received: July 27th, 2014
Accepted for publication: September 15th, 2014

tions of variable blood flow [4], [5]. The natural degradation of elastin and collagen fibres occurs as a result of biochemical cascade reactions accompanying the development of various diseases, including aneurysms and atherosclerosis [19], [24] as well as due to ageing of the organism [20]. Consequently, disturbances occur in the process of transfer of loads through the walls of blood vessels.

Understanding the significance of structural components and defining their contribution to the process of transfer of loads is critical for the description of biomechanics of a blood vessel operating under conditions of variable mechanical loads. So far, three different research concepts have been used to describe the issue in question. Most time has been devoted to the testing of mechanical properties of tissues subjected to prior selective removal of elastin or collagen fibers. Selective digestion for human external iliac arteries was carried out for the first time by Roach and Burton [23]. Currently, there is a large choice of procedures for selective digestion of connective tissues of proven efficacy, with selective removal of elastin still posing particular problems [7], [21]. Another research concept involves chemical fixation of the examined tissue by solutions containing crosslinking agents (i.e., glutaraldehyde and formaldehyde) under mechanical load conditions directly during the strength test [27]. It should be pointed out that crosslinking may influence in various ways the tissue structure [6], [28]. After fixation, the tissues are subjected to standard imaging procedures, such as electron microscopy, histological staining or histo- and immunohistochemical staining. This results in an image of the tissue microstructure fixed at a particular strain value. Hybrid techniques were also used, where during tests of uniaxial and biaxial stretching of unfixed tissues simultaneous observations were conducted on the behaviour of load-bearing components using light microscopes and Raman spectroscopes [10], [12], [22]. The last two concepts, even though they give an important insight into the biomechanics of load transfer at the structural level, do not allow us to specify the mechanical properties of the systems of load-bearing fibres naturally present in blood vessel walls. On the other hand, the technique used in this study, involving selective digestion of collagen or elastin from samples of human thoracic aorta, made it possible to define the mechanical properties of respective spatial arrangements of load-bearing fibres (separately for collagen and elastin fibres), which is the purpose of this work.

2. Material and methods

Research material

The research material consisted of 22 segments of thoracic human aortas without macroscopically visible atherosclerotic lesions, obtained post-mortem at the Department of Forensic Medicine, Wrocław Medical University, from 22 different individuals ranging in age from 28 to 62 years old (average: 48 ± 7 years old). The storage time of the preparations from collection to testing did not exceed 12 hours. The samples (n = 42) were excised from the research material in the circumferential direction relative to the long axis of the vessel. All samples were excised using a puncher, which made it possible to maintain a constant sample width of 5 mm.

Selective digestion

The samples were randomly assigned to age-matched and equinumerous groups. A selective digestion was conducted using trypsin or autoclaving preceded by incubation in formic acid. In order to degrade elastin, the test samples were digested in 0.01 M phosphate buffer saline at pH 8.0 containing 2 mg of trypsin (AppliChem, A3964, 0001) per ml of buffer. The vessels were incubated at 37 °C for 3, 5 and 22 hours. In order to remove collagen from the tissue, segments of arteries were incubated in 90% formic acid at 45 °C for 5, 12 and 22 hours. Then, the vessels were autoclaved four times in distilled water at 1.05 bar pressure and 121 °C for 22 min. The efficacy of digestion was assessed immunohistochemically. Digestion procedures were subjected to optimization (data not presented here) primarily to select the time of tissue exposure to the factor degrading extracellular matrix proteins. Therefore, a 22 hour incubation with trypsin was used to remove elastin from the aortic wall as well as autoclaving followed by incubation in formic acid for 12 hours in order to remove collagen from tissue.

Immunohistochemical examination

Small pieces of digested and native aortas were fixed in formalin and embedded in paraffin. Serial 5 μm tissue sections were deparaffinised. Immunolocalization of collagen types I, IV and elastin was performed by the avidin-biotin complex method (LSAB®2 System, HRP, DAKO) preceded by revelation of antigens by proteolytic digestion with Proteinase K (S3020, Dako). Antibodies to elastin (anti-elastin (BA-4):

sc-58756, Santa Cruz), collagen type I (collagen I antibody COL-1, Novus Biologicals) and collagen type IV (anti-collagen type IV, clone FH-7A, Sigma-Aldrich) were used in the working dilutions 1:50, 1:50 and 1:2500, respectively. At the end, the sections were counterstained with hematoxylin.

Mechanical measurements

Testing of mechanical properties was performed on a testing machine (Synergie 100, MTS®). After mounting the samples, the unloaded reference length (l_0) was determined at first positive indication of the force sensor, not more than 0.01 N. All samples were subjected to pre-stretching; they were loaded 3 times to 2 N (native samples and samples digested with trypsin) or 0.05 N (samples digested with formic acid) and unloaded to zero stress. Pre-stretched and pre-loaded samples were uniaxially stretched until failure at a constant rate of 5 mm/min.

During mechanical testing, all geometrical dimensions of the samples: length (l), width (s) and thickness (g) were measured without contact using a video-extensometer (ME 46-350, Messphysik®) and the stretch ratio λ was calculated at each data point. The components of the in-plane Green–St. Venant strain tensor (ε) with the assumption of negligible shear components of the deformation gradient tensors were determined using (1)

$$\varepsilon = \frac{1}{2(\lambda^2 - 1)} \tag{1}$$

where ε – in-plane Green–St. Venant strain tensor [–], λ – stretch ratio.

The Cauchy stress (σ) was calculated as the applied force (F) normalized by the deformed cross-sectional area

$$\sigma = \frac{F}{s \cdot g} \tag{2}$$

where F – force [N], s – sample width [mm], g – sample thickness [mm].

Stress–strain curves were determined for each sample. The values of three basic parameters were analyzed: tensile strength (σ_{MAX}) and maximum tangential modulus for low (E_1) and high strain values (E_2) (Fig. 1). Tensile strength (σ_{MAX}) was defined as maximum tension prior to failure. The ranges with the determined maximum tangent elastic moduli were determined based on the method proposed by Holzapfel [13].

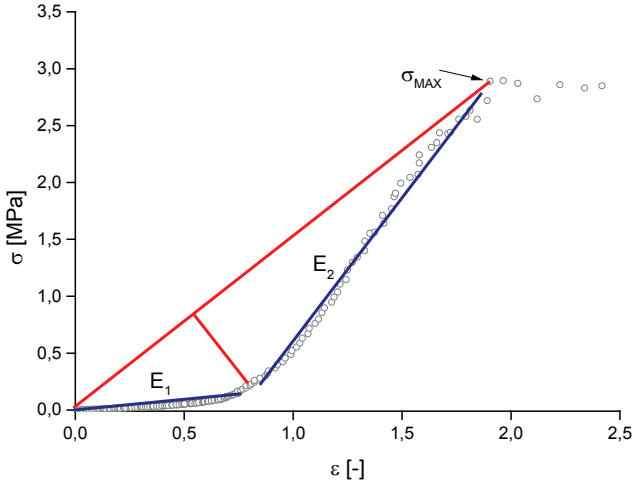

Fig. 1. The diagram for determining the mechanical parameters based on the stress–strain characteristics

Statistical analysis

The statistical analysis was performed using the non-parametric Kruskal–Wallis one-way analysis of variance by rank test and post-hoc test by Dunn (Statistica 10.0, StatSoft). The tests were carried out assuming the limit significance level (p) of 0.05.

3. Results

The effectiveness of digestion of the aortic wall was evaluated on the basis of immunohistochemical reactions. The assessment covered the intensity of sample staining and location of the colour reaction (Fig. 2). On the basis of an analysis of images obtained for the preparations subjected to a procedure based on autoclaving it can be concluded that both types of collagen were successfully removed. A relaxation of the structure of the arterial wall and numerous empty spaces throughout the thickness of the wall (Fig. 2, row 2) can be observed at the same time. Intensive brown staining of the preparations stained for the presence of elastin antigen proves that elastin is present in the arterial wall after digestion with autoclaving.

Digestion based on treatment with trypsin caused a complete removal of elastin in the middle of the aortic wall, as shown by the total absence of colour reaction to the presence of elastin. On the other hand, a low-intensity colour reaction to the presence of elastin was observed on the borders of the media and adventitia layers as well as the media and intima layers. In the case of immunostaining collagens, the pres-

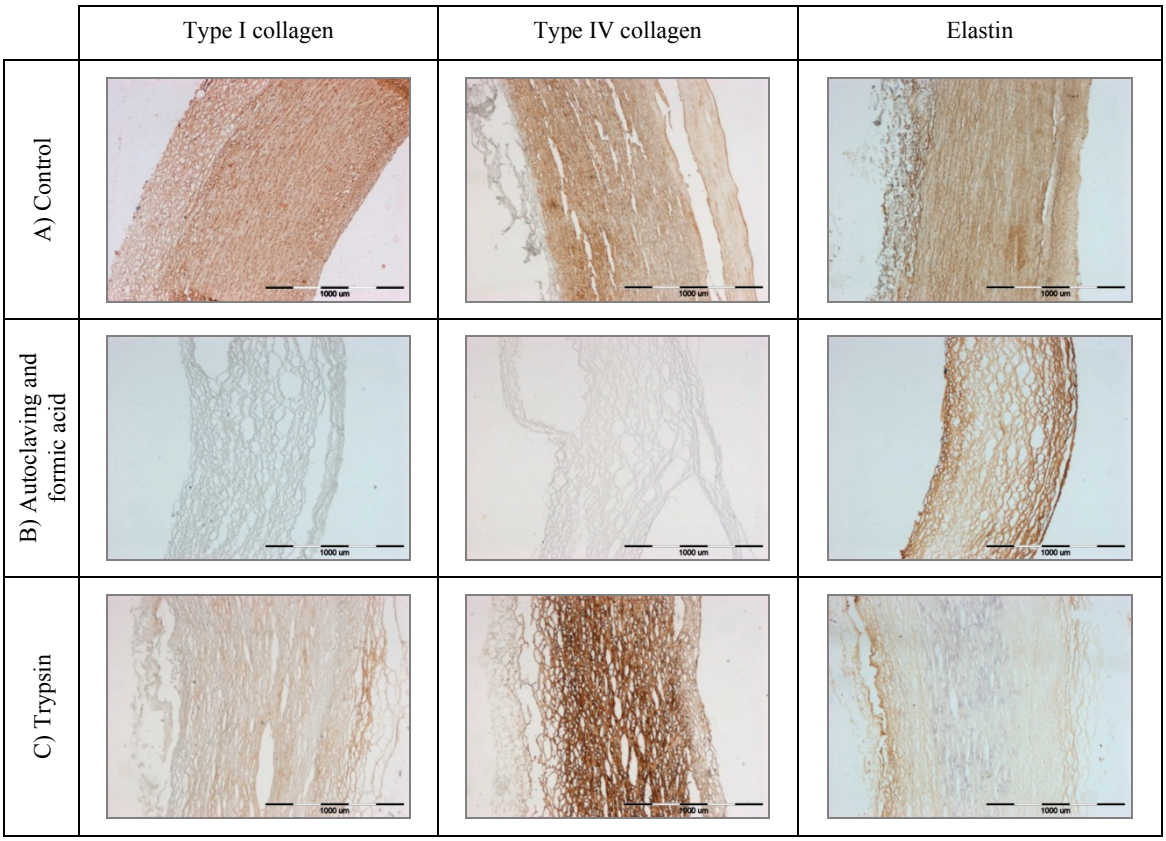

Fig. 2. Immunostaining for the type I collagen, the type IV collagen and elastin digested samples. Control native samples (row A), autoclaving followed by incubation in formic acid (row B), digestion with trypsin (row C)

ence of type I and IV collagen was demonstrated. The intensity of colour reactions to the presence of the type I collagen is lower compared to the intensity observed for native samples. Thus, the applied procedure of elastin digestion may also lead to violation of integrity of the type I collagen (fibrillar collagen) structure, and even partial digestion (decomposition) of this protein (Fig. 2, row 3). However, at pH 8.0 trypsin should be nonspecific to collagen. Consequently, further type IV collagen (non-fibrillar collagen) are exposed and a stronger intensity of colour reactions to the presence of the type IV collagen is observed compared to the control group.

Native aortic samples and aortic samples subjected to selective elastin and collagen digestion using, respectively, incubation techniques with trypsin and autoclaving followed by incubation in formic acid were tested by means of uniaxial stretching. The obtained stress-strain curves differ significantly as to their course and as to the range of stress and strain values (Fig. 3). The stress-strain curves for samples digested with trypsin and for native samples have a non-linear course typical of connective tissues. The maximum tangential moduli were determined for the range of low and high strain values, which were defined separately for each sample. Quasi-linear characteristics were obtained for samples digested by autoclaving preceded by incubation with formic acid. For these latter conditions, on the basis of the curve obtained for native samples, were estimated the ranges of strain in which both maximum tangential moduli were determined.

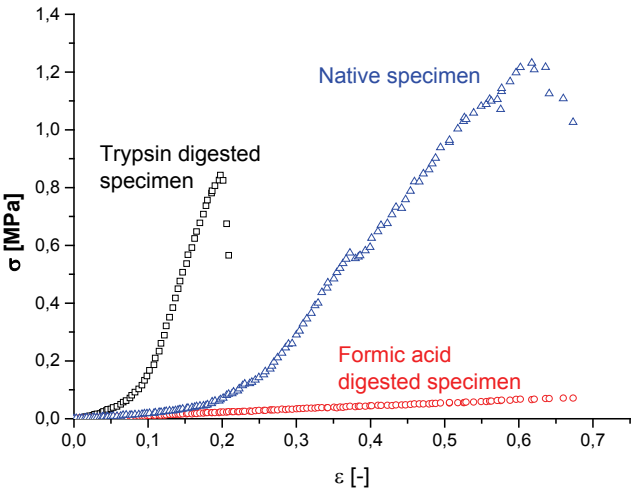

Fig. 3. Examples of stress-strain curves for native and digested samples

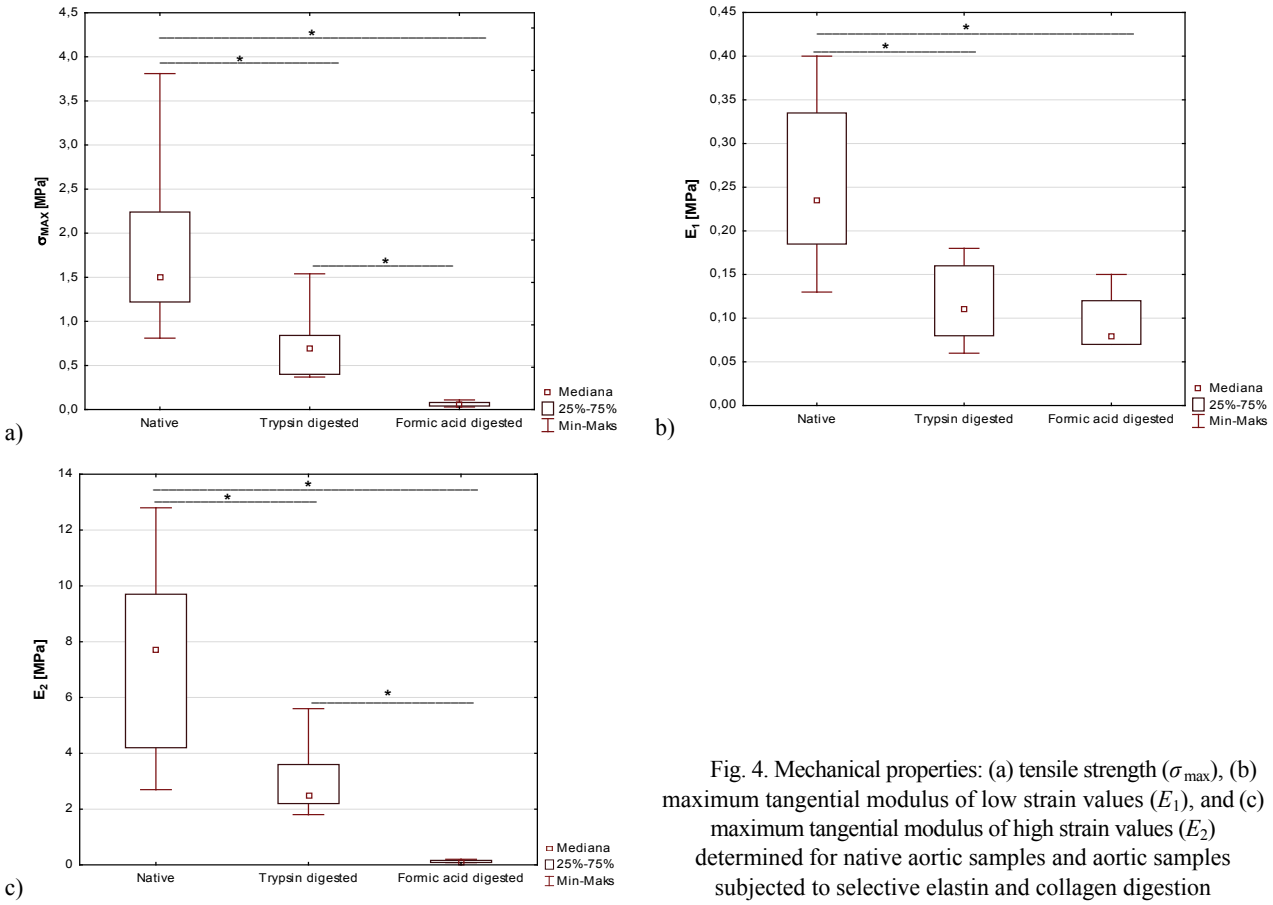

Fig. 4. Mechanical properties: (a) tensile strength (σ_{max}), (b) maximum tangential modulus of low strain values (E_1), and (c) maximum tangential modulus of high strain values (E_2) determined for native aortic samples and aortic samples subjected to selective elastin and collagen digestion

In the case of samples digested with, either autoclaving preceded by formic acid treatment or acting of trypsin, the recorded mechanical properties were significantly lower than in native samples (Fig. 4). The tensile strength of samples subjected to digestion is statistically significantly lower than in native samples (Me = 1.5 MPa), with the strength of samples digested with formic acid (Me = 0.06 MPa) being significantly lower than in samples treated with trypsin (Me = 0.7 MPa). The tangent elastic modulus for low strain values is comparable in the digested samples (Me = 0.08 MPa for formic acid and Me = 0.11 MPa for trypsin). The maximum tangent elastic modulus for high strain values is significantly lower in samples digested with trypsin (Me = 2.5 MPa) and samples digested with formic acid followed by autoclaving (Me = 0.1 MPa) than in native samples (Me = 7.7 MPa). As expected, in the case of samples digested with formic acid, the maximum tangent modulus of elasticity did not change significantly in either strain range.

4. Discussion

There are two families of fibres taking part in the process of transfer of mechanical loads, i.e., elastin fibres and collagen fibres. Their number, spatial arrangement and specific properties determine the capacity of a blood vessels to resist mechanical loads resulting from the impact of blood on vessel walls. Although the mechanical properties of elastin and collagen fibres are well described, knowledge about the behaviour of natural arrangements of load-bearing fibres under the influence of mechanical loads is insufficient. Therefore, selective digestion procedures were used in order to determine the mechanical properties of the respective arrangements of collagen and elastin fibres. Such procedures cause selective degradation of elastin and collagen fibres.

The elastic fibre is composed of two entities: amorphous elastin and highly cross-linked microfibrillar networks on which elastin is deposited [8], [22]. Crosslinking results in elastin which is extremely stable, insoluble, chemically inert and resistant to hydrolysis [8], [31]. Due to the biochemical properties of elastin, the currently used digestion techniques are mainly based on enzyme-mediated elastin removal by elastase [18], [26]. Elastase is a constituent of the crude trypsin used initially for elastin degradation [23]. Pure trypsin does not include elastase, but in suitable conditions, i.e., at pH 8.0, it digests elastin and is non-specific to colla-

gen. Another method, i.e., alkali digestion at room temperature, removes cells and elastin and leaves intact collagen [29].

Collagen is a group of proteins whose structure includes specific amino acid sequences rich in glycine, proline and hydroxyproline. Fibre-forming collagens of types I and III are prevalent in the vascular wall. They accompany each other and perform mechanical functions imparting tensile strength. They are located in all layers of the arterial wall, i.e.: intima, media and adventitia [14]. Non-fibrillar collagens, including type IV which is the most widespread collagen in the artery, participate in anchoring and organizing the ECM meshwork. Type IV collagen occurs in the endothelial basement membrane where it forms a scaffold with laminin, entactin and perlecan and the basal lamina surrounding the SMC [17]. It is not present in the adventitia except for the vasa vasorum walls. The remaining collagen types V, VI and VIII present in the arterial wall have no significant impact on the biomechanics of the aortic wall. Selective digestion of collagen from connective tissue structures currently uses various techniques [7], [22] enabling elastin purification. The methods used to isolate elastin rely on the chemical inertness and insolubility of the highly crosslinked elastin networks [11]. The methods of elastin purification based on the action of temperature over 60 °C are characterized by very high efficacy [7], [11] and are preceded by the formic acid treatment or the cyanogen bromide (CNBr)-formic acid treatment procedure due to its effectiveness in removing non-elastin (cells, collagen and proteoglycan) components from arterial tissues [18], [26], [31]. Other common elastin purification procedures using hot alkali are focused on the removal of collagen and proteoglycans that can produce fragmentation of the elastic network [11]. Use of collagenase digestion of native tissues to remove collagen is another published method to obtain elastin left in tissue, particularly from samples containing low amounts of elastin as compared to tissues like the aorta.

Selectivity and effectiveness of digestion of both enzymatic and non-enzymatic extracellular matrix proteins is highly dependent on the process conditions. The most important factors that can significantly affect efficacy of digestion but can also contribute to modification of other extracellular matrix components are the process temperature, the time of exposure to the degrading factor and its concentration. As shown in previous studies, the quality of selective digestion methods varies both in terms of their efficacy and selectiveness. However, direct and unambiguous comparison of commonly used techniques of selective digestion is hampered by the fact that different criteria are used to verify the effectiveness of the process. The authors rely mainly on the assessment of preparations of digested tissues [11], [18], [25], [27] stained with histological techniques that differentiate elastin and collagen fibres without the possibility of differentiation of the respective types of collagen. Modification of the structure after digestion can be so large that histological staining is ineffective despite the presence of protein in the structure [25]. It is therefore advisable to use more specific verification techniques, such as the technique of immunohistochemical protein staining used in this study. The use of antibodies directed at unique antigens of individual proteins allowed an analysis of efficacy and selectivity of the applied digestions with respect to elastin, type I fibrillar collagen (stained also during histological staining) and type IV collagen, which does not form fibrils, and thus it is not stained in standard histological procedures.

Selective digestion of the aortic wall allowed scaffold-like structures (simplifying: scaffolds) to be obtained whose composition resembled natural arrangements of fibres in the native tissue. Both types of the obtained scaffolds (collagen and elastin) are characterized by reduced mechanical properties compared to the native tissue. The strength of the samples subjected to digestion in formic acid and autoclaving (elastin scaffolds) is 25-fold lower compared to the native tissue. Lu et al. [18] obtained a decrease of the tensile strength of the elastin scaffold, but only 5-fold, compared to the native aorta. They also noted that the distensibility of the elastin network was similar to that of native aorta, which is consistent with the results presented and stems from the properties of the elastin macromolecule. Elastin is a protein that can work at borderline high strains without signs of failure. Most of the samples digested by means of autoclaving preceded by formic acid treatment failed at strains of the order of 60÷90%. However, these values are lower than those recorded for elastin fibres not subjected to chemical modification, for whom the course of the stress-strain curve up to 300% strain is linear [9]. In this range, Young's modulus for elastin fibres is about 500 kPa [1]. The maximum tangential moduli for high and low strain values for elastin networks were about 100 kPa. A similar result at strain below 5% was obtained by Zou et al. [31]; however, tangent modulus at 5% of strain increased to 200 kPa. Moreover, Gundiah et al. [11] showed that elastin scaffolds prepared by autoclaving have a trend for higher modulus (900.79 ± 678.02 kPa) as compared to hot alkali treatment (417.74 ± 162.23 kPa).

The removal of elastin resulted in collagen scaffolds with lower tensile strength, distensibility and maximum tangential moduli than those of native aorta. The strength of samples treated with trypsin (collagen scaffolds) is more than 2-times lower than in native tissue. The properties of tissue digested with trypsin are significantly lower than those determined previously for the collagen fibres. Collagen fibres are destroyed at low strain levels of about 3÷4% [3], while the obtained collagen network fails at strains of the order of 16÷22%. Young's modulus of collagen fibres and collagen scaffold amounts to, respectively, about 130 MPa [1] and 2.5 MPa. Lower tensile strength and lower maximum tangential modulus for high strain values of collagen networks as compared to native aorta and native collagen fibre are indicated by partial degradation of type I collagen. There are also other hypotheses on the causes of reduced mechanical properties of collagen scaffolds, such as, e.g., insufficient alignment and lack of proper orientation of ECM molecules remaining after digestion [18].

To characterize the mechanical properties of elastin and collagen networks, a stress-strain analysis was performed. The tissues subjected to autoclaving preceded by formic acid treatment are characterized by a quasi-linear course of the stress-strain curve. In contrast, the course of the stress-strain curve for trypsin-digested samples is similar to the curves for native samples. Stress-strain characteristics for soft tissues are typically non-linear [2], [30]. The present research in this regard confirms the existing theory on the participation of the respective families of fibres in the process of transfer of mechanical loads. Elastic elastin fibres transfer loads at low load values (of pressures or forces) [3], whereas collagen fibres gradually take over the function of load transfer at increasingly higher values of forces. The unloaded collagen is gradually included into the process of transfer of mechanical loads, resulting in highly non-linear load characteristics [2], [30]. Additional information in this regard was provided by studies conducted by Sokolis et al. [27], where light microscopy examination disclosed that the elastic lamellae gradually unfolded at low stresses and were almost straight at physiologic and high stresses, while collagen fibres reoriented in the longitudinal axis at low stresses, started uncoiling at physiologic ones, and straightened massively at high stresses. A biaxial tensile test performed by [31] comparing elastin scaffolds vs intact aorta showed that elastin is mainly responsible for the linear elastic response of the arterial wall at lower strains. The mechanical response of our scaffolds suggests that elastin becomes load-bearing at low to physiologic stresses and collagen at physiologic to high stresses. Efficient functioning of the aortic wall in such a wide range of strains is determined by its complex and hierarchical structure. Elastin provides high deformability of the wall while collagen gives it its mechanical strength. As demonstrated by the digestions conducted, disturbances in the form of even partial elimination of one family of fibres interfere with the behaviour of the aortic wall under the influence of mechanical loads. Such phenomena are observed in the course of many diseases, such as, e.g., aortic aneurysm [15] or atherosclerosis, where in severe cases calcium deposits are formed [16].

Acknowledgements

This publication is part of the project "Wrovasc – Integrated Cardiovascular Centre", co-financed by the European Regional Development Fund, within Innovative Economy Operational Program, 2007–2013.

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