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Quantification of Elastin, Collagen and Advanced Glycation End Products as Functions of Age and Hypertension

Milena Atanasova¹, Aneliya Dimitrova², Boryana Ruseva³,
Angelina Stoyanova⁴, Miglena Georgieva⁵ and Emiliana Konova^{5,6}

¹*Department of Biology, Medical University of Pleven, Pleven,*

²*Department of Pathophysiology, Medical University of Pleven, Pleven,*

³*Department of Physiology, Medical University of Pleven, Pleven,*

⁴*Department of Chemistry, Medical University of Pleven, Pleven,*

⁵*Medical Center "Clinical Institute for Reproductive Medicine", Pleven,*

⁶*Center for Reproductive Health; Medical University of Pleven, Pleven,
Bulgaria*

1. Introduction

Elastin and collagen are the major extracellular matrix (ECM) proteins that make up the framework of the elastic arteries structure. These two fibrous proteins are the main structural components of arterial walls, and they provide the strength and resilience needed by the aorta to accommodate the pressure and volume variations during each heartbeat (Coquand et al., 2011). Elastin is a polymer of linear polypeptide chains and constituting more than 50% of the dry weight of the proximal parts of the aorta. It is the main provider of tissue elasticity, while collagen acts to stiffen the wall and to limit its extensibility (Samila & Carter, 1981). During aging the aorta diameter and stiffness increases because of degenerative changes in elastin, resulting in a transfer of stress to less extensible collagenous components of the aorta (Coquand et al., 2011).

Elastin production takes place during late gestation and before the end of childhood, but expression in very low rate persists in adulthood. The turnover rate for collagen and elastin is low in healthy arteries, but vascular pathology upsets the regulatory pathways that maintain this balance. In response to hypertension, the overexpression of both proinflammatory and proteinase-inhibitory molecules dramatically increases arterial ECM synthesis (Arribas et al., 2006; Jacob et al., 2001; Jacob, 2003). Elastin and elastic fibers are progressively degraded by enzymatic processes involving an age-related imbalance between anti-proteases and proteases. In particular, elastin degrading enzymes, i.e. elastases, include several matrix metalloproteinases (MMP), such as MMP-2 and MMP-9 (Jacob, 2003; McNulty et al., 2005). Imbalance in matrix metalloproteinase/tissue inhibitors of metalloproteinases may contribute to alteration in collagen turnover and extracellular matrix remodelling. However, the ECM proteins synthesized in response to hypertension have a three-dimensional architecture that is functionally less optimal than those deposited during fetal development and may play an important role in determining the modulus of pathological

elastin tissue (Arribas et al., 2006; Jacob et al., 2001; Jacob, 2003). The increase of aortic stiffness is not only because of enzymatic degradation of elastin, but also is due to other mechanisms mainly including age-dependent increase in the collagen content and arterial wall thickening, non-enzymatic glycation of proteins (elastin and collagens), leading to the formation of deleterious advanced glycation end-products (AGEs) and related molecular cross-links which modify the tissue mechanical properties (Gibbons & Dzau, 1994; Corman et al., 1998; Lakatta, 2003; O'Routke, 2007).

Advanced glycation is a major pathway for the posttranslational modifications of tissue proteins and begins with non-enzymatic addition of sugars to the primary amino groups of proteins. These early glucose-derived Schiff bases and Amadori products undergo a series of inter- and intramolecular rearrangement, dehydration, and oxidation-reduction reactions and produce the late products termed advanced glycation end products (AGEs). Excessive accumulation of AGE on tissue proteins has been implicated in the pathogenesis of many of the sequels of diabetes and normal aging. Protein-linked AGEs act to crosslink connective tissue proteins and to chemically inactivate nitric oxide activity and thus are associated with endothelial dysfunction. They also act as a recognition signals for AGE receptor systems that are present on diverse cell types (Yang et al., 1991; Vlassara, 1994). Accumulation of AGEs in vascular walls increases intimal medial thickening, collagen is impaired, more cross-linked and elastin fibrils are broken. Large arteries are stiffen and mechanical stress increases, leading to hypertension and all its deleterious consequences (Dart & Kingwell, 2001; Ziemann & Kass, 2004)

Excessive accumulation of AGEs on tissue proteins changes their structure and respectively functions, reduces their susceptibility to degradation and none of the last place immunogenicity. Specifically, the interaction of AGEs with vessel wall components increases vascular permeability, the expression of procoagulant activity and the generation of ROS (Yan et al., 1994). Glycated proteins form common immunological epitopes which raise formation of population of anti-AGE autoantibodies (AGEAb). These antibodies recognize and react with AGE-epitopes regardless of the proteins they have been formed on. Assessment of the levels of these antibodies shows that they are present at low titres even in sera of healthy subjects, perhaps as a part of homeostatic mechanism which clears glycated structures via *in situ* destruction or via opsonization of the glycated proteins and products of their degradation (Baydanoff et al., 1996). However, in the conditions of increased non-enzymatic glycation the homeostatic control is inefficient and the generation of these antibodies increases (Baydanoff et al., 1996).

The aim of our study was to investigate the effects of age and hypertension on quantity and quality of elastin and collagen in the aortic wall of the rats. In order to reach this aim we used the spontaneously hypertensive rats (SHR), that are appropriate genetical model for studying essential hypertension compared to normotensive Wistar-Kyoto rats (WKR) at 2-, 4- and 8-months of age.

2. Materials and methods

2.1 Animals

Female and male Spontaneously Hypertensive Rats (SHR, n=30) and Wistar Kyoto Rats (WKY, n=33) were used. Animals were born and raised under conventional conditions in

the animal facility of the Medical University of Pleven and were allowed free access to tap water and a standard laboratory chow. Animals were housed and kept under a normal 12 h light/dark cycle at $22 \pm 2^\circ\text{C}$. They were divided into 6 groups: 2-month-old SHR (2mSHR, n=7, 4 female and 3 male); 4-month-old SHR (4m SHR, n=9, 5/4); 8-month-old SHR (8mSHR, n=14, 9/5); 2-month-old WKY (2mWKY, n=12, 5/7); 4-month-old SHR (4mWKY, n=9, 5/4); 8-month-old WKY (8mWKY, n=12, 5/7). Our experimental design was approved by the Animal Care and Use of Laboratory Animals group of the Ethical Committee of the University, based on the principles described in the Guide for the Care and Use of Laboratory Animals (Bayne, 1996).

2.2 Blood collection

At the end of the 2nd, 4th and 8th month, following overnight fasting, the abdominal cavity of rats was opened under pentobarbitone sodium anesthesia (26 mg/kg body weight, i.p.). Blood was collected from the bifurcation of the aorta and put at 37°C to clot. After separation 0.02% NaN_3 was added to each serum sample and stored at -20°C prior to use.

2.3 Quantification of elastin in the thoracic aorta

The quantity of elastin was measured after dissection of the descending thoracic aorta and cleaning of blood and surrounding adipose tissue. Length and width (both sides) of the vessels were recorded by using a grid in the eyepiece, after opening of the vessel in length.

Elastin was then quantified by using a protocol deriving from a previously described method (Wolinski, 1972) with small modifications. Briefly, after delipidation in acetone/diethyl ether (1:1, vol/vol) and drying, the dry weight was recorded by using a Kern ALS 120-4 balance (precision: 0.01 mg). Cell proteins were extracted by gentle agitation in 0.3% SDS for 24 h and then 3 times in 5 M guanidinium chloride with preservative 0.02% sodium azide for 2 h. After washing 3 times in distilled water the extracellular proteins, other than elastin remaining in the aortic segments, were solubilized by three 15-minute extractions in 1 ml of 0.1 M NaOH in a boiling water bath. Elastin was quantified by determining the dry weight of the residue as percent dry weight of the aorta or mg/cm.

2.4 Quantification of collagen in the thoracic aorta

The content of the aortic collagen was assayed by determination the hydroxyproline presented in NaOH solution. The solution was evaporated to dryness and hydrolyzed in 6N HCl under vacuum conditions for 24 hours at 110°C . A colorimetric assay according Woessner (1961) was applied for determination of the hydroxyproline. Assuming that collagen contains 12.77% hydroxyproline by weight its quantity was presented as mg collagen per cm aorta (Keeley et al., 1984).

2.5 Direct determination of advanced glycation end-products (AGEs) formed *in vivo*

Soluble α -elastin was obtained from the descending thoracic aorta by the method of Partridge (1955). Insoluble elastin was hydrolyzed 5 times in 0.25 M oxalic acid in boiling

water bath, and then dialyzed against phosphate buffered saline (PBS). Protein content of each sample was determined by measurement of the absorption of UV light at 280 nm wavelength and calculated according to a standard curve, constructed on the basis of different dilutions of rat α -elastin (EPC, St. Louis, USA).

Maillard reaction-related fluorescence (FC), representative of AGEs formed *in vivo*, was measured as an index of advanced glycation in 360/450 nm excitation/emission (Baydanoff et al., 1994; Monnier et al., 1984) with Corning-EEL fluorimeter. Quinine sulfate 1 μ M in 0.1N H₂SO₄ was used as a standard. The levels of AGEs were expressed as arbitrary fluorescence units (AU) per mg protein.

2.6 Measurement of circulating AGEAb

2.6.1 Glycation of KLH

Keyhole Limpets Hemocyanin (KLH) (Sigma) - 20mg/ml was glycated *in vitro* with 3.33 M glucose in 0.4 M phosphate buffer, pH 7.5 with preservative 0.04% NaN₃, at 37°C, for 12 weeks. The formation of advanced glycated end product of KLH (AGE-KLH) was determined via measuring the fluorescence at 360/440 nm excitation/emission. After dialyzing against phosphate buffer, the obtained AGE-KLH was used as antigen in home-made ELISA.

2.6.2 ELISA for determination of circulating anti-AGE antibodies

AGEAb were assessed by home-made ELISA, as described in Dimitrova et al. (2009). The assay was performed as follows: Microtiter 96 well plates (Greiner Microlon) were coated with 100 μ l of AGE-KLH per well in carbonate buffer (pH 9.6) with concentration 5 μ g/ml. The plates were incubated 2 hours at 37 °C and then overnight at 4 °C to complete binding. Then the plates were washed three times with PBS with 0.05 % Tween 20 (PBS-Tween) before their blocking with 0.1 % BSA and incubation for 1 h at 37 °C. The next step was addition of 100 μ l of tested rat serum, diluted 1:10 with PBS-Tween. Bound antibodies were reacted with anti-mouse IgG peroxidase conjugate, diluted 1:3200 in 1% human serum albumin for 1 h at 37 °C (BulBio - Sofia, Bulgaria). Ortho-phenylene diamine was used as colorimetric substrate. The reaction was terminated by 50 μ l 8N H₂SO₄ and the absorbance (A) was read at 492 nm on automatic micro-ELISA plate reader. Serum samples were analyzed in triplicate and the average calculated.

2.7 Statistics

Comparisons of SHR and control rats were assessed using one- or two-way ANOVA followed when necessary by Fisher's least significant difference test (LSD) for paired value comparisons. The results are presented as mean values \pm SEM, and p values \leq 0.05 were considered as statistically significant. The correlations between investigated parameters were tested by the method of Pearson. The statistical package used was SPSS v. 15.

3. Results

3.1 Elastin content in the thoracic aorta

Figure 1 shows elastin content in the thoracic aortas of both rat strains aged 2, 4 and 8 months. When the elastin quantity was presented as percentage of the dry weight of the

aorta (fig. 1A) SHR at different ages did not differ significantly, whereas in the WKR groups factor "age" was found to have a significant effect ($p < 0.05$) – elastin quantity decreased with age. Elastin content was significantly larger ($p < 0.032$) in 8-month-old hypertensive animals compared to age matched normotensive group. When the elastin quantity was presented as milligrams per centimeter of aorta (fig. 1B) the absolute amount of elastin in SHR at different ages did not differ from age matched WKR.

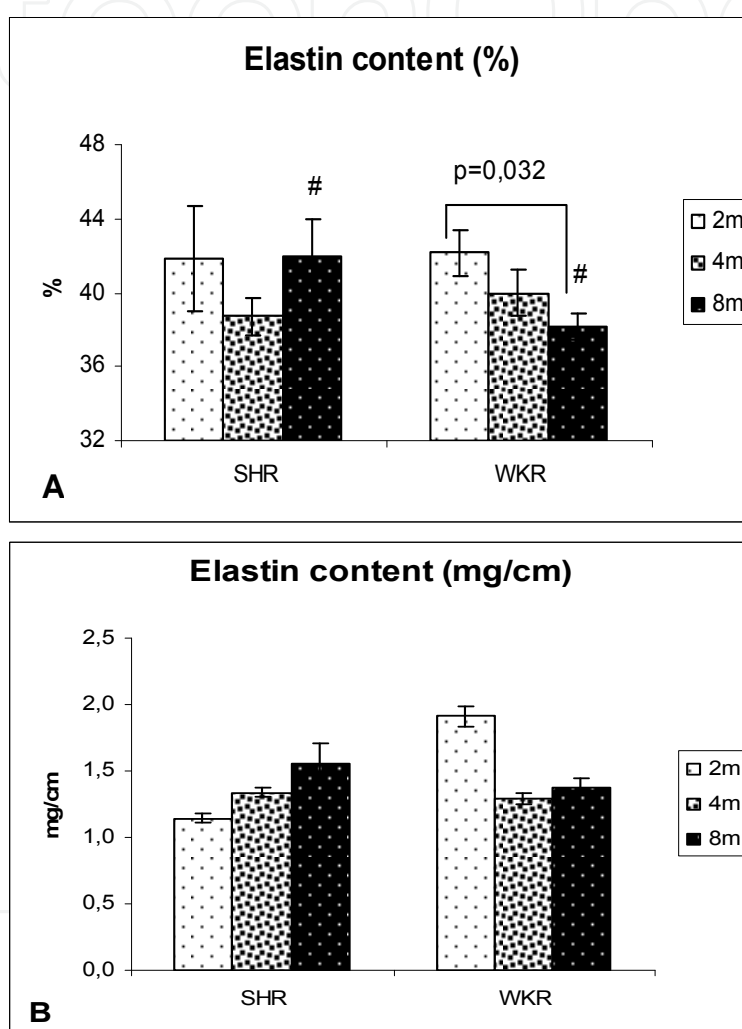


Fig. 1. Quantity of elastin in the thoracic aorta of SHR and WKR at three different ages, expressed as (A) percent dry weight of the aorta and (B) mg elastin per cm aorta. # Significant difference between 8-months-old SHR and WKR ($p = 0.032$).

3.2 Collagen content in the thoracic aorta

The collagen quantity showed a trend towards increase with age, but without reaching the statistically significant threshold (fig. 2). SHR at 2 and 4 months of age had significantly larger collagen content than age matched WKR. The groups of 8-month-old animals did not differ significantly. Hypertension was found to have significant effect ($p = 0.019$) on the collagen content of the thoracic aorta of the investigated rats, but age was not.

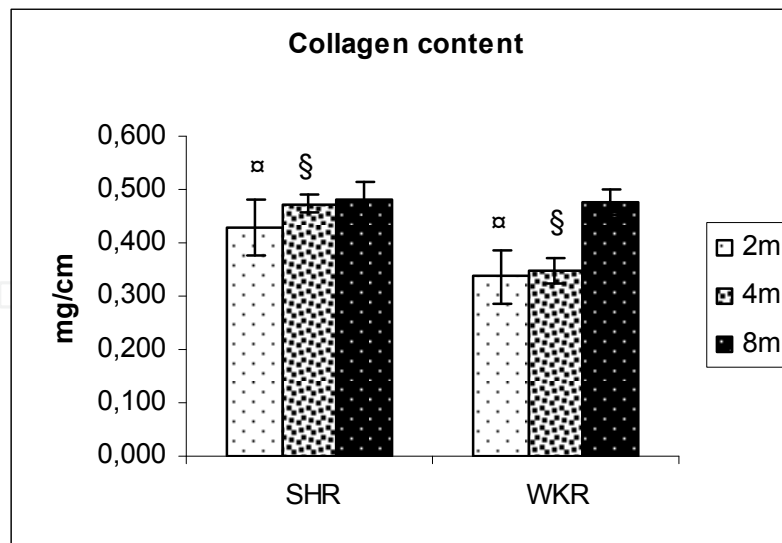


Fig. 2. Quantity of collagen in the thoracic aorta of SHR and WKR at three different ages, expressed as mg collagen per cm aorta. α - Significant difference between 2-months-old SHR and WKR ($p = 0.056$); § - Significant difference between 4-months-old SHR and WKR ($p = 0.007$).

3.3 Direct determination of AGEs formed *in vivo*

Results obtained by direct determination of AGEs formed *in vivo* are presented in AU per mg elastin (fig. 3). The direct measurement of Maillard reaction-related fluorescence of obtained aortic α -elastin was found to increase with age in both strains and to be significantly higher ($p=0.036$) in elastin obtained by 8-month-old SHR samples compared to WKR at the same age.

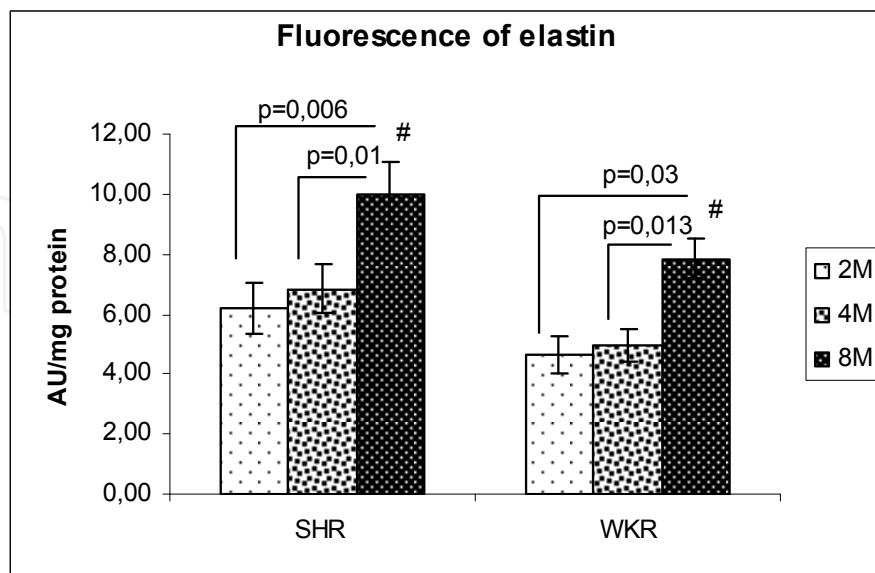


Fig. 3. Direct determination of AGEs formed *in vivo*. Maillard reaction-related fluorescence, measured for the soluble α -elastin obtained from the three aged groups of SHR and WKR. #- Significant difference between 8-months-old SHR and WKR ($p = 0.036$). AU - arbitrary units

ANOVA analysis showed that fluorescence of the purified elastin was influenced significantly by the age and hypertension ($p=0.001$ and $p=0.0026$).

3.4 Circulating anti-AGE antibodies

AGEAb were presented in all the sera tested. The serum levels of these antibodies (fig.4) in SHR substantially increased with age. Their mean values in WKR showed a trend towards increase with age, but without being significant. The youngest animals group from the two strains did not differ in contrast with the other two age groups in which antibody levels were significantly higher in hypertensive animals, compared to normotensive ones. The correlation (Pearson coefficient) between the serum levels of anti-AGEAb and the age and hypertension was highly significant ($r = 0.553$, $p = 0.001$ for age and $r = - 0.440$, $p = 0.009$ for hypertension).

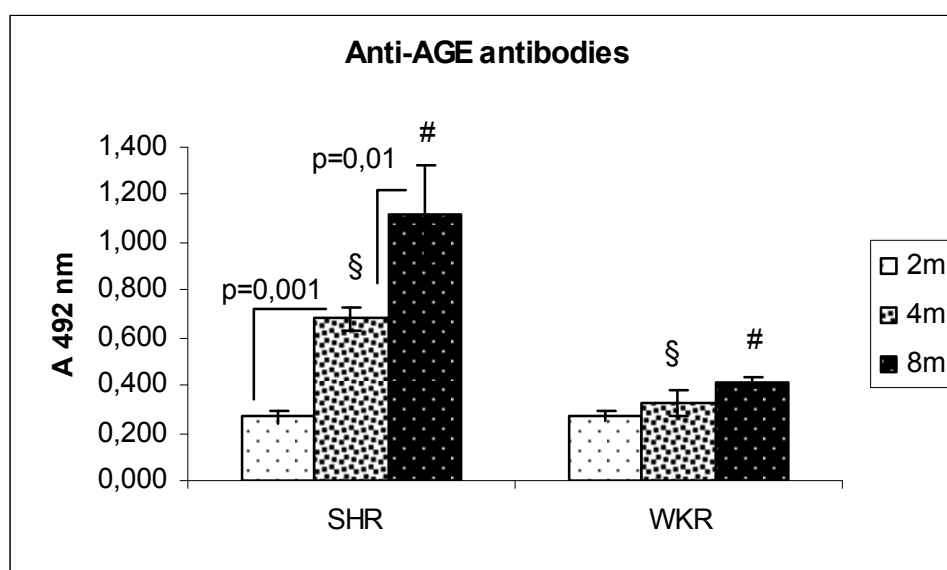


Fig. 4. Serum levels of AGEAb, measured by indirect home-made ELISA. § - Significant difference between 4-months-old SHR and WKR ($p = 0.012$). #- Significant difference between 8-months-old SHR and WKR ($p = 0.001$).

4. Discussion

Elastin and collagen fibrils are the structures that endow the vascular wall with elasticity and strength. But similarly to most of the connective tissue proteins they are proved to be pliable of cross-linking and thus their physiological turnover is changed and they become resistant to hydrolytic degradation (Zieman 2003). Altered by non-enzymatic glycation they accumulate in the vessel matrix in unorganized and non-functional pattern (Bailey 2001). This structural disorganization affects mainly the media and leads to mechanical alteration of the aortic wall especially in the condition of hypertension (Laurent, 1995; Bezie et al.,1998). Marque et al., (1999) explained age-linked aortic stiffening in SHR with more rapid increase with age of the aortic collagen content in and accelerated accumulation of advanced glycation end products on elastin and collagen fibers.

In the present study, we compared the elastin and collagen content of thoracic aortas from Wistar-Kyoto rats (WKR) and spontaneously hypertensive rats (SHR) at three different ages. In

addition, glycation of purified aortic elastin and antibodies against AGEs were determined. The investigated parameters were compared as functions of age and hypertension.

Elastin production was investigated at the protein level, through the measurement of aortic elastin content. The relative elastin content (expressed as a percentage of dry weight) was significantly lower in the 8-month-old WKR, compared to the 2-month-old WKR and 8-month-old SHR. However, no significant differences were observed when the elastin content in the aorta was expressed as weight per aorta length (mg/cm). This can be explained with variations in body size and, thus, aortic dry weight among individual rats from both sexes, the absolute amount of elastin varied widely (Sauvage et al., 1999). The expression of elastin content relative to dry weight eliminates considerations of these large individual variations in aortic dry weight. It appeared that age had no general significant effect on the joint distribution of elastin content ($p > 0.05$), but there is a slight decrease with age in aortic elastin content (-9%) in the oldest WKR (Fig. 1).

In the literature there is not clear age-related trend of decrease or increase of both elastin and collagen quantity and during hypertension development. Tsoporis et al., (1998) followed the biosynthesis of collagen and elastin during the development of spontaneous hypertension in SHR. They found that both collagen and elastin synthesis (as revealed by specific hydroxyproline activity) exceeded WKR control levels in the prehypertensive period (at the age of 4 weeks), decreased in the development of hypertension (to the age of 14-16 weeks), and increased again in the period of the established hypertensive state (beyond the age of 16 weeks). Several studies showed that this second increase in connective tissue proteins may be prevented, depending on the choice of antihypertensive therapy (Tsoporis et al., 1998; Han et al., 2009).

From the two main collagen types present, synthesis of collagen type III exceeded that of type I in the prehypertensive period (at the age of 4 weeks) and this relation was reversed during the period of established hypertension. They suggested that the vascular connective tissue metabolism in SHR differs from that in strain-matched controls, and the reverse rate of collagen type III to collagen type I synthesis during hypertension development may be considered an adaptive response to the increasing pressure load which may alter the mechanical properties of the vessel wall (Deil et al., 1987).

Han et al (2009) investigated medial and adventitial layers of the thoracic aorta 4- and 8-month-old SHR and WKR. They discovered that compared with WKR, SHR exhibited greater collagen and elastin content in the media, but decreased collagen and elastin content in the adventitial layer. Both medial and adventitial collagen and elastin content increased significantly with age in both strains and was greater in 8-month-old rats compared to 4-month-old rats (Han 2009).

It has been shown that the elastin network plays a major role in the maintenance of aortic elastic properties in adult SHR, not through variations of its total amount but through increases of the extent of its anchorage to the smooth muscle cells (Laurent, 1995, Bezieet al., 1998). Our data also supported the finding that quantity of elastin is not generally changed in the conditions of hypertension that is why we tested the Maillard reaction-related fluorescence to check the index of advanced glycation.

Hypertension is characterized by insulin resistance, and a number of studies have suggested that it plays a major role in its etiology (DeLano & Schmidt-Schonbein, 2007).

Other study of DeLano & Schmid-Schönbein (2008) states emphatically that in hypertensive rats, proteases cleave extracellular portions of several protein receptors, such as the insulin receptor, so that insulin can no longer bind and facilitate normal metabolism of glucose. In insulin resistance, alterations in glucose and lipid turnover lead to the production of excess AGEs (Vasdev et al., 2007; Potenza et al., 2005). AGEs are complex group of compounds, and the structure of a lot of them is identified - N-carboxymethyl-lysine (CML), pentosidine, imidasolones, pirraline, etc. Although the chemistry of AGEs is not fully discovered, increased levels of circulating and tissue AGEs have been demonstrated in both animal and human studies in pathologies and aging (Brüel & Oxlund, 1996; Ziemann & Kass, 2004). Increase of non-enzymatic glycation with aging is due to the life-long exposure to glucose even in normoglycemia. In pathology or aging conditions, when non-enzymatic glycation of proteins is increased, the capacity of normal homeostasis seems to be inefficient. This way AGEs are accumulated and contribute to the development of long-term aging process especially in long-lived proteins (Konova et al., 2004). Our results from the fluorescence studies of obtained aortic α -elastin showed increase with age especially in the hypertensive animals. The highest fluorescence was measured in the group of 8-month-old SHR which was significantly higher ($p=0.036$) in comparison to WKR at the same age. These results suggest an increase of non-enzymatic glycation with aging, even in normoglycemic animals and are in agreement with our previous investigations of age-dependent glycation of human aortic elastin (Konova et al., 2004). Moreover, we demonstrated the accelerating role of hypertension for the increase of formation of irreversible late advanced glycation end products in long-lived connective tissue proteins.

We did not find significant differences in AGEAb levels between SHR and WKR rats at 2 months of age. At about two months of age in arterial walls of SHR occur alterations that are consequences of developed hypertension. Observed vascular wall hypertrophy also plays an important role in hypertension by reducing tissue flexibility and elasticity (Mecham & Davis, 1994). During the early phases of hypertension development, wall distensibility might even be augmented in the SHR rats but long-term hypertension decreases arterial distensibility (Zanchi et al., 1997). Formed AGEs affect the biochemical and physical properties of proteins and ECM, including the charge, hydrophobicity, turnover and elasticity of collagen and elastin (Baydanov et al, 1994), and the cell adhesion, permeability and pro-inflammatory properties of the ECM (Baines, 2001). These structural and functional changes alter the antigenicity and immunogenicity of vascular elements. This provokes the immune system to react by producing different types of antibodies including AGEAb, directed to their own but altered structures. In our study AGEAb were presented in all the investigated rat sera but their levels were in positive correlation to the increasing age only in hypertensive animals. We observed significantly higher levels of AGE Abs in 8-month-old SHR compared to other age groups and age matched WKR. Our previous studies in patients with occupational vegetative polyneuropathy of upper limbs and in male SHR demonstrated similar results (Dimitrova et al., 2009, 2010).

5. Conclusion

In general, there were not clearly drawn age-related differences in the elastin and collagen quantity of the thoracic aortas of the investigated rats. But there were significant differences in the late products of glycation of the purified elastin and serum levels of antibodies

against them. The quantity of obtained elastin did not change but the quality did. It appeared that the investigated factors had a generally significant effect and we observed a substantial increase of AGEs and AGEAb with age especially in the condition of hypertension.

Although AGEs in proteins are probably correlative, rather than causative, with respect to aging, they accumulate to high levels in tissues in age-related chronic diseases, such as atherosclerosis, diabetes, arthritis and neurodegenerative diseases. Inhibition of AGE formation in these diseases may limit oxidative and inflammatory damage in tissues, retarding the progression of pathophysiology and improve the quality of life during aging. It is necessary to investigate genetic, pharmacologic, and nutritional factors, which could decrease the levels of AGEs, to break cross-links in the glycated structures of ECM and to restore the turnover balance in order to improve the functionality of the vessel walls.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
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Unit 405, Office Block, Hotel Equatorial Shanghai
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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