FACTA UNIVERSITATIS
Series: **Medicine and Biology** Vol. 16, No 1, 2014, pp. 31–35 UC 611.61

HISTOCHEMICAL AND MORPHOMETRIC ANALYSIS OF CONNECTIVE TISSUE IN HUMAN GLOMERULES DURING AGING

Vesna Stojanović**, Ivan Jovanović, Slađana Ugrenović, Braca Kundalić, Miljana Pavlović

University of Niš, Faculty of Medicine, Department of Anatomy, Serbia

Abstract. *During aging there are alterations in human glomerules followed by increase of the connective tissue amount, accumulation of glomerular basal membrane and mesangial matrix on account of the rest of cell population. The aim of the study was quantification of the present connective tissue in mesangium of glomerules which morphologically did not show signs of sclerosis and determination of its part in glomerular structure. Investigation included 30 tissue samples of human kidneys of cadavers of both sexes, aged 20-85. Tissue samples were routinely stained with Mallory trichrome stain and analyzed by light microscope under 400× magnifications. Images were analyzed with ImageJ software. Statistical analysis was performed with NCSS-PASS software. Cluster analysis was performed for the classification of glomeruli into 3 age groups, first with average age of 29, second with 44 and third with average of 71 years old. Histochemical investigations indicated the growth of connective tissue in mesangium of human glomerules during aging. It was also demonstrated morphometrically showing significant increase ($p<0.05$) of mean connective tissue area and its percentage in glomerule. In first, the youngest group the connective tissue was present in 17.33%, while in second group there was statistically significant increase (32.11%). The most significant growth compared to other age groups appeared in third group, where there was 40.66% of connective tissue in glomerule. Results of morphometrical and statistical analysis suggest that during aging process there is significant increase of area and percentage of connective tissue in the glomerule followed by reduction of cell size.*

Key words: *Human glomerules, mesangium, connective tissue, aging*

Introduction

Aging may be considered as gradual deterioration of cell function which is based on biophysical and biochemical changes of cell content, gradual loss of cell capacity for reproduction and regeneration of structural elements [1–3]. Very important problem in studying the aging process is also how to distinguish aging from disease. Aging is connected with physiological, morphological and functional changes in the kidney. Many of these changes, such as loss of nephrons, may be interpreted as an attempt of protection in aging process which influences renal homeostasis. The disorders call into question renal backup and reduce renal capacity which leads to new insults [4,5]. Etiological and other factors such as cytokines, growth factors, proliferation, pro-apoptotic, transcription and other factors [6,7], are involved in renal aging.

Age-related changes in kidney are similar to those verified in chronic renal disease and experimental models with chronic renal deficiency. Old age in humans is followed with renal mass loss. The loss is primarily developed in cortex, while medulla is relatively spared, which is shown by some studies whose results suggest that even half of neurons may disappear until old age [8–10]. It is known that a man is born with definitive number of nephrons (except in premature birth), and that its morphological and functional maturation lasts till sexual maturity, on account of corpuscle's size, amount of vascular and connective tissue and increased length and tortuosity of tubules [4,5,11–13]. Aging is accompanied with nephron loss [4,11,13,14], increase of sclerotic glomerules percentage, changes in blood vessels with obliteration of the lumen, decrease of glomerular cells number and higher interstitial tissue content in cortex and medulla [15,16]. All of these changes which appear during aging affect function of corpuscles and kidney. Human glomerular aging includes progressive loss of glomerules which is directly related to birth mass, presence of shunts between the afferent and efferent arteriole, growth of mesangial matrix followed with onset of glomerulosclerosis and increase in number of globally sclerotic glomerules [17–20]. Previous studies showed that "physiological" deterioration of glomerules normally begins during intrauterine life and continues during child's growth and development.

The aim is to quantify the presence of connective tissue in the mesangium of the glomerules which do not show morphological signs of sclerosis and to determine its part in glomerular structure during aging.

Correspondence to: Vesna Stojanović, MD, PhD
Faculty of Medicine, Dept. of Anatomy,
81 Dr Zoran Đinđić Blvd., 18000 Niš, Serbia
Phone: +381 18 45 70 029 Fax: +381 18 423 87 70
E-mail: vesna@medfak.ni.ac.rs

Material and Methods

The material was human right kidney tissue of 30 cadavers, obtained during routine autopsies at the Institute for Forensic Medicine in Niš. Their age ranged from 20 to 85 years. During autopsy kidney damage or congenital anomalies were not observed. Cadavers were without previously diagnosed kidney disease, diabetes, hypertension, or any other systemic disease.

Tissue specimens were fixed in 10% buffered formalin for 12 hours and then embedded in paraplast. The tissue was then cut into 5 μm thick sections and routinely stained with Mallory trichrome stain. Histological slices were analyzed under 400× magnification. Images of histological slices were captured with digital camera (5 megapixels resolution).

Glomerules were analyzed with ImageJ software (http://rsbweb.nih.gov/ij/) which was spatially calibrated with object micrometer (1:100). Glomerular tuft area (AG), Feret's diameter (DF), perimeter (BG), diameter along main (DM) and secondary axis (Dm), total number of cells per glomerular area unit (Nn), and glomerular connective tissue area (ACT) were measured. Seven sclerotic glomeruli, if noticed, then non-sclerotic glomeruli located subcortically, juxtamedullary and columnary, seven of each, selected by unbiased method, were analyzed per one case, therefore there was a maximum of 28 glomeruli per one case. In total, 743 glomeruli were analyzed in all 30 cases. Glomerular images were additionally processed for connective tissue area measurement. Glomerular tuft image was first manually selected by polygonal selection tool and extracted from the other parts of histological slice image. Selection of its connective tissue, which was green stained on Mallory trichrome stained sections, was performed by "color-based treshholding" option. Its application was based on green colored sample of glomerular tuft image. Afterwards, only green stained parts of glomerule remained on image, which was further converted into binary image. Binary image was used for connective tissue area measurement. Green colored samples were taken at three different localizations in each glomerular tuft image. Connective tissue area was measured for each sample. Average connective tissue area was then calculated from three obtained values for each glomerular tuft. Glomerular connective tissue percentage was obtained from the ratio between glomerular connective tissue area and total glomerular area. Average values of morphometric parameters were calculated for each of all 30 evaluated cases.

Statistical analysis was performed with NCSS-PASS software (http://www.ncss.com/). Cluster analysis by the k-means method was performed for the classification of glomeruli into age groups according to their morphometric characteristics. One-way ANOVA was used for the comparison of more than two groups. In cases where data did not have normal distribution Kruskal-Wallis one-way ANOVA was used for the comparison of more than two groups. Statistical significance test was performed for $p<0.05$.

Results

Morphological analysis

During histological analysis of renal preparations stained by trichrome staining – modification by Mallory, renal corpuscles are classified into three groups based on size, cellularity and presence of connective tissue. The first group includes sclerotic corpuscles, the second group includes morphologically normal ones and the third is composed of hypertrophic corpuscles.

In the group of sclerotic renal corpuscles (Fig. 1, A–B) we noticed collapse of capillaries, intensive accumulation of extracellular matrix, while the glomerular capillary network almost disappeared and was replaced by connective tissue. Inside of sclerotic glomerule rare capillaries (Fig. 1, A) and scarce nuclei (Fig. 1, B) may be seen. External leaf of Bowman's capsule was not clearly separated from internal leaf so there was relatively narrow urinary space only in places. Around described corpuscles there were numerous proximal and distal tubules, while in the interstitial connective tissue there were poorly observable capillaries with clearly expressed interstitial sclerosis. Majority of these corpuscles was noticed in the oldest persons.

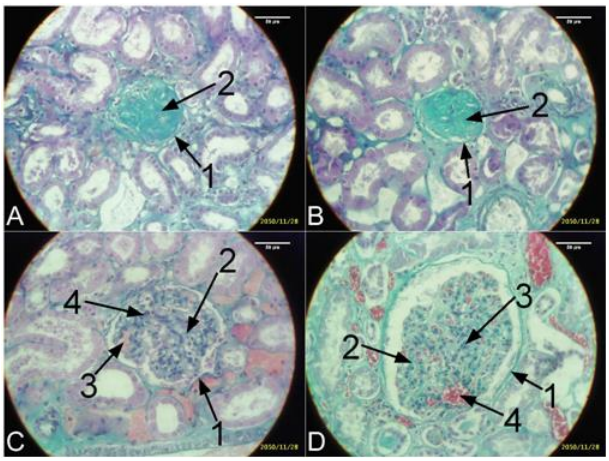

Fig. 1. A — Sclerotic renal corpuscle of a male, aged 48; B — Sclerotic renal corpuscle of a female, aged 39; C — Normal renal corpuscle of a male, aged 48; D — Hypertrophic renal corpuscle of a male, aged 78. 1, Bowman's capsule; 2, connective tissue; 3, blood vessels; 4, nuclei. Mallory's trichrome staining, 400×.

Morphologically normal corpuscles (Fig. 1, C) were the most numerous in the middle-aged and the youngest persons. They possess clearly defined external and internal leaf of Bowman's capsule which lies next to glomerular capillary network. Between these two leaves there is urinary space with relatively constant width in all parts of glomerule. Glomerular capillary network consists of clearly visible capillary loops surrounded by mesangial matrix with scarce connective tissue and numerous nuclei of mesangial cells which are small, dark and centrally positioned. There are proximal and distal

tubules around glomerules, with relatively narrow interstitial space and clearly noticeable capillaries inside.

Hypertrophic capillaries (Fig. 1, D) were considerably larger than previous ones. The external leaf of Bowman's capsule was thickened in almost all parts, while the internal one was sporadically discontinuous and urinary space was much expanded in some parts. Glomerular capillaries were multiplied; mesangial matrix was considerably replaced by connective tissue, while cellularity in glomerule was increased. In interstitial space there were tubules, capillaries and increased content of connective tissue. The presence of these corpuscles is higher in middle-aged and older persons.

Morphometric analysis of glomerules

As a result of the analysis we obtained three groups of cases. The first group consists of the youngest cases, six in total, who were aged 24–33 years, average 29. Eleven older cases, aged 40–49 years, average 44, were in the second group. In the third group there were 13 oldest cases aged 65–76 years, average 71 (Table 1).

Mean glomerular area of investigated cases shows changes which were not statistically significant, while mean connective tissue area and its percentage in glomerule rise significantly ($p<0.05$) during aging (Table 1; Fig. 2). In the first, the youngest group mean connective tissue area is 2601.18 μm², which is 17.33% of glomerular area. In the second, older group, there was statistically significant increase of connective tissue area. Its value is 4468.52 μm², which makes up 32.11% of glomerule. The most significant increase of connective tissue area is present in the third, the oldest group, compared to other two ($p<0.05$). Its mean value is 4468.52 μm², or 40.66% of glomerular area (Table 1; Fig. 3).

Mean perimeter, mean diameter along main axis of glomerule and mean Feret's diameter show the same trend, as well as mean glomerular area during human aging, while mean diameter along secondary axis of glomerule shows continuous decrease from first to third age group (Table 1; Fig. 4). Changes of these parameters are not statistically significant which indicates that size and shape of investigated glomerules are not in correlation with aging process.

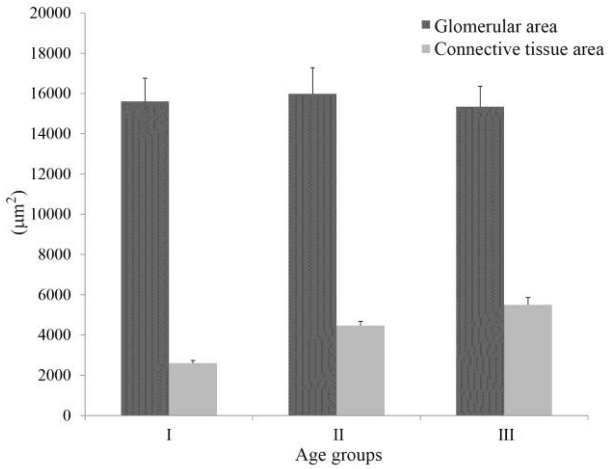

Fig. 2 Mean glomerular area and mean glomerular connective tissue area of groups obtained by cluster analysis

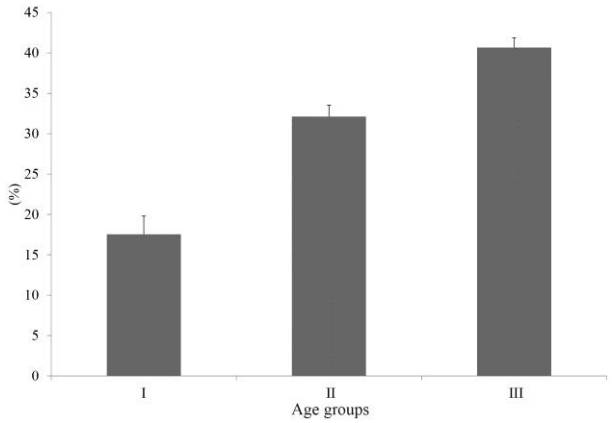

Fig. 3 Mean percentage of connective tissue per glomerule of groups obtained by cluster analysis

Table 1. Morphometric features of glomerular groups obtained by cluster analysis

	A_G (μm²)		B_G (μm)		D_M (μm)		D_m (μm)		D_F (μm)		A_{VT} (μm²)		VT%		$N_n(1/\mu m^2) \times 10^{-3}$	
Cluster	I (n = 114)															
Parameter	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md
Velue	22478.81	21894.61	564.24	556.76	189.36	185.69	149.84	149.53	197.22	193.01	6774.02	6406.87	30.66	31.49	5.9	5.9
SE	314.27		4.69		1.53		1.30		1.58		115.15		0.45		0.1	
95% LCL	21859.05	21188.06	554.99	546.10	186.36	183.13	147.27	146.19	194.11	189.62	6546.94	6207.42	29.76	30.54	5.7	5.8
95% UCL	23098.56	22664.15	573.50	568.77	192.37	189.62	152.40	151.94	200.33	197.60	7001.10	6588.96	31.55	32.46	6.0	6.1
	II (n = 430)															
Cluster	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md
Parameter	22478.81	21894.61	564.24	556.76	189.36	185.69	149.84	149.53	197.22	193.01	6774.02	6406.87	30.66	31.49	5.9	5.9
Velue	314.27		4.69		1.53		1.30		1.58		115.15		0.45		0.1	
SE	21859.05	21188.06	554.99	546.10	186.36	183.13	147.27	146.19	194.11	189.62	6546.94	6207.42	29.76	30.54	5.7	5.8
95% LCL	23098.56	22664.15	573.50	568.77	192.37	189.62	152.40	151.94	200.33	197.60	7001.10	6588.96	31.55	32.46	6.0	6.1
	II (n = 430)															
Cluster	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md	$\bar{X}$	Md
Parameter	22478.81	21894.61	564.24	556.76	189.36	185.69	149.84	149.53	197.22	193.01	6774.02	6406.87	30.66	31.49	5.9	5.9
Velue	314.27		4.69		1.53		1.30		1.58		115.15		0.45		0.1	
SE	21859.05	21188.06	554.99	546.10	186.36	183.13	147.27	146.19	194.11	189.62	6546.94	6207.42	29.76	30.54	5.7	5.8
95% LCL	23098.56	22664.15	573.50	568.77	192.37	189.62	152.40	151.94	200.33	197.60	7001.10	6588.96	31.55	32.46	6.0	6.1

Mean perimeter, mean diameter along main axis of glomerule and mean Feret's diameter show the same trend, as well as mean glomerular area during human aging, while mean diameter along secondary axis of glomerule shows continuous decrease from first to third age group (Table 1; Fig. 4). Changes of these parameters are not statistically significant which indicates that size and shape of investigated glomerules are not in correlation with aging process.

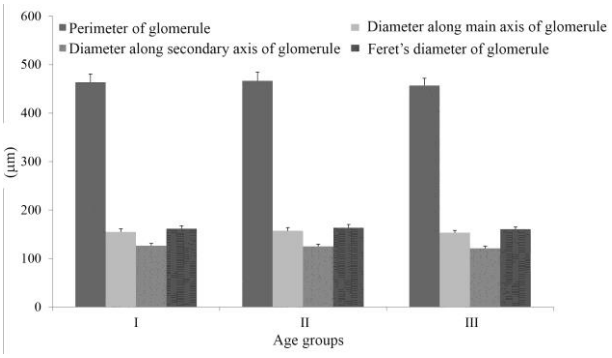

Fig. 4 Mean perimeter, mean diameter along main axis, main diameter along secondary axis and mean Ferret's diameter of glomerules of groups obtained by cluster analysis

Mean cell number per area unit significantly increases ($p < 0.05$) during aging (Table 1; Fig. 5). There is decrease of 11.60% in the second group compared to the first, decrease of 16.39% and 26% in the third compared to the second and the first, respectively. During aging process the glomerules show higher values of mean connective tissue area and percentage which is followed by decrease of cell number.

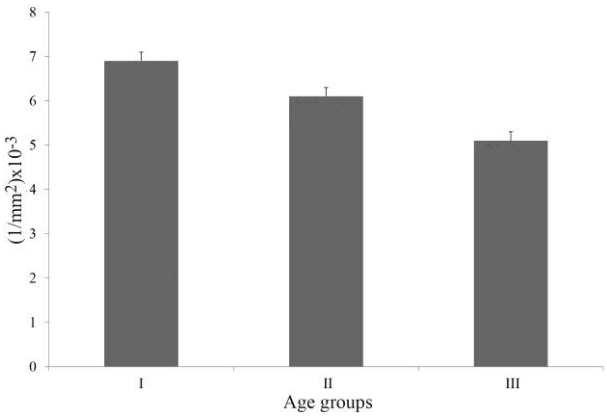

Fig. 5 Mean cellularity of glomerules of groups obtained by cluster analysis

Discussion

On the basis of abundant literature data about the changes in glomerules which precedes either focal (FSGS) or global glomerulosclerosis of kidney [10,13,21], in the first part of our study we estimated the increase of glomerular connective tissue area, as well as its areal fraction (percentage) per one glomerule. Additionally, we analyzed the relationship between the age and latter cited parameters in order to determine dynamics of connective tissue production during the aging and its influence on glomerular cellularity. Opposed to earlier investigations which mostly suggest the increase of sclerotic glomerules percentage during aging [22–24], our results showed increase of connective tissue even in the glomerules with non-manifested morphologically visible sclerosis and intensity of that increase. In the third, the oldest group connective tissue occupied 40.66% of total glomerular area, in the second 32.11%, and only 17.33% in the first group, whereby this process was at the same time followed with significant decrease of glomerular cell number which was the most prominent in the third group and somewhat milder in the second age group. Similar attempts of quantification of connective tissue were not found in available literature, but we tried to link it with the data about age-related alterations of the structural elements of the glomerule which might take part in the production of glomerular connective tissue. It was Vechner [25] who determined the rise of mesangial cells part up to 6.2% of glomerular volume in middle age and 10.4% in old age. Sorensen [26] investigated relation between mesangial cells, mesangial matrix, endothelial cells and other glomerular structures. His results showed that mesangial and endothelial cells occupied proportionally largest part of glomerule. If we take into account that proliferation of these cells leads to increased production of extracellular matrix and its connective tissue, then we can find correlation between age and increased percentage of connective tissue that we obtained. However, decreased cell number per area unit, followed by increased connective tissue percentage, may indirectly indicate that the glomerules may enter the process of global sclerosis, especially after age of 50, according to our results. One of the most interesting current findings is discovery of extreme variability in number of the glomerules in different individuals. Research by Neugarten et al. [17] showed that number, size of glomerules and kidney weight decreased with aging. Numerous researchers determined that the growth of mesangial matrix caused by collagen deposition, capillary obliteration and immune-mediated glomerular inflammation lead to progressive reduction of total cell number, particularly after age of 60 [13,20–22], which is also in accordance to our results. Mesangial matrix changes its protein structure and proliferates in initial phase enlarging the size of the glomerule that eventually leads to the accumulation of connective tissue in the glomerule and decrease in its size. All these alterations significantly affect development of age-related glomerulosclerosis [13]. The results of Kasiske's [23] morphological study demonstrated positive correlation between number, i.e. percentage of sclerotic glomerules and aging, as well as intrarenal vascular disease. Aging and intrarenal vascular disease together (with or without hypertension) directly correlate with glomerulosclerosis, particularly in outer parts of the cortex.

Glomerular hypertrophy is an important feature of FSGS, diabetic nephropathy, membranous glomerulonephritis, hypertension and obesity-related nephropathy. While glomerular hypertrophy may be useful for renal function in the short term, it seems that its presence becomes detrimental over a long period of time [27,28]. It is supposed that enlarged glomerules increase the risk of the onset of sclerosis as a consequence of hyperperfusion and high glomerular capillary pressure. Considering the inflammatory nature of glomerusclerosis in many renal diseases, it would be rational to expect that glomerular hypertrophy precedes glomerulosclerosis and that it also appears in the population that does not suffer from renal disorders.

Conclusion

From this point of view, our results, which suggest significant growth of connective tissue in glomerules during aging in the individuals that have not developed the signs of renal diseases, gain in importance.

Acknowledgments. The authors would like to thank the contract grant sponsor Ministry of Science and Technological Development of Republic of Serbia (nos. 41018 and 175092).

References

1. Kalinovskaia EG. Močevidateljnaia sistema. Biologia starenia. Nauka: Leningrad, 1982. (Russian)
2. Kowald A, Kirkwood TB. A network theory of ageing: the interactions of defective mitochondria, aberrant proteins, free radicals and scavengers in the ageing process. Mutat Res 1996; 316:209–236.
3. Lata H, Walia L. Ageing: Physiological aspects. JK Science 2007; 9:111–115.
4. Mackenzie HS, Taal MW, Luyckx VA, Brenner BM. Adaptation to nephron loss. In: Brenner BM (ed) Brener and Rector's the kidney, 6th ed. Saunders: Philadelphia, 2000; pp 1901–1942.
5. Zhou XJ, Rakheja D, Yu X, Saxena R, Vaziri ND, Silva FG. The aging kidney. Kidney Int 2008; 74:710–720.
6. Percy C, Pat B, Poronnik P, Gobe G. Role of oxidative stress in age-associated chronic kidney pathologies. Adv Chronic Kidney Dis 2005; 12:78–83.
7. Peppa M, Uribarri J, Vlassara H. Aging and glycoxidant stress. Hormones (Athens) 2008; 7:123–132.
8. Palmer BF, Levi M. Effect of ageing on renal function and disease. In: Brenner BM (ed) Brener and Rector's the kidney. Saunders: Philadelphia, 1996; pp 2274–2296.
9. Rodríguez-Puyol D. The aging kidney. Kidney Int 1998; 54: 2247–2265.
10. Martin JE, Sheaff MT. Renal aging. J Pathol 2007; 211:198–205.
11. Lindeman RD. Overview: renal physiology and pathophysiology of aging. Am J Kidney Dis 1998; 16:275–282.
12. Beck LH. Changes in renal function with aging. Clin Geriatr Med 1998; 14:199–209.
13. Silva FG. The aging kidney: A review – Part I. Int Urol Nephrol 2005; 37:185–205.
14. Mimran A. [Renal function and aging]. Nephrologie 1990; 11: 275–280 (French)
15. Epstein M. Aging and the kidney. J Am Soc Nephrol 1996; 7: 1106–1122.
16. Long DA, Mu W, Price KL, Johnson RJ. Blood vessels and the aging kidney. Nephron Exp Nephrol 2005; 101:95–99.
17. Neugarten J, Gallo G, Sibiger S, Kasiske B. Glomerulosclerosis in aging humans is not influenced by gender. Am J Kid Dis 1999; 34:884–888.
18. Tracy ER, Parra D, Eisaquirre W, Torres Balanza RA. Influence of arteriolar hyalinization on arterial intimal fibroplasia in the renal cortex of subjects in the US, Peru, and Bolivia, aplicable also to other populations. Am J Hypertens 2002; 15:1064–1073.
19. Marcantoni C, Ma LJ, Federspiel C, Fogo AB. Hypertensive nephrosclerosis in African Americans versus Caucasians. Kidney Int 2002; 62:172–180.
20. Stojanović V, Jovanović I, Ugrenović S, Pavlović S. Quantification of sclerotic renal glomeruli during the aging process in humans. Med Pregl 2010; 61:775–778.
21. Korbet SM. Primary focal segmental glomerulosclerosis. J Am Soc Nephrol 1998; 9:1333–1340.
22. Zheng F, Plati AR, Potier M, et al. Primary focal segmental glomerulosclerosis in B6 mice disappears after menopause. Am J Pathol 2003; 162:1339–1348.
23. Kasiske BL. Relationship between vascular disease and age-associated changes in the human kidney. Kidney Int 1987; 31: 1153–1159.
24. Schwartz MM, Churchill M, Bidani A, Churchill PC. Reversible compensatory hypertrophy in rat kidneys: Morphometric characterization. Kidney Int 1993; 43:610–614.
25. Wehner H. Stereologiche Untersuchungen am Mesangium normaler menschlicher Nieren. Wirchows Arch Abt A Pathol Anat 1968; 344:286–294.
26. Sorensen FH. Quantitative studies of the renal corpuscles III: The influence of postmortem delay before taking renal tissue samples and of the duration of tissue fixation. Acta Path Microbiol Scand A 1975; 83:251–258.
27. Basgen JM, Rich SS, Mauer SM, Steffes MW. Measuring the volumen density of the glomerular mesangium. Nephron 1988; 50:182–186.
28. Fogo AB. Progression versus regression of chronic kidney disease. Nephrol Dial Transplant 2006; 21:281–284.