

# STEREOLOGICAL STUDY OF COLLAGEN IN THE AGING OF URINARY BLADDER IN RATS

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## ABSTRACT

**Background:** The ageing process is inevitable and culminates in functional worsening of multiple systems, including the urinary bladder. Functional alterations of the bladder are recognised, as well as the importance of connective tissue and collagen to normal bladder functions. However, little is known about which are the structural alterations suffered by bladder in the aging process. **Purpose:** To describe and to quantify histological alterations related to collagen types I and III through stereology in rats submitted to the process of the ageing urinary bladder. **Methods:** Seventy-two Wistar rats were divided into six groups. Each group was sacrificed in different ages: 3, 6, 9, 12, 18 and 24 months. It was removed the urinary bladder and performed stereological and histological analysis of the bladder tissue's collagen (volumetric density of collagen types I and III, and their relative frequencies). **Results:** Between groups 1 (3 months) and 2 (6 months) there was a statistically significant increase in collagen type III. When compared groups of younger rats (3-12 months) to older rats (18-24 months), a significant decrease in volumetric density of collagen type III ( $p < 0.05$ ) is noticed. As for collagen type I, the highest relative frequency occurred at 12 months and suffered a decrease in the older groups 5 and 6 ( $p < 0.05$ ). **Conclusions:** There are significant alterations in volumetric densities of collagen types I and III with the ageing of the urinary bladder. We observed a decrease in volumetric density of collagen type III when compared younger to older rats.

**KEYWORDS** Aging, Urinary Bladder, Histology, Rats.

## Introduction

The events that take living beings from conception to birth, sexual maturing and adulthood eventually cease to be progressive and renewing as the ageing process takes its course [1].

The urogenital tract, including the urinary bladder, is primarily affected by ageing mechanisms. This organ must play the role of storing socially adequate volumes of urine while keeping constant pressure, to finally be emptied voluntarily under low pressure and low resistance to urinary flow [2,3].

The bladder is a hollow organ, and the structure of its wall consists of four distinct parts: a) an inner layer, delineated by the urothelium; b) the lamina propria, composed mainly by irregular and dense connective tissue, fibroblasts and a thin layer of muscularis propria surrounded by c) an external layer of smooth muscle (detrusor muscle), with fibers well organized in bundles and connective tissue rich in collagen and elastin involving its perimysium and d) an adventitious external layer of loose connective tissue [4,5].

The many components of the bladder play specific and integrated roles for the correct functioning. The connective tis-

sue and the collagen are intrinsically connected to the elastic properties of the bladder and adaptation of bladder to different pathophysiological situations, with implications in fundamental features of the bladder like accommodation of volume and complacency [2,6-8].

Some experimental studies are trying to correlate morphological, structural and histological alterations in urinary bladder to events related to ageing demonstrating that the aged urinary bladder shows functional alterations like reduced complacency, an increase of after post-volume residues as well as detrusor hyperactivity, impaired contractility or the combination of both [9-12]. Although the functional alterations of the bladder with ageing are recognized, as well as the importance of connective tissue and collagen to normal bladder functions, little is known about which are the structural alterations suffered by bladder in the aging process along with the correlation between these alterations to clinical and functional events related to the urinary bladder aging.

This article aims to describe and quantify the histological modifications related to collagen types I and III in the process of the ageing urinary bladder.

## Materials & Methods

### Experimentation

The conduction of the present study was previously approved by the Ethics Committee of the institution as demanded by the Canadian Council on Animal Care. Seventy-two albino male Wistar rats (*Rattus norvegicus albinus*, Rodentia, Mammalia) were used. The test subjects were evenly distributed in 24 cages (3 animals per cage) of polyethylene measuring 60 x 50 x 22 cm. Humidity and temperature (22 °C) were controlled and kept constant for the study's duration. The animals were subjected to a 12-hour bright/dark cycle and were sheltered from external noise. They received ad libitum filtered water and commercial feed specific for their species.

The rats were divided into six groups of 12 animals, which were sacrificed in chronological order of age: group 1 with three months, group 2 with six months, group 3 with nine months, group 4 with 12 months, group 5 with 18 months and group 6 with 24 months.

At each predetermined point in time, the animals' biometric data were obtained (weight, stature, and body mass index applying the formula with weight in grams and stature in cm<sup>2</sup>).

Afterwards, the animals were put under anaesthesia by applying, intraperitoneally, 1mL/1000g (of living weight a solution) of ketamine (57.67 mg/ml) associated with 2% of Xylazine Hydrochloride(0.2g/10 mL). Under anaesthesia, the animals were then fixed on a surgical board and submitted to abdominal and thoracic antiseptis followed by a thoracic and abdominal medium incision. The right atrium was then incised leading to death by exsanguination. Immediately after the cardiorespiratory arrest, the urinary bladder, as well as other organs (used in other studies) like kidneys, testicles, liver, penis, brain, heart and the aorta, were removed.

After removal, the bladder was opened longitudinally, submerged and fixated in boudin (picric acid, formol and acetic acid) for 24 hours. Afterwards, the pieces remained in alcohol 70%, and then dehydrated in a decreasing series of xylol and alcohols (solution of xylol 1, solution of xylol 2, solution of absolute ethylic alcohol and xylol, absolute alcohol 1, 2 and 3, alcohol 95%, alcohol 90%, alcohol 80%, alcohol 70%, running water, distilled water, 1 to 2 hours in each solution).

After total dehydration, the urinary bladders were then submerged in paraffin and transformed in blocks that were subsequently submitted to successive cuts using a microtome (American Optical, Spencer AO 820). The cuts (5 µm thickness) were then put together in slides and stained using the Picrosirius method (solution of Sirius Red F3BA in 0,1% and saturated aqueous solution of picric acid by approximately 60 minutes) to identify the collagen fibres of the bladder and be stereologically analysed.

The thicker collagen fibres, strongly birefringent, that represent collagen type I was coloured in different shades of orange, yellow and red, while the thinner and more dispersed fibres, collagen type III, were coloured in green. Other elements of the bladder wall were coloured in black.

The area occupied by the collagen fibres was analyzed with an optical microscope of polarised light, biologic plan 0.1 (Polaris – B.photonics) bound to a B-Xtreme Intel G2030 Dual core processor computer coupled to a digital colour camera with CMOS and Hoe Mirage I image capture systems.

Images of 10 random histological fields (400x) of each histological slide were captured. Posteriorly, these images were analysed quantitatively for collagen fibres (coloured with Picrosirius Red) using the windows program Image Pro-plus, version 4.5 of cybernetics® on a Pentium III computer.

All values obtained were transformed in percentages (%) to find the relative volumetric densities of collagen in the studied structures.

### Statistical Analysis

The medium values of collagen density of each rat were considered as the average density of the ten histological fields analysed. The medium values of each group were described using summary measures such as average, standard deviation, median, minimum and maximum values. To compare the quantitative variables between the groups, analysis of variances (ANOVA) and multiple comparisons of Tukey were used. Values with  $p < 0.05$  were considered statistically significant.

## Results

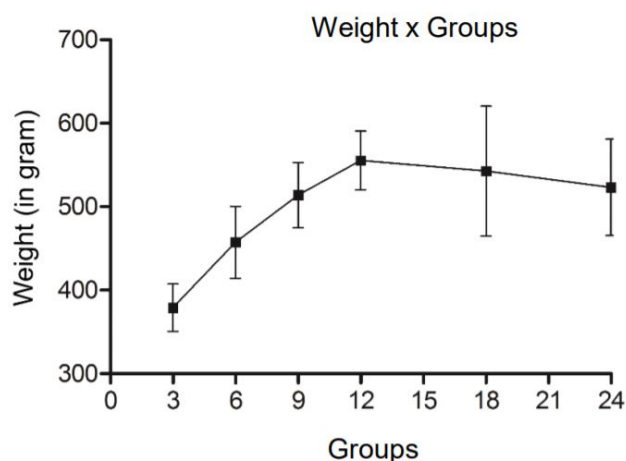
There were no losses during the study. The older animals showed clear signs of ageing such as hair rarefaction and decreased activity. The biometric data shows that the animals had an increase in weight until 12 months of age and a subsequent discreet decrease which led to a stable body mass index in the groups older than 12 months (Graphic 1).

Table 1 shows the obtained volumetric density values of collagen type I and III. The highest volumetric density of collagen type I occurred in group 4 (12 months of age) with an average of 19.81% of the composition of the bladder wall. On the other hand, the lowest volumetric density value occurred in group 2 (6 months of age) with an average of 8.08%. When it came to collagen type III, the highest volumetric density average, 3.62%, was found in group 3 (9 months of age). A sharp drop in volumetric densities was then recorded in the groups 5 and 6, reaching the minimum average of 0,12% in group 6 (24 months of age). These differences in the collagen type III between the younger and the older groups among maximum volumetric density (9-months group) and minimum values (24-months groups) are exemplified in Figure 1.

Graphs 2 and 3 visually illustrate the results presented in Table I.

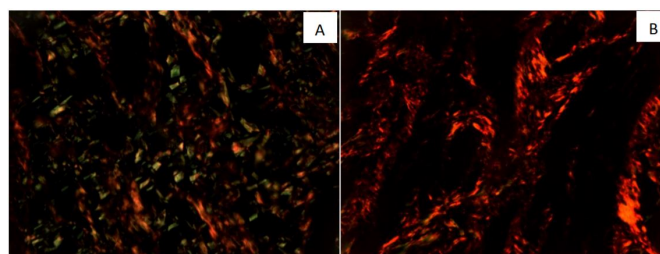
**Table 1** Description of average values of collagen according to groups and results of ANOVA.

	Group	Average %	SD	Median	Minimum	Maximum	N	p
<b>Type-I Collagen</b>	3 months	14,82	4,57	15,19	7,68	20,70	12	<0,001
	6 months	8,08	3,27	6,34	4,55	13,87	12	<0,001
	9 months	8,62	3,82	6,98	4,55	14,47	12	<0,001
	12 months	19,81	5,49	19,03	12,02	34,01	12	<0,001
	18 months	10,29	1,24	10,78	7,89	11,91	12	<0,001
	24 months	8,87	2,02	9,14	4,93	11,21	12	<0,001
<b>Type-III Collagen</b>	3 months	2,13	1,27	1,91	0,36	4,27	12	<0,001
	6 months	3,39	0,97	3,30	2,34	5,82	12	<0,001
	9 months	3,66	1,16	3,48	2,34	5,82	12	<0,001
	12 months	3,42	1,17	3,39	1,09	4,88	12	<0,001
	18 months	0,68	0,32	0,62	0,36	1,25	12	<0,001
	24 months	0,12	0,24	0,01	0,00	0,84	12	<0,001



**Graphic 1:** Weight versus groups results, showing subsequent increase in the weight of the animals from the group 3 to 12 months, and then a slightly decrease in older rats.

In Table 2, the relative frequencies of collagen type I and III are presented through multiple comparisons between the groups. Between the groups 1 and 2 there was a statistically significant increase in collagen type III. An increase of collagen type III between the groups 2 and 3 and a slight decrease in group 4 (12 months) was also noted; however, there was no statistical significance. When comparing groups of younger rats (3-12 months) to older rats (18-24 months), a significant decrease in volumetric density of collagen type III ( $p < 0.05$ ) is noticed. For collagen type I, the highest relative frequency occurred at 12 months and suffered a decrease in the older groups 5 and 6 ( $p < 0.05$ ).



**Figure 1:** Microscopic analysis under polarized light of bladder wall structure. Green points represent collagen type III. Collagen type III on 9-months group (A), at maximum volumetric density, versus minimum volumetric density seen on 24-months group (B)

## Discussion

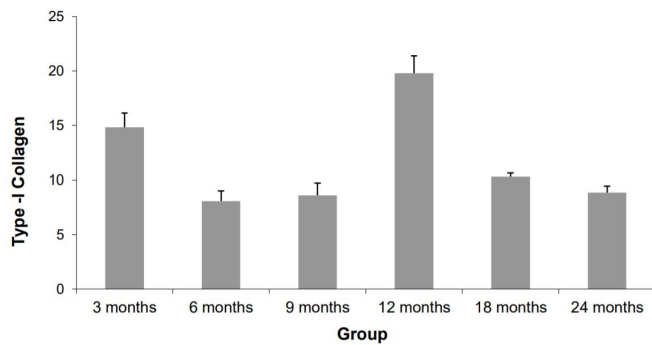
The median age of the world's population is increasing, and the factors implied in this shift are related to the decline in fertility and a 20-year increase in the average life. The average lifespan is expected to extend another ten years by 2050 [13]. In Brazil, the number of ageing adults increased from 3 million in the 1960's to 14 million in 2002 (a 500% growth in 40 years), and this population is estimated to reach 32 million people in 2020 [14]. The growing number of senior citizens increases the demand for the public health system and social services [15].

Almost every physiologic system has its functions diminished with the ageing process [16,17], and the urinary tract is no exception [18]. Clinical urodynamic studies have demonstrated that advanced ages are associated with a reduced bladder capacity, an increase in uninhibited contractions, a decreased urinary flow rate, and an increased post-void residual urine volume [19,20]. The ageing bladder specifically may be narrowed down to detrusor hyperactivity, impaired contractility, or a combination of both [21].

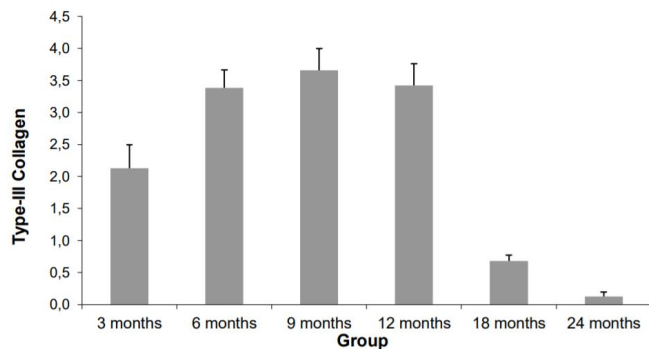
Zhao et al., using Fischer/Brown Norway rats, revealed that

**Table 2** Multiple comparisons of average values of collagen among groups.

	Comparison		Average difference	Standard error	p	CL 95% Inferior	CL 95% Superior
<b>Type- I</b>	3 months	6 months	6,74	1,51	<0,001	2,31	11,17
<b>Collagen</b>	3 months	9 months	6,20	1,51	0,002	1,77	10,63
	3 months	12 months	-4,99	1,51	0,018	-9,42	-0,56
	3 months	18 months	4,54	1,51	0,042	0,11	8,97
	3 months	24 months	5,95	1,51	0,003	1,52	10,38
	6 months	9 months	-0,54	1,51	0,999	-4,97	3,89
	6 months	12 months	-11,73	1,51	<0,001	-16,16	-7,30
	6 months	18 months	-2,21	1,51	0,688	-6,64	2,22
	6 months	24 months	-0,79	1,51	0,995	-5,22	3,64
	9 months	12 months	-11,19	1,51	<0,001	-15,62	-6,76
	9 months	18 months	-1,67	1,51	0,877	-6,10	2,76
	9 months	24 months	-0,25	1,51	>0,999	-4,68	4,18
	12 months	18 months	9,52	1,51	<0,001	5,09	13,95
	12 months	24 months	10,94	1,51	<0,001	6,51	15,37
	18 months	24 months	1,42	1,51	0,935	-3,01	5,85
<b>Type- III</b>	3 months	6 months	-1,26	0,39	0,022	-2,40	-0,12
<b>Collagen</b>	3 months	9 months	-1,53	0,39	0,003	-2,67	-0,40
	3 months	12 months	-1,30	0,39	0,017	-2,44	-0,16
	3 months	18 months	1,45	0,39	0,005	0,31	2,59
	3 months	24 months	2,00	0,39	<0,001	0,86	3,14
	6 months	9 months	-0,28	0,39	0,980	-1,41	0,86
	6 months	12 months	-0,04	0,39	>0,999	-1,18	1,10
	6 months	18 months	2,71	0,39	<0,001	1,57	3,85
	6 months	24 months	3,26	0,39	<0,001	2,12	4,40
	9 months	12 months	0,24	0,39	0,990	-0,90	1,38
	9 months	18 months	2,98	0,39	<0,001	1,84	4,12
	9 months	24 months	3,54	0,39	<0,001	2,40	4,68
	12 months	18 months	2,74	0,39	<0,001	1,61	3,88
	12 months	24 months	3,30	0,39	<0,001	2,16	4,44
	18 months	24 months	0,55	0,39	0,709	-0,58	1,69



**Graphic 2:** Average values of collagen type I and respective deviation patterns.



**Graphic 3:** Average values of collagen class III and respective deviation pattern according to groups.

even when external factors remain constant, the onset of the ageing process is accompanied by, an increase in bladder capacity, post-residual volume and micturition frequency, and a decrease in micturition pressure, when comparing old versus young rats. These findings are consistent with the physiological alterations present in ageing humans. This validates the animal model in bladder ageing studies [22]. In the presented study, the animals of the last group's lifetime were 720 days, therefore based on their life cycle, they can be considered elderly rats [23]. The development of the animals judging by their weight was deemed to be adequate when compared to the pattern curves found in the literature [24].

Even though we know that the viscoelastic properties of the bladder are intimately related to the Types I and III collagen [2,4,25], we do not know precisely how these structural components behave during the ageing process. Albeit some studies tried to describe the alterations these structural components go through during pathologic processes.

Previous studies have demonstrated that collagen synthesis and deposition are increased in organ fibrosis disorders such as myocardial fibrosis and fibrotic diseases of the skin [26,27], and it is generally accepted that in the early stages of a fibrotic lesion there is enhanced synthesis of collagen type III, while type I collagen synthesis predominates at later stages [4]. Histologic and biochemical studies have shown that non-compliant bladder is characterised by an abnormal deposition of extracellular matrix proteins in the muscular layer of the bladder wall [28,29]. Deveaud and colleagues, in a molecular analysis of collagens, in a pediatric subset of the population with "non-compliant" bladders (neurogenic and non-neurogenic) detailed that in these pathological conditions. There is an increase in total collagen

deposition, an increase in the type III:type I ratio (from 1:3 in normal bladders to 1:2 in non-compliant bladders), and an apparent increase in the type III collagen fibers quantity, of almost 48%, mainly by infiltrating the detrusor muscle layer. It shows that not only is there an increase in the amount of collagen type III, but also a modification in the distribution of this collagen, being hypothesised a significant role of collagen type III in the bladder dysfunctions [4]. Using a model of the obstructed bladder in young rabbits, Tekgul et al. reported an increase in deposition of type-I collagen between the muscular bundles and increased staining for type-III collagen in and around the muscular bundles [30].

The mechanism by which collagen accumulates has not been completely elucidated. Collagen deposition may be the result of increased protein synthesis, decreased degradation, or a combination of both. Kaplan et al., studying pediatric patients with urodynamically proven non-compliant bladders, using a reverse transcription-polymerase chain reaction technique to quantify messenger RNA (mRNA) have shown that type III collagen mRNA levels are increased in these fibrotic sets, stating that the accumulation of type III collagen protein is transcriptionally regulated [2]. Deveaud et al. found that while there are increased levels of mRNA of both procollagen types I and III in non-neurogenic non-compliant bladders, only an increase deposition of type III collagen was shown, with no alteration on protein levels of type I collagen in this subset of patients. It suggests that besides the genetic mechanisms regulating fibrotic deposition in non-compliant non-neurogenic bladders, there is a post-transcriptional mechanism of protein synthesis regulation [4]. So, further studies are required to elucidate the mechanisms in the cell that are responsible for the altered collagen expression and deposition.

It is worth mentioning that in these studies, the structural alterations of the bladder wall were described during pathologic phenomena of the bladder (obstruction, neurogenic bladder). Our study observes the quantitative alterations that collagen types I and III go through during the natural ageing process, which is not necessarily pathologic in nature [1]. This demonstrates the importance of our study in expanding new horizons in the discussion of the ageing mechanisms of the urinary bladder and urogenital tract.

Alterations in the volumetric densities of collagen type I and III occurred along the age curve of the studied groups. Although it was not possible to establish a pattern that may predict quantitative alterations of collagen type I, such pattern may have been established when analysing type III collagen quantities.

Between groups 1 and 2 (3 and 6 months of age), there was a statistically significant increase in collagen type III; an increase of collagen type III between groups 2 and 3 (6 and 9 months of age) and a discrete reduction of the type III collagen relative frequency between groups 3 and 4 were noticed. However, these alterations were not statistically significant which may denote quantitative stability of collagen type III in this age range (between 6 and 12 months).

In the older rats groups (18 and 24 months) there was an abrupt drop in the volumetric density of type III collagen, with the statistically significant difference between the older and younger groups (<12 months). This suggests that the decrease of collagen type III volumetric density occurs sometime after 12 months of age. Therefore in ageing rats, not necessarily affected by pathological processes of the urinary bladder there is a reduction in the deposition of type III collagen.

It was not the goal of this article to correlate the alterations of collagen types I and III with eventual functional alterations of the urinary bladder in ageing, and with the data showed either is possible to establish this correlation. However, there is a new vision in the discussion about the structural alterations that follow the process of ageing.

## Conclusion

There are significant alterations in the volumetric densities of collagen type I and more notably III that accompany the ageing process in the urinary bladder of rats. We observed a decrease in the volumetric density of collagen type III when comparing younger versus older rats. This decrease occurs after 12 months of life.

## Authors' Statements

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## References

1. Hayflick L. How and why we age. *Exp Gerontol* 1998;33(7-8):639-53.
2. Kaplan EP, Richier JC, Howard PS, Ewalt DH, Lin VK. Type III collagen messenger RNA is modulated in non-compliant human bladder tissue. *J Urol* 1997; 157:2366-69.
3. Chang SL, Howard PS, Koo HP, Macarak EJ. Role of type III collagen in bladder filling. *Neurourol Urodyn* 1998; 17(2):135-45.
4. Deveaud CM, Macarak EJ, Kucich U, Ewalt DH, Abrams WR, Howard PS. Molecular analysis of collagens in bladder fibrosis. *J Urol*. 1998; 160(4):1518-27.
5. Andersson KE, Arner A. Urinary bladder contraction and relaxation: physiology and pathophysiology. *Physiol Rev*. 2004; 84(3):935-86.
6. Macarak EJ, Howard PS: The role of collagen in bladder filling. *Adv Exp Med Biol* 1999; 462:215-223.
7. Chang SL, Chung JS, Yeung MK, Howard PS, Macarak EJ. Roles of the lamina propria and the detrusor in tension transfer during bladder filling. *Scand J Urol Nephrol Suppl* 1999;201:38-45.
8. Stevenson K, Kucich U, Whitbeck C, Levin RM, Howard PS. Functional changes in bladder tissue from type III collagen-deficient mice. *Mol Cell Biochem* 2006;283(12):107-14.
9. Resnick NM, Yalla SV. Detrusor hyperactivity with impaired contractile function. An unrecognized but common cause of incontinence in elderly patients. *JAMA* 1987;257:3076-3081.
10. Ameda K, Sullivan MP, Bae RJ, Yalla SV. Urodynamic characterization of nonobstructive voiding dysfunction in symptomatic elderly men. *J Urol* 1999;162:142- 146.
11. Siroky MB. The aging bladder. *Rev Urol* 2004; 6 Suppl 1:S3-7.
12. Hashim H, Abrams P. Overactive bladder: an update. *Curr Opin Urol* 2007;17:231.
13. United Nations. Report of the Second World Assembly on Aging. Madrid, Spain: United Nations, April 8-12, 2002.
14. Lima-Costa MF, Veras R. Envelhecimento e saúde pública. *Caderno de Saúde Pública do Rio de Janeiro* 2003; 19(3): 700-1.
15. Centers for Disease Control and Prevention (CDC). Trends in aging—United States and worldwide. *MMWR Morb Mortal Wkly Rep* 2003; 52(6):101-4, 106.
16. Geokas MC, Contreas CN, Majumdar AP. The aging gastrointestinal tract, liver, and pancreas. *Clin Geriatr Med* 1985;1:177-205.
17. Egashira K, Inou T, Hirooka Y, et al. Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. *Circulation* 1993;88:77-81.
18. Diokno AC, Brock BM, Brown MB, Herzog R. Prevalence of urinary incontinence and other urological symptoms in the noninstitutionalized elderly. *J Urol* 1986;136:1022-1025.
19. Diokno AC, Brown MB, Goldstein NG, Herzog AR. Urinary flow rates and voiding pressures in elderly men living in a community. *J Urol* 1994;151:1550-1553.
20. Madersbacher S, Pycha A, Schatzl G, et al. The aging lower urinary tract: a comparative urodynamic study of men and women. *Urology*. 1998;51:206-212.
21. Resnick NM, Yalla SV. Detrusor hyperactivity with impaired contractile function. An unrecognized but common cause of incontinence in elderly patients. *JAMA*. 1987.
22. Zhao W, Aboushwareb T, Turner C, et al. Impaired Bladder Function in Aging Male Rats. *J Urol* 2010; 184(1): 378-385.
23. Quinn R. Comparing rat's to human's age: how old is my rat in people years? *Nutrition* 2005; 21(6): 775-7.
24. Cossio-Bolaños M, et al. Reference curves for assessing the physical growth of male Wistar rats. *Nutr Hosp* 2013;28(6):2151-6.
25. Chung JM, Jung MJ, Lee SJ, Lee SD. Effects of Prolyl 4-Hydroxylase Inhibitor on Bladder Function, Bladder Hypertrophy and Collagen Subtypes in a Rat Model With Partial Bladder Outlet Obstruction. *Urology* 2012;80(6):1390.e7-1390.e12.
26. Weber K, Sun Y, Guarda E, Katwa L, Ratajska A, Cleutjens J, Zhou G. Myocardial fibrosis in hypertensive heart disease: an overview of potential regulatory mechanisms. *Eur Heart J* 1995;16:2:4.
27. Rockwell, W., Cohen, I. and Ehrlich, H.: Keloids and hypertrophic scars: a comprehensive review. *Plast. Reconst. Surg* 1989; 84: 827.

28. Landau EH, Jayanthi VR, Churchill BM, Shapiro E, et al. Loss of elasticity in dysfunctional bladders: urodynamic and histochemical correlation. *J Urol* 1994;152:702.
29. Shapiro E, Becich MJ, Perlman E, Lepor H. Bladder wall abnormalities in myelodysplastic bladders: a computer assisted morphometric analysis. *J Urol* 1991;145:1024.
30. Tekgul S, Yoshiino K, Bagli D, et al. Loss of elasticity in dysfunctional bladders: urodynamic and histochemical correlation. *J Urol* 1994;152(2 Pt 2):688-691.