Influence of a hypercholesterolemic diet on the collagen composition of the bladder wall extracellular matrix in rats

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Summary. Purpose: To investigate the effects of hypercholesterolemic diet on the collagen composition of urinary bladder wall. Materials and methods: Forty-five female 4-week-old Wistar rats were divided into three groups: 1) control group fed a normal diet (ND); 2) model of bladder outlet obstruction (BOO) group fed a ND; and 3) group fed a HCD (1.25% cholesterol). Total serum cholesterol, LDL cholesterol and body weight were assessed at baseline. Four weeks later, group 2 underwent a surgical procedure resulting in a partial BOO, while groups 1 and 3 underwent a sham similar surgical procedure. Six weeks later, all animals had their bladders removed; serum cholesterol and LDL cholesterol levels and body weights were measured. Morphological and morphometric analysis was performed by Picrosirius staining and collagen types I and III were identified by immunofluorescence. Statistical analysis was completed and significance was considered when p<0.05. Results: Rats fed an HCD exhibited a significant increase in LDL cholesterol levels (p<0.001) and body weight (p=0.017), when compared to the groups fed a ND during the ten-week study period. Moreover, the HCD induced morphological alterations of the bladder wall collagen, regarding thin collagen fibers and the amounts of type III collagen when compared to the control group (p=0.002 and p=0.016, respectively), resembling the process promoted in the BOO model. Conclusions: A hyper-cholesterolemic diet in Wistar rats promoted morphological changes of the bladder types of collagen, as well as increases in body weight and LDL cholesterol.

Key words: Collagen, Extracellular matrix, Urinary bladder, Dietary cholesterol, Wistar rats

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Introduction

The primary function of the bladder is the storage and emptying of an adequate urine volume. A complex neurological pathway including the brain, spinal cord and peripheral nerves is responsible for controlling the urethral and bladder smooth muscle activity (Torrens, 1987; de Groat et al., 1993; Yoshimura and de Groat, 1997). Bladder compliance and its intrinsic myogenic properties are responsible for maintaining the bladder at low pressure during its filling (Abrams et al., 1988). Many pathological conditions can lead to bladder dysfunction, such as neurological disorders, abnormalities in detrusor muscle cells and interstitial alterations. Bladder extracellular matrix is composed predominantly of collagen (50%) and elastin (2%), and lies on a bed of proteoglycans, blood and lymph vessels and nerves (Chancellor and Yoshimura, 2002). The most prevalent types of collagen found in the bladder are types I, III and V (Macarak et al., 1995; Chang et al., 1998); they are able to change their conformation in response to bladder wall stretching when the intravesical volume increases, resembling a coil (Chang et al., 1998; Chancellor and Yoshimura, 2002). Type III collagen is the first type of collagen to be synthesized in the bladder fibrosis process (Teodoro and Yoshinari, 2009).

Chronic partial bladder outlet obstruction is a prevalent condition that may lead to bladder dysfunction and, over the long term, irreversible bladder fibrosis (Uvelius et al., 1991; Sutherland et al., 1998; Hanai et al., 2002, 2003; Li et al., 2006). Ischemia followed by reperfusion is the primary pathophysiological mechanism of bladder fibrosis (Azadzoi et al., 1999; Ghafar et al., 2002; Shabsigh et al., 2002), although reperfusion is the most aggressive causative factor (Bratslavsky et al., 2003). Many other conditions cause bladder fibrosis, such as diabetes, aging and dislipidemia. Dislipidemia may consist of hyper-

triglyceridemia, hypercholesterolemia or both.

Hypercholesterolemia is a risk factor in many cardiovascular diseases and may also affect the genitourinary tract. Experimental models of hypercholesterolemia have demonstrated prostate enlargement, detrusor overactivity and erectile dysfunction (Rahman et al., 2007; Son et al., 2007; Xie et al., 2007). Although hypercholesterolemia has been associated with bladder dysfunctions, there is no strong scientific evidence associating this metabolic alteration to structural changes of the bladder wall. These structural changes may lead to impairment of both bladder compliance and detrusor contractile function. The purpose of this study was to verify if a hypercholesterolemic diet promotes changes of collagen types I and III in rat detrusor layer.

Materials and methods

The entire experiment was conducted at the Bioterism Centre, Medical Investigation Laboratory of Rheumatology and Medical Investigation Laboratory of Urology, at the School of Medicine of the São Paulo University, Brazil. The animals received humane care in compliance with the *Guide for care and use of laboratory animals* (NIH Publication 85-23, revised 1985) and was approved by the Ethical Committee of our institution.

Experimental protocol

Forty-five female four-week-old Wistar rats were divided into three groups, each consisting of 15 rats: 1) control group fed a normal rat diet (ND); 2) model of bladder outlet obstruction (BOO) group fed a ND; and 3) group fed a hypercholesterolemic diet (HCD; 1.25% cholesterol). Initially, all rats underwent biochemical tests to determine serum total cholesterol and LDL cholesterol levels. Body weight was also assessed. Four weeks later, all animals underwent surgery: group 2 underwent a partial BOO, while groups 1 and 3 underwent a similar, incomplete operation that did not result in a BOO. Under general anesthesia, a midline longitudinal incision was made. The urethra was dissected and isolated. In groups 1 and 3, the procedure was concluded at this time. In group 2, an unabsorbed 5-zero Nylon suture was passed and tied loosely around the urethra with a 22G needle, leaving approximately 1 mm of residual lumen (Steers et al., 1991). All groups were still fed their particular diet (groups 1 and 2 with the normal diet and group 3 with the HC diet). After six more weeks, for a total of ten weeks of study, biochemical analyses and body weight measurements were performed again in all groups. Surgery was performed again and all animals had their bladder removed and were then euthanatized in a CO_2 chamber.

After fixation, the tissue was embedded in paraffin. Three-micrometer-thick slices were obtained by means of a microtome and then stained with hematoxylin/eosin and Picrosirius (Junqueira et al., 1979; Vidal et al., 1982) (Bio-Optica Milano s.p.a., Milan, Italy).

Collagen evaluation

The bladder collagen remodeling was evaluated at the detrusor layer to characterize the ECM deposition. Collagen was stained in a 0.2% solution of Sirius Red (Direct Red 80,C.I. 35780, Aldrich, Milwaukee, WI) dissolved in aqueous saturated picric acid, and observed under polarized light microscopy. The enhancement of collagen birefringence promoted by the Picrosirius polarization method is specific for collagen structures composed of aggregates of oriented molecules. The analysis of the reactions was observed in polarized light with an Olympus microscope (Montes and Junqueira, 1991; Montes, 1996).

Immunofluorescence assays for collagen I and III

For collagen types I and III detection the paraffin-embedded samples were mounted on slides of aminosylane (Sigma Chemical Co., St Louis, Missouri, USA). The slides were soaked in xylene and dehydrated in decreasing ethanol concentrations. The immunogenical sites were exposed by enzymatically treating bladder samples with bovine pepsin 4 mg/ml (10,000 unit/tissue dray (UTD); Sigma Co., St Louis, Missouri, USA) in acid buffer, pH 2.2, for 30 min at 37°C, followed by incubation in phosphate-buffered 5% milk, pH 7.0. After this process, the slides were incubated overnight at 4°C with rabbit polyclonal anti-collagen types I and III antibodies and diluted to 1:50 and 1:100, respectively, in phosphate-buffered saline (PBS). The cross reactivity of types I and III antibodies with other collagen types was evaluated by "immunoblot" (Teodoro et al., 2003). Next, all slides were washed several times in PBS-0.05% Tween 20 and incubated for 90 min with a secondary antibody (fluorescein-conjugated rabbit anti-IgG, Sigma, St Louis, Missouri, USA) diluted to 1:50 in PBS containing 0.006% Evans blue. The slides were again washed several times in PBS-0.05% Tween 20 and mounted with buffered glycerin (Teodoro et al., 2003, 2004). The evaluation of the reaction was observed in an Olympus fluorescence microscope.

Confocal microscopy

We used laser-scanning confocal microscopy (LSCM) for the 3D image reconstruction to evaluate type I collagen in muscular layer sections, comparing the different groups. These sections were examined with a LSCM (LSM 410; Carl Zeiss) using a 340 objective. Images were stored in the laser-scanning microscope, formatted, and stored in a computer (LSM Image Browser software, ver. 2,50.0929; Carl Zeiss) as tagged information file format files. A series of 45 or 50 optical sections were produced with a 0.5 mm distance.

Tridimensional (3D) reconstruction of collagen types I was obtained by immunofluorescence microscopy, as previously described, and observed with a Zeiss LSM 510 META/UV confocal laser-scanning microscope.

Collagenous fibers histomorphometric analysis

Characterization of the bladder collagenous fibers was considered only at the muscular layer and its extracellular matrix, excluding blood vessels and nerves. The background in the optical field was standardized. All the fields were acquired on the same day, in order to provide adequate tissue analysis of thick and thin collagen fibers under Picrosirius polarized light microscopy, obtained with a 400 magnification.

Polarized light was used to intensify the normal birefringence of collagenous fibers and to determine the location of collagen-containing fibers. The number of collagen fibers in the bladder wall was determined by an image analysis system in an optical microscope equipped with a light polarizer coupled to an image analyzer. The system consisted of a Q-Color 5 camera, coupled to an Olympus microscope, from which the images could be visualized on the monitor. The images were processed through a digital system installed in a computer (Pentium 4, 300 Mhz) using the Image-Pro-Plus, version 6.0 software. The enhancement of collagen birefringence promoted by the Picrosirius polarization method is specific for collagenous structures composed of aggregates of orientated molecules. The threshold for collagenous fibers was established for each slide after enhancing the contrast up to a point at which the fibers were easily identified as birefringent (collagen) bands. The color of collagen fibers stained with Picrosirius red and viewed with polarized light depends upon fiber thickness; as fiber thickness increases, the color changes from green-yellowish to orange-reddish (Junqueira et al., 1982; Hiss et al., 1988). The area occupied by the fibers was determined by digital densitometric recognition, by adjusting the threshold level of measurement to all the fibers of the collagenous system. The collagen content was measured in the muscular layer and expressed as a relationship between the quantities of collagen fibers divided by the total area of bladder studied, expressed as a fraction.

The amount of collagen types I and III were quantified by immunofluorescence using the same method.

Statistical analysis

Numerical values were presented as average ± standard deviation and statistical analysis was performed using the SPSS version 12.0 software for Windows. The categorical variables were tested through the ANOVA method and group comparisons through the one-way ANOVA method. When their suppositions were satisfied Tukey multiple comparisons were performed (Singer and Andrade, 2000). Statistical significance was considered when the p-value was <0.05.

Results

All but one animal from group 2 completed the experiment. In order to compare the metabolic changes, regarding the serum total and LDL cholesterol levels and body weight gain, groups 1 and 2 (rats fed a ND) were combined to compare to the group fed a HCD. However, twelve animals from groups 1 and 2 were not included in these tests because of a lack of data due to storage problems. All biochemical and weight gain parameters over the ten-week study period are summarized in Table 1. Otherwise, in morphological analysis, all groups were compared independently and all animals were considered.

Biochemical analysis

No difference was found in the serum total cholesterol levels between the animals fed a HCD or ND (p=0.376). On the other hand, LDL cholesterol was significantly higher in the HCD group relative to the ND groups (p=0.041).

Body weight

The group fed a HCD presented a weight gain significantly higher than the groups fed a ND (p=0.017).

Morphological analysis

The mean thickness of the muscle layer of each group was 33.43±3.41 μm in group 1, 36.99±5.42 μm in group 2 and 33.71±6.54 μm in group 3, with no statistical difference among them (p=0.147).

Picrosirius-red staining detected a similar amount of thick collagen fibers (p=0.311), but a significant difference of thin collagen fibers among the three groups (p=0.002). This difference was due to a larger quantity of type III collagen fibers in group 3 relative to group 1 (p=0.002, Δm 0.852 μm; CI 95% 0.292-1.412). Group 2 also showed a larger quantity of these fibers when compared to group 1, although it did not reach statistical significance (p=0.055, Δm 0.560 μm; CI 95% -0.010-1.130). Figures 1 and 2 illustrate these data.

Immunohistochemistry for types I and III collagen showed a statistical difference between the groups. In type I collagen analysis, this difference (p=0.005) was due to a larger quantity of this collagen in group 2 when compared to group 1 (p=0.003, Δm 1.439 μm; CI 95% 0.444-2.434). The other comparisons were not significantly different (group 1 vs. group 3: p=0.225, Δm 0.687 μm; CI 95% -0.308-1.682; and group 2 vs. group 3: p=0.170, Δm 0.752 μm; CI 95% -0.243-1.747). In type III collagen analysis, both groups 2 and 3 presented statistically significant larger quantities of collagen when compared to the control group (group 1 vs. group 2: p=0.002, Δm 3.055 μm; CI 95% 1.135-4.975; and group 1 vs. group 3: p=0.016, Δm 1.559 μm; CI 95% 0.262-2.857). Groups 2 and 3 were also similar in this analysis (p=0.211, Δm 1.496 μm; CI 95% -0.586-3.577). Figures
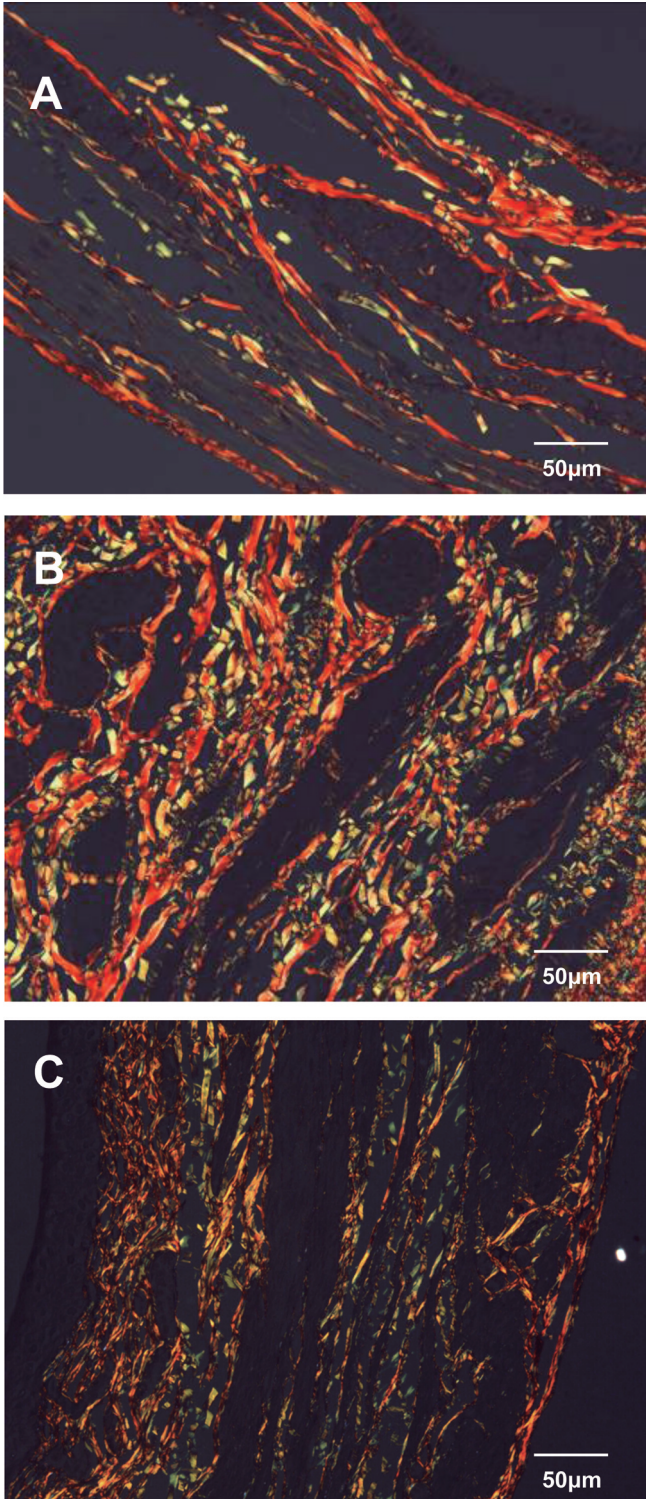
Fig. 1. Urinary bladder wall section of rats stained with Picrosirius red observed under polarized light. The fragments showed thick collagen fibers on orange-reddish and thin collagen fibers on green-yellowish birefringence color. **A.** Control group fed a normal diet (ND). **B.** Model of bladder outlet obstruction (BOO) group fed a ND. **C.** Group fed a hypercholesterolemic diet (HCD) with 1.25% cholesterol. x 400

3 and 5 illustrate these data. After immunofluorescence, the 3D reconstruction by confocal microscopy (Fig. 4) showed no inherent fluorescence of the tissue in all three groups, i.e., fluorescence was actually gained from the reaction with the studied collagen types.

Discussion

Rats fed a hypercholesterolemic diet showed higher levels of LDL cholesterol and more significant body weight gain than rats fed a normal diet for ten weeks. Moreover, rats fed a HCD had morphological changes in the bladder wall extracellular matrix, concerning types I and III collagen composition, similar to changes presented in the partial bladder outlet obstruction model fed a ND.

Interestingly, total cholesterol was not statistically different between rats fed a ND and those fed a HCD (87.6 ± 8.7 mg/dL x 91.0 ± 21.2 mg/dL, respectively; p=0.376). Other studies have presented different serum total cholesterol results (Son et al., 2007), using a HCD
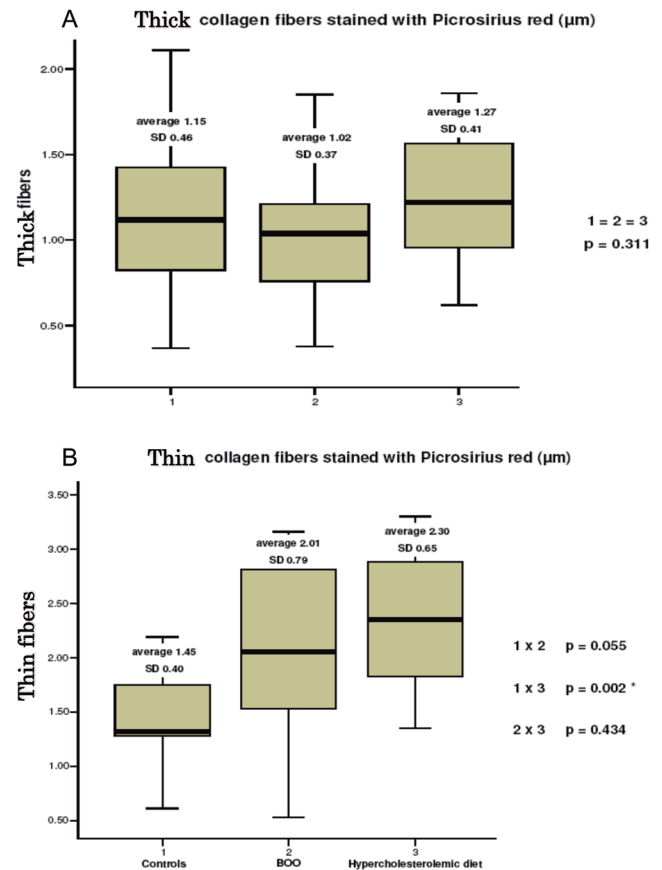

Fig. 2. Box-whisker plots of data of thick **(A)** and thin **(B)** collagen fibers of rats urinary bladder wall by Picrosirius red (*μ*m) in control group fed a normal diet, bladder outlet obstruction group (BOO) and group fed a hypercholesterolemic diet with 1.25% cholesterol (HCD) (ANOVA One-Way and Tukey's method).

emulsified in 10% olive oil (Asahina et al., 2005) or a HCD enriched with coconut oil (Hiss et al., 1988). All these experiments (Asahina et al., 2005; Son et al., 2007; Zulet et al., 2007) utilized older male Sprague-Dawley rats, and gender and race differences might account for this discrepancy in total cholesterol increase (Savolainen et al., 1991; Dietschy et al., 1993). In the present study, female rats were used to prevent a possible intraprostatic urine reflux and local inflammation, as well as the effect of the HCD on the prostate structure. In the same way, young rats were chosen to minimize the bias of natural bladder aging. These choices may be responsible for the

metabolic differences found in prior studies, since both female and younger rats have a more efficient metabolic homeostasis compared to older male rats, minimizing the changes expected from the experimental diet.

On the other hand, the significant increase of LDL cholesterol in the group fed a HCD compared to the groups fed a ND (70.9 ± 20.4 mg/dL x 48.2 ± 8.5 mg/dL, respectively; $p<0.001$) was concordant with the results presented in other studies (Sérougne et al., 1995; Asahina et al., 2005; Zulet et al., 2007). Similarly, body weight gain was significantly higher in the group fed a HCD than in the groups fed a ND (281.5 ± 30.3g x

Table 1. Biochemical tests (serum total cholesterol and LDL-cholesterol) and body weight measurement data of groups fed a normal rat diet (groups 1 and 2) and fed a hypercholesterolemic diet (group 3).

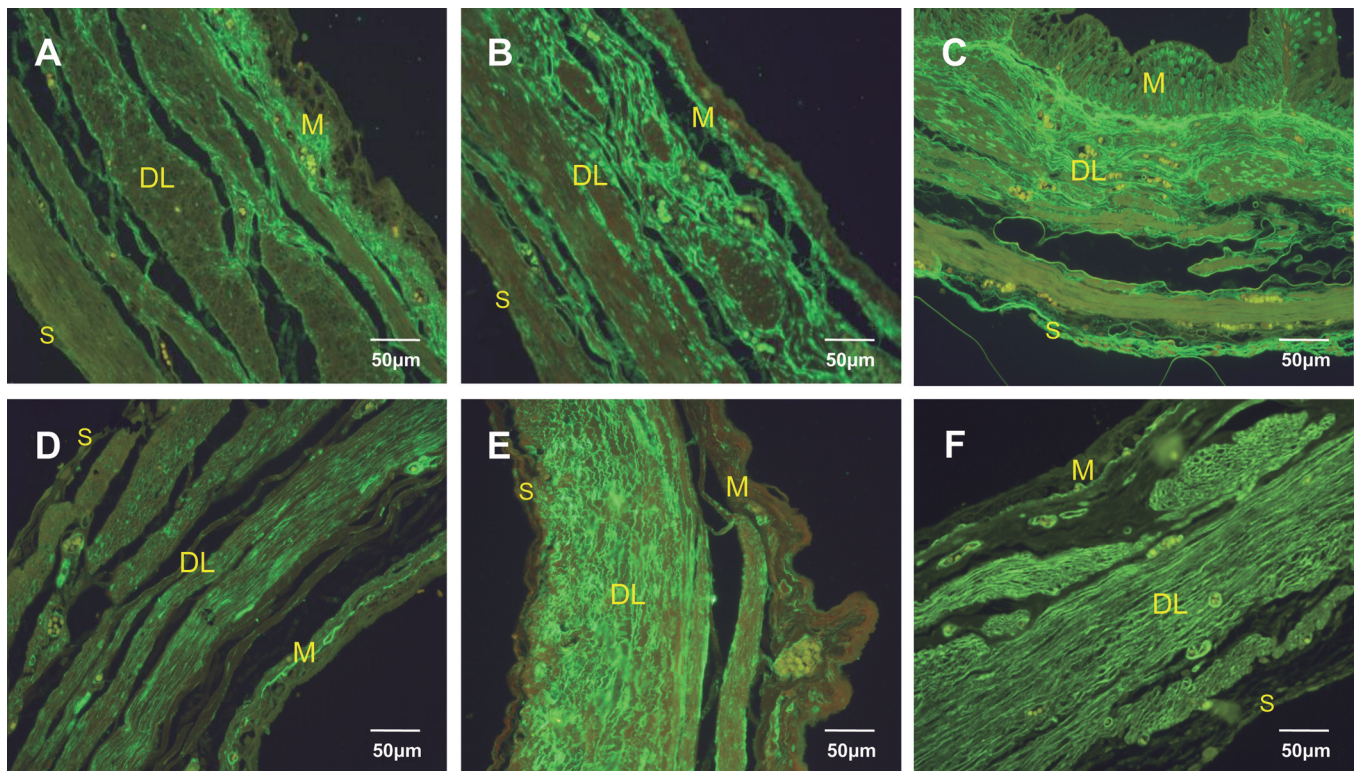| Groups	N	Time	Total cholesterol (mg/mL)		LDL-cholesterol (mg/mL)		Body weight (g)	
			Mean	SD	Mean	SD	Mean	SD
Normal diet	17	Initial	80.88	15.82	48.41	11.35	83.24	15.43
		10 weeks	87.62	8.74	48.15	8.54	239.77	29.63
HC diet	15	Initial	85.67	24.22	53.71	22.34	94.47	16.63
		10 weeks	91.00	21.20	70.90	20.36	281.47	30.27
p			0.376		0.041*		0.017*	

Fig. 3. Immunofluorescence to types I (**A, B, C**) and III (**D, E, F**) collagen in rats urinary bladder wall section of control (**A, D**), BOO model (**B, E**) and HCD (**C, F**). There is an evident higher amount of collagen in (**B**), whereas (**A**) and (**C**) showed a similar pattern in type I collagen, while there is an evident higher amount of the type III collagen in (**E**) and (**F**), relative to (**D**). BOO: Model of bladder outlet obstruction; HCD: Group fed a hypercholesterolemic diet (HCD) with 1.25% cholesterol. x 400

239.8±29.6g, respectively; p=0.017), as previously reported by other authors (Sérougne et al., 1995; Son et al., 2007; Zulet et al., 2007).

Bladder dysfunctions have been demonstrated previously in hypercholesterolemic models of detrusor overactivity (Rahman et al., 2007) and micturition alterations (Son et al., 2007). In the former study

(Rahman et al., 2007), this finding was associated with bladder wall thickness and an increase in purinergic receptors in rats fed a HCD. In the latter study with micturition alterations (Son et al., 2007), no morphological alteration was identified by hematoxilin-eosin or Masson's trichromic staining. However, these are not specific stains for collagen detection (Rich and
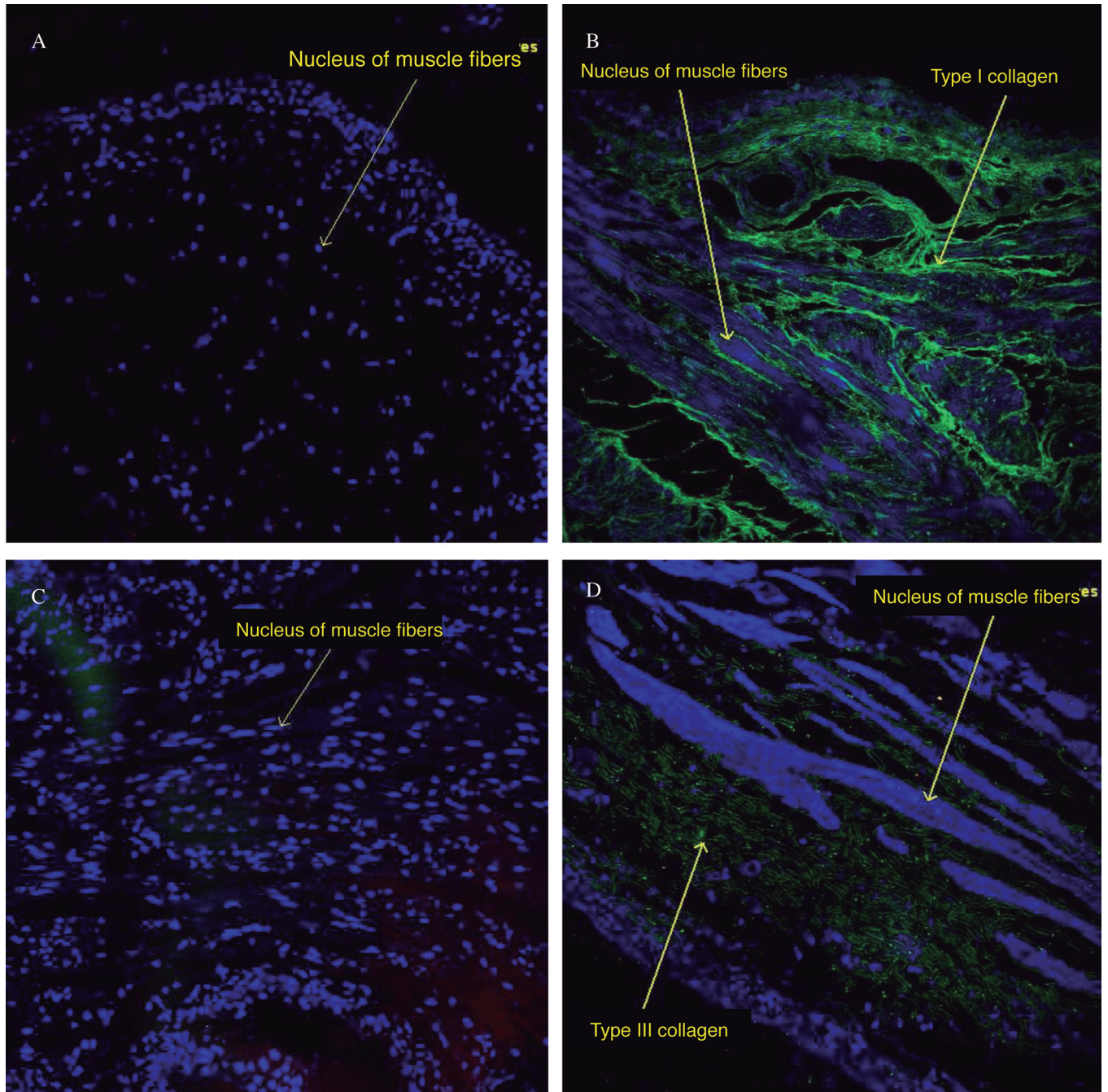

Fig. 4. Three-dimensional image reconstruction show collagen types I (**A, B**) and III (**C, D**) in rat urinary bladder wall section. Note that collagen the type I collagen fibers confirm thickness and dramatic morphological disorganization when compared with reddish birefringence stained by Picrosirius in bladder wall. x 400

Whittaker, 2005).

This study, focusing on morphological analysis, detected important extracellular matrix differences among the three groups. Picrosirius-red staining showed a similar distribution of thick collagen fibers (p=0.311) among the groups, while the amount of thin collagen fibers was significantly higher in the HCD group when compared to group 1 (p=0.002). Group 2 also presented a larger quantity of these fibers in comparison to the control group, but with only borderline statistical significance (p=0.055). This may reflect a recent deposition of collagen fibers, related to the hypercholesterolemic diet.

In order to evaluate the collagen types involved in this process, we analyzed by immunostaining types I and III collagen, and detected differences among the three groups (p=0.005 and p=0.001, respectively). In type I collagen analysis, this difference was due to a larger

quantity of this collagen in the BOO group (group 2) when compared to group 1 (p=0.003). Group 3 showed a similar amount of type I collagen to both groups 1 and 2. On the other hand, type III collagen analysis of both groups 2 and 3 showed significantly larger quantities of this collagen than in group 1 (p=0.002 and p=0.016, respectively), denoting an increased deposition of this type of collagen in rats fed a HCD, similar to that which occurred in the BOO model fed a normal diet.

Hypercholesterolemia is increasingly considered a worldwide public health problem. In addition to the known cardiovascular disorders that may result from hypercholesterolemia, it also seems to induce structural changes in the bladder wall that possibly affect the physiological and functional bladder mechanisms, as suggested by previous studies, which emphasize the importance of its prevention and early treatment.

The mechanisms by which hypercholesterolemia induces morphological changes in the bladder are somewhat different from those occurring after BOO, though the final result is similar and is enough to make it clinically relevant and subject to further investigation. A hypothesis to be considered is that ischemia caused by hypercholesterolemia can chronically lead to morphological changes, also characterized by collagen deposition in the bladder wall. Therefore, the model we used will play an important role in the further studies, assuming the fact that a hypercholesterolemic diet generates this metabolic deviation.

Conclusions

Herein, we showed that a hypercholesterolemic diet promotes changes in the collagen composition of the bladder wall extracellular matrix in rats. This fact is similar to that observed in bladder outlet obstruction and justifies future research on this matter, such as dietary influences on the BOO model, bladder function studies and possible effect of pharmacological prevention of hypercholesterolemia. Although likely, we cannot infer that a high LDL cholesterol level itself promotes an increase in bladder collagen type III, since weight gain was also observed in these rats.

Fig. 5. Box-whisker plot of data of immunofluorescence to types I **(A)** and III **(B)** collagen (μm^2) in rats urinary bladder wall of control group fed a normal diet, bladder outlet obstruction group (BOO) and group fed a hypercholesterolemic diet with 1.25% cholesterol (HCD) (ANOVA One-Way and Tukey's method).

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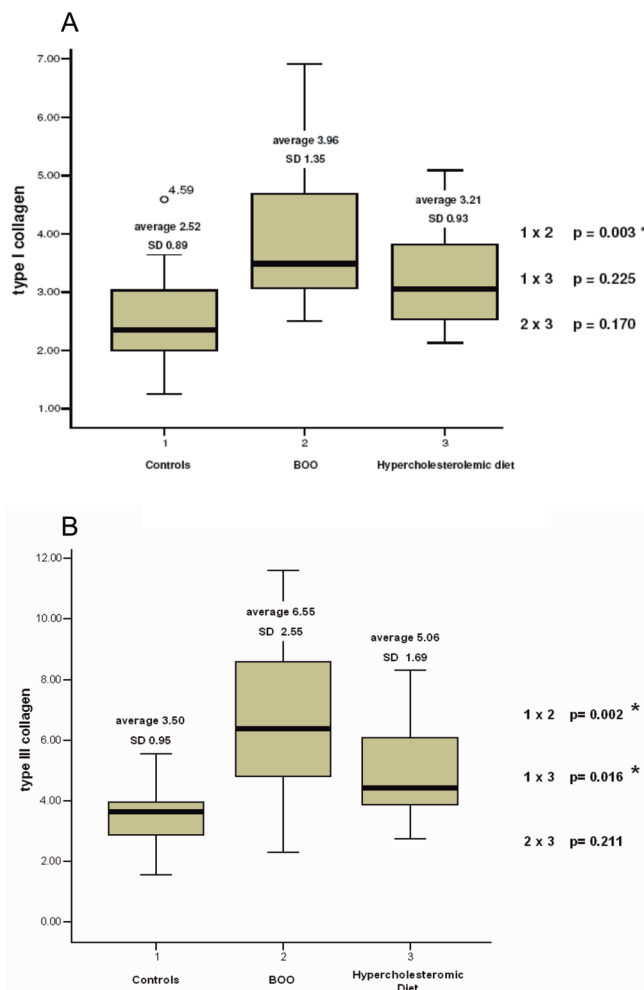Azadzoi K.M., Tarkan T., Siroky M.B. and Krane R.J. (1999). Atherosclerosis-induced chronic ischemia causes bladder fibrosis and non compliance in rabbits. J. Urol. 161, 1626-1635.

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