Effects of prepubertal corticosterone treatment on urinary bladder[1]

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ABSTRACT

PURPOSE: The aim of this work was to analyze the bladder wall modifications after a chronic treatment with high doses of corticosterone in prepubertal rats.

METHODS: This study included 26 male rats assigned into four groups: T30 was treated with corticosterone until 29 days of age and killed at day 30, while T65 group received the same treatment but was killed at day 65. Each group had its own control group (C30 and C65). For treated animals, daily intraperitoneal injections of corticosterone (20 mg/Kg) were administered between 7th and 29th day of life. Bladders were removed and collagen, smooth muscle, elastic fibers system, vascular density and epithelium were analyzed by morphometrical methods, immunofluorescence, and biochemistry.

RESULTS: Vascular density in lamina propria was reduced by 40% ($p<0.05$) in group T65. Collagen organization was altered in T30 and T65, although total collagen concentration was unchanged. The T65 group had an increase in elastic system fibers. There was no difference in epithelial height and cell density between the groups. Concerning the smooth muscle fibers density we observed a 19% increase ($p<0.05$) in the T65 group.

CONCLUSION: Prepubertal administration of corticosterone induces structural modifications in the bladder of rats in a medium term analysis.

Key words: Bladder; Corticosterone; Morphometry; Prepubertal; Rats.

INTRODUCTION

Glucocorticoids (GC) regulate with a high degree of specificity cellular differentiation in several tissues, but GC excess can exert negative effects on body growth, negatively influencing cell proliferation [1]. Furthermore it was also suggested that GC can accelerate the aging process [2]. Early exposure to GC can accelerate or delay the functional organic maturation, depending on the dose and exposure time. Data obtained by Seckl [3] suggests that both pharmacological and physiological prenatal exposures to GC excess are able to cause cardiovascular, neuroendocrine and metabolic disorders in the adult life. At both prenatal and pubertal phases, GC helps normal body growth and the formation and maturation of genital organs, functioning together with thyroidian hormones, adrenal androgens and sexual hormones [4]. However, exposure to excess GC inhibits spontaneous secretion of such hormones [5].

In previous work [6, 7], we found, using morphometrical evaluation, important alterations in the mean diameter of cervical ganglion neurons from GC-treated rats during the postnatal eight days. Other studies also show that GC present in the cellular microenvironment is a critical determinant of cellular morphological and biochemical differentiation [8].

High doses of GC are used to prevent or treat inflammation in newborn babies with chronic lung disease. Despite short-term side effects, such as bleeding from the stomach or bowel, higher blood pressure and difficulty tolerating glucose, there are little studies concerning long-term and/or urinary tract complications [9].

The aim of this work was to analyze the bladder wall after a treatment with high doses of corticosterone in a short and medium term rat model.

METHODS

Twenty six newborn male Wistar rats were used in this study. The animals were kept with their mothers during all treatment period. Only male pups were used in the study. The rats were kept in a room with controlled temperature (25 ± 1 C) and with artificial dark–light cycle (lights on from 7:00 am to 7:00 pm) and mothers were fed standard rat chow and water ad libitum. The rats were weighed daily until the day of death.

All experiments were done according to the Brazilian law for scientific use of animals, and this project was formally approved by the Animal Care and Use Committee of the Biology Institute of State University of Rio de Janeiro (protocol no. CEA/250/2008).

The rats were randomly assigned into four groups, treated animals sacrificed on 30th postnatal day (T30, n=6) and respective control group (C30, n=5), treated animals sacrificed on 65th postnatal day (T65, n=7) and respective control group (C65, n=8).

Animals from the groups T30 and T60 received intraperitoneal injections of corticosterone Sigma® (2 mg/100g of body height) daily. Treatment was administrated from the 7th to the 29th day after conception (22 days of treatment). The injections were always performed in the morning, between 8:00 and 10:00 am, according to previously established protocols [10].

Animals from groups C30 and C60 were kept in the same conditions, with the exception of the intraperitoneal injections of corticosterone.

At 30 (T30 and C30) or 65 (T65 and C65) days of life, rats were sacrificed by anesthetic overdose and the bladder was dissected under magnification, weighted and fixed by immersion in 10% formaldehyde solution (pH 7.4).

Specimens were processed for paraplast embedding and sectioned to obtain 5 μm slices which were stained by different methods as follows. Picrosirius red stained slices were used to distinguish different types of collagen [11]. Masson´s trichrome stained slices were analyzed for smooth muscle density, blood vessels density and epithelial height and cellular density.

Smooth muscle density in the bladder wall was estimated by superimposing a 100 points grid with over the previously outlined bladder wall (with all of its layers) with software ImageJ. Images with final magnification of 200X were used for this purpose. Results were expressed as percentage, calculated by the number of points which superimpose smooth muscle layer.

The blood vessels density was assessed only in the lamina propria of the bladder, which was defined as the loose connective tissue between the urothelial basement membrane and the inner edge of the detrusor layer. Using the ImageJ software version 1.42 (NIH, Bethesda, Maryland, USA) in sections captured at a final magnification of X200, a continuous segment of lamina propria was outlined and its surface area was measured. Thus, the number of blood vessels in the outlined segment of lamina propria was counted and divided the previously measured area of this segment. For each animal, a total of 25 evenly spaced segments of lamina propria were used for these quantifications. Results were expressed as number of blood vessels per mm^2.

The epithelial height and cellular density were measured at a final magnification of X1000 in 25 different fields per animal using a calibrated ImageJ program. Epithelial height, expressed in μm, was measured in 10 different locations per field, resulting

in 250 measures per animal. Cellular density was estimated by counting nuclei in a previously outlined continuous area of epithelium using a specific function of the ImageJ program, and the results were expressed as number of nuclei per mm^2.

Also, elastic fibers were assessed in anti-elastin indirect immunofluorescence labeld slices. Antigen retrieval was carried out prior to incubating the sections with the primary antibody by treating dewaxed sections with a ready-made pepsin solution (Digest-All Kit, solution 3, Zymed Laboratories, San Francisco, CA, USA), according to the manufacturer's instructions. The primary antibody used was rabbit polyclonal anti-elastin (ab21610, Abcam, Cambridge, MA, USA). And secondary antibody was Alexa Fluor 488 goat anti-rabbit IgG (H+L) (A11008, Invitrogen, Carlsbad, CA, USA).

All micrographs were captured by a DP71 camera coupled to BX51 microscope with conventional, polarized and fluorescent light sources (Olympus, Tokyo, Japan).

For biochemical analysis, a small fragment of the bladder was fixed in cold acetone and kept in this fixative for 24 h at 4° C. The samples were then finely minced and submitted to two changes of 24 h each in 40 mL of chloroform:methanol (2:1, v/v) at room temperature. The solvent was then decanted, and after incubation at 60°C for 30 min, a preparation of dry and defatted bladder tissue was obtained and weighed. The concentration of total collagen in the tissue was determined by a colorimetric hydroxyproline assay. Thus, 5–14 mg of dry, defatted bladder were hydrolysed in 6M HCL for 18 h at 118°C as previously described [12]. The assay was then carried out in the neutralized hydrolysates using a chloramin T method [13]. Results were expressed as micrograms of hydroxyproline per milligram of dry, defatted bladder.

The Student's-t-test was used for mean comparisons. In all cases, significance was set at a probability value of 0.05. All analyzes were performed using GraphPad Prism software.

RESULTS

Blood vessels density was significantly reduced in group T65 when compared to C65 by 40.83% (p=0.0015). In animals sacrificed with 30 days, we found a non statistically significant reduction in the treated animals.

Smooth muscle density increased by 19.4% in the T65 group in comparison to C65 (p=0.0239). Differences among T30 and C30 were not statistically significant, although treated group showed a small increase.

The analysis of the elastic system fibers by immunofluorescence showed an increasing in elastic fibers in the treated animals T65 when comparing to its control group C65 (Figure 1).

The analysis of collagen in picrosirius red stained sections observed under polarization microscopy showed that treated animals (both 30 and 65 days old) had a more homogenous distribution of red and green colored collagen fibers which indicates the presence of different types of collagen (Figure 2).

The concentration of total collagen in bladder obtained by biochemical analysis showed no significant difference among the groups.

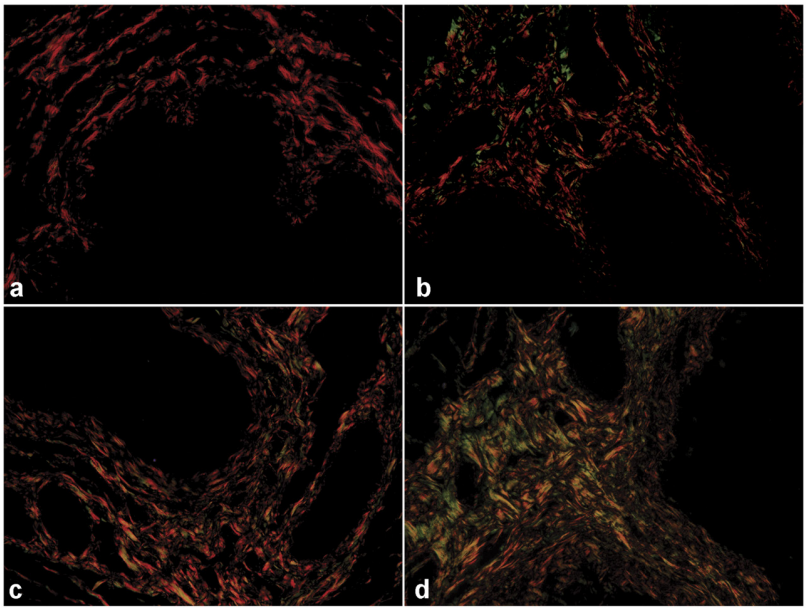

Figure 1. Qualitative analysis of collagen in Picrosirius Red stained sections. a) C30, b) T30 c) T65; d) T65. In *a* and *c* there is a predominance of red or reddish color. In *b* and *d* most fibers are stained in green. X 400.

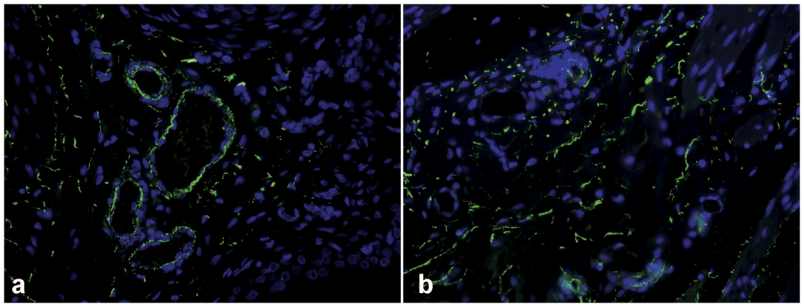

Figure 2. Anti elastin (elastic system fibers) imunolabeled sections observed in green (Alexa 488 probe). a) C65; b) T65 . In *b* a substantial increase in fluorescence was observed. X 400.

Regarding epithelial height and cellular density no difference was found between the groups studied.

No differences in the final weight were observed between control and treated groups after 30 and 65 days.

All numerical data are presented in Tables 1 and 2.

Table 1. Numerical data from rats control (C30) and treated with corticosterone (T30) and sacrificed with 30 days old.

	C30	T30	P value
Body weight (g)	81.5 ± 7.241	80.62 ± 5.399	> 0.05
Smooth muscle density (%)	53.53 ± 9.593	55.93 ± 4.078	> 0.05
Blood vessels density (b.v./mm^2)	75.16 ± 10.96	60.10 ± 15.66	> 0.05
Epithelial height (µm)	28.19 ± 7.469	34.90 ± 4.207	> 0.05
Epithelial cellular density (nuclei/ mm^2)	5736 ± 1056	5122 ± 775	> 0.05
Total collagen (µg OH-pro/mg d.t.)	27.74 ± 6.595	29.81 ± 8.202	> 0.05

Data expressed as mean ± SD; b.v.: blood vessels; OH-pro: hydroxyproline; d.t.: dry tissue.

Table 2. Numerical data from rats control (C65) and treated with corticosterone (T65) and sacrificed with 65 days old.

	C65	T65	P value
Body weight (g)	276.7 ± 39.33	262.6 ± 50.37	> 0.05
Smooth muscle density (%)	41.53 ± 2.94	49.60 ± 5.423	0.0239
Blood vessels density (b.v./mm^2)	94.12 ± 13.86	55.69 ± 16.89	0.0015
Epithelial height (µm)	25.8 ± 4.768	29.34 ± 11.35	> 0.05
Epithelial cellular density (nuclei/mm^2)	5304 ± 1155	4984 ± 939.4	> 0.05
Total collagen (µg OH-pro/mg d.t.)	46.24 ± 11.37	54.31 ± 12.80	> 0.05

Data expressed as mean ± SD; b.v.: blood vessels; OH-pro: hydroxyproline; d.t.: dry tissue.

DISCUSSION

GC are widely used in the treatment of several newborn diseases [14, 15]. GC excess, however, leads to alterations in various organs and tissues such as skin, bones, muscles and brain [2, 16]. However there are few reports regarding the impact of GC on the bladder. To characterize possible effects of GC on the bladder, we used supraphysiological doses of corticosterone in prepubertal rats and assessed the effects of the drug in the short and medium term in the bladder wall. We observed a change in the vascularization of the bladder lamina propria characterized by a significant decrease in the number of blood vessels in treated animals. However these changes were only observed in animals after 65 days of life. Therefore, we can assume that these changes will manifest themselves only in a medium term period after the treatment.

The mechanical properties of the bladder wall depend on the resistance, viscosity and elasticity of the detrusor muscle and connective tissue [17]. In this experiment, GC promoted a 19% increasing in detrusor muscle density. This finding was also seen only in animals with 65 days, with no alterations in the short term analyzed animals. An abnormal increase in the detrusor occurs frequently in the bladder with partial obstruction and includes progressive hypertrophy of smooth muscle and changes in collagen and elastic fibers system [18].

The collagen and elastic fibers are the main components of the extracellular matrix. They are present in the bladder wall and are closely related to bladder compliance . Wognum *et al.* [19] showed that the bladder responds to periods of high pressure by increasing their compliance through the interaction between the young collagen and the elastic fibers synthesis. An abnormal increase in the amount of collagen and elastic fibers occurs most commonly in congenital or acquired disease as in obstructive uropathy, possibly leading to a loss of strength and elasticity of the bladder wall [20].

The fluorescence microscopic analysis showed a higher concentration of elastic system fibers in the lamina propria of the

treated groups after 65 days, suggesting a decrease in distensibility and bladder compliance in animals treated with corticosterone.

Collagen quantification performed by biochemical analysis showed no difference between groups. However, the qualitative observation by polarizing microscopy in the sections stained by picrosirius red [11] showed different colors on the lamina propria of the bladder. These results permits to suppose, by the predominance of green color, observed in sections from groups of 30 and 65 days treated with corticosterone, and the predominance of red color observed in the sections from control groups, that there is a difference between the types and organization of collagen present in the sections. In the treated groups, one type of collagen, possibly collagen type III, is predominant in relation to another type of collagen that is characterized by a color ranging from reddish to orange, which is predominant in the control group and is usually associate with collagen type I. It seems therefore that the vesical stroma is undergoing a significant turnover, with an active process of collagen formation in the treated group.

In conclusion, prepubertal administration of corticosterone induces structural modifications in the bladder of rats witch are only seen in a medium term analysis. Future studies regarding clinical or morphological alterations on bladder of persons who received GC in early infancy are desired.

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