International Braz J Urol
Official Journal of the Brazilian Society of Urology
Vol. 31 (6): 562-568, November - December, 2005

STEREOLOGICAL AND MORPHOMETRIC ANALYSIS OF COLLAGEN AND SEMINIFEROUS TUBULES IN TESTES OF PATIENTS WITH CRYPTORCHIDISM SUBMITTED OR NOT TO TREATMENT WITH HUMAN CHORIONIC GONADOTROPHIN

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ABSTRACT

Objectives: Quantify the distribution of collagen and analyze the seminiferous tubules diameter in the testis of patients with cryptorchidism, to verify if the previous use of human chorionic gonadotrophin (hCG) affects these structures.

Material and Methods: Samples of parenchymal tissue of cryptorchid testis obtained during peroperative biopsies were collected from 26 patients. Sixteen samples were embedded in paraffin and stained with picrosirius red to evidence fibers of collagen system. The quantification of these fibers was determined by stereological methods, using a test system M-42. To obtain seminiferous tubules diameter we used 10 of the 26 samples. These samples were embedded in Epon and the analyses were carried out in semi-thin sections, stained with toluidin blue. The selected results of each group were statistically analyzed and compared by the student's t and Tukey-Kramer's tests.

Results: The testicular interstitium and lamina propria of patients treated with hCG showed statistically significant less collagen system fibers, when compared to the testes of patients nontreated (0.30% versus 0.39%, $p = 0.0079$). The seminiferous tubules diameters were not statistically significant different between the testes of patients treated and nontreated with hCG (67.5 versus 59.35 µm, $p = 0.0609$).

Conclusions: hCG use in the cryptorchidism could delay, at least temporarily, a progressive growth of fibers of collagen system. We did not find statistically significant difference in the seminiferous tubular diameters between treated and nontreated patients.

Key words: testis; cryptorchidism; human chorionic gonadotrophin; collagen; seminiferous tubules; infertility
Int Braz J Urol. 2005; 31: 562-8

INTRODUCTION

Cryptorchidism is the most frequent anomaly caused by a flaw in testicular migration. Failure to treat cryptorchidism would result in testicular atrophy and infertility.

The most common histological findings in cryptorchidism are: a) germinative cells number decrease in various maturation stages; b) variation in seminiferous tubules diameter, with a thickening in lamina propria and c) peritubular fibrosis (1,2).

There are studies demonstrating histological alterations in cryptorchid testis in the first year of life, as Leydig and germinative cells decrease. Later on, the seminiferous tubules would develop atrophy in various degrees, the Sertoli cells would degenerate and hyalinosis and peritubular fibrosis may develop. Temperature increase in the cryptorchid testis would be an important factor in the genesis of histological alterations in advanced ages (3).

Besides surgery, hormonal therapy is the only medical therapy known for non migrated testes. Currently, there are 2 types of hormonal therapy: human chorionic gonadotrophin (hCG) and gonadotrophin releasing hormone (GnRH). hCG is used based on the premise that stimulation of Leydig cells will result in an increase of plasmatic testosterone, causing testicular migration. GnRH would stimulate endogen secretion of LH and FSH (4).

Currently, hormonal treatment has been indicated with better results and safety to patients with the following characteristics: non migrated palpable testes (unilaterally or bilaterally), ages ranging from 6 months to 2 years and without associated hernia. Nevertheless, some authors support its use in early ages, even if not matching the above listed characteristics, with or without orchiopexy. Orchiopexy would be combined to hormonal treatment as a way to prevent the deleterious effects of androgen insufficiency, common in cryptorchidism (5,6). Others use it as a way to facilitate surgery, since its use would increase testicular volume and improve its vascularization (7).

The alterations caused by cryptorchidism in germinative cells are widely known and described with details in the literature; conversely, little information is known about the alterations in interstitium and in seminiferous tubules and their respective consequences.

The objectives of this work is to characterize, through stereology, if hCG use in cryptorchidism affects collagen distribution in the testes, and characterize possible morphologic alterations in cryptorchid testes, comparing seminiferous tubules diameter in patients submitted or not to hCG treatment previously to surgery.

MATERIALS AND METHODS

We analyzed 26 testes, 11 of patients who were treated with hCG before orchiopexy and 15 from patients nontreated with hCG.

The hCG was given by intramuscular injection twice a week for 5 weeks, according to following protocol: patients until 2 years old received injections of 250 UI (total - 2500 UI), patients between 2 and 6 years old received injections of 500 UI (total - 5000 UI) and patients over 6 years old received injections of 1000 UI (total - 10000 UI) (8).

Free and informed consent was granted for all cases in this study. The research protocol was assessed and approved by the Bioethics Committee of the hospital where the orchiopexies were performed.

Testicular samples were obtained through a 5 mm long incision in the tunica albuginea, near the superior pole of the testis. The material was fixed in 10% formalin buffered and embedded in paraffin or fixed in 2.5% glutaraldehyde and embedded in Epon. Both were maintained in the fixative solution at 4^{o}C for 24 hours.

Seminiferous tubules diameter assessment was performed in 10 patients. Four patients had unilateral cryptorchidism and 6 patients had bilateral cryptorchidism. Five patients had been treated with hCG previously to orchiopexy and 5 had not. Semithin sections were obtained from the material and embedded in Epon. From each sample, 10 sections were obtained with a 30 μm interval and were stained with toluidin blue. The diameters were determined using the Image Pro, Media Cybernetics computer-assisted morphometry software.

Collagen quantification was performed in sections of the material embedded in paraffin and stained with picrosirius red. Five sections of each fragment were selected and in each section, 5 random fields were observed with a total of 25 test areas studied in each testis. The images for analysis and quantification of collagen were obtained with a magnification of 400X in an Olympus optic microscope coupled to a Sony CCD video camera and transferred to a Sony KX 14-CP1 monitor (9). The selected histological areas were quantified using test-grid system on the digitized fields on the screen of a color

monitor. From stereological principles in isotropic tissue, the area distribution of a given structure, as determined on a two-dimensional section of the structure, is proportional to the volume distribution of the structure. The volume density of the histological components was calculated as Vv = Pp/Pt, where Vv is the volume density, p is the tissue component under consideration, Pp is the number of test points associated with p, and Pt is the number of points of the test system (9).

The results obtained were analyzed and compared in the Graphpad prism software (Graphpad) through Kolmogorov-Sminorv test to verify if each group was under a normal distribution. The non-paired student's t test was used to determinate if the differences between the groups were significant.

RESULTS

Table-1 indicates the location of the testes included in the study.

The results of seminiferous tubules diameters in patients treated and nontreated with hCG did not show statistically significant difference (67.5 versus 59.35 µm, p = 0.0609), Figures 1 and 2, Table-2.

Table 1 – *Location of the testes studied and treated (hCG +) or nontreated (hCG -) patients with human chorionic gonadotrophin previously to surgery.*

	Inguinal Canal	External Ring (supra scrotal)	Abdominal
hCG +	6	3	2
hCG -	6	5	4

The testicular interstitium and lamina propria of patients treated with hCG showed statistically significant less collagen system fibers, Figures 3 and 4, when compared to the testes of patients nontreated (0.30% versus 0.39%, p = 0.0079).

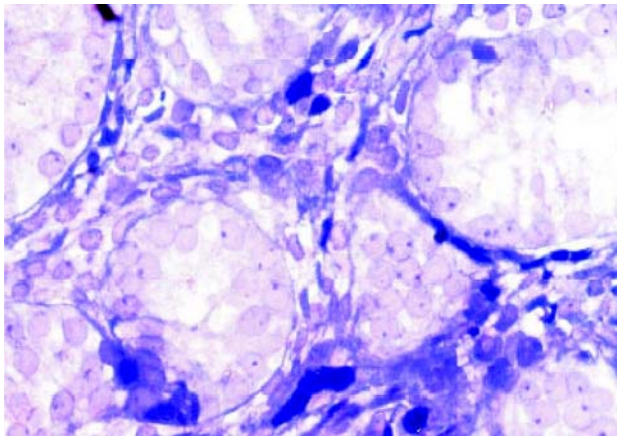

Figure 1 – *Photomicrography of testicular parenchyma of a nontreated patient (Toluidin blue, X40).*

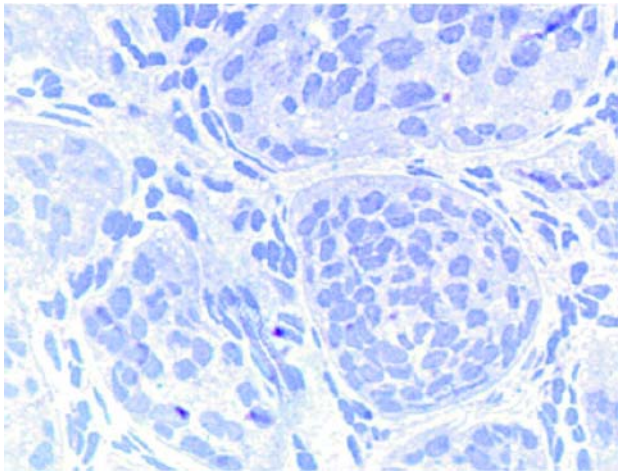

Figure 2 – *Photomicrography of testicular parenchyma of a treated patient (toluidin blue, X40).*

Table 2 – *The seminiferous tubules diameters in 5 patients treated and 5 patients nontreated with hCG.*

Age (years)	Diameter (µm)	
	Treated	Nontreated
4	75.76	60.29
5	62.19	59.39
5	60.81	
7	75.79	56.64
7		55.65
8	63.06	64.79

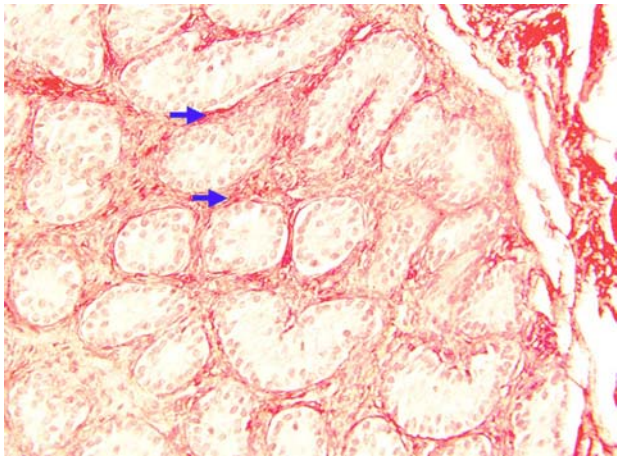

Figure 3 – *Photomicrography of testicular parenchyma of a nontreated patient (picrosirius red, X400).*

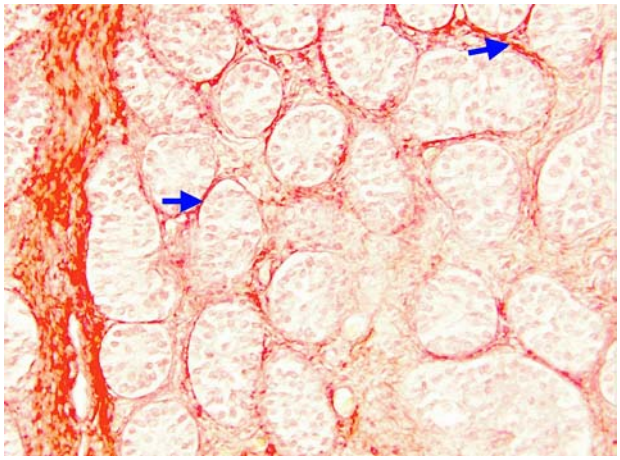

Figure 4 – *Photomicrography of testicular parenchyma of a treated patient (picrosirius red, X400).*

COMMENTS

The efficiency of hCG as therapy for cryptorchidism has been broadly discussed in its clinical and histological aspects. The testicular migration process have been studied by some authors, most of them focusing on the consequences of the changes that occur in the germinative epithelium (5,10,11). Most authors believe that on this aspect, the hCG use would be beneficial to patients.

Most studies on this subject relate a substantial collagen increase in the testes of elderly patients, accompanying testicular atrophy expected in this age, independently from cryptorchidism being or not a previous condition (12). Nevertheless, some studies challenge this report as a general rule, showing some cases in which 80 year old men presented histologic testicular parameters similar to those of young men (13).

The literature describes an interaction between extracellular matrix, tubular wall and germinative cells, being very important to their normal development. (12,14).

In cryptorchid testes, some studies have been demonstrated an important increase in collagen quantity, resulting in interstitial fibrosis and in a thickened lamina propria of seminiferous tubules (3,15). Treatment with hCG would cause temporary histologic changes in testis, nevertheless, without determining permanent damage (16).

Analyses of the groups with and without treatment with hCG showed a significant difference between then, compatible to a possible prevention of collagen increase in patients submitted to hCG, reducing interstitial fibrosis and thickening of the lamina propria of the seminiferous tubules and, consequently, avoiding alterations in germinative cells (3,5).

The question is if the collagen alteration contributes in any way to prevent germinative cell alterations and, consequently, any worsening in fertility levels in cryptorchid patients without treatment. Another possibility to prove the hCG efficiency in treatment of cryptorchid testes is the determination of seminiferous tubules diameter (13,17). We compared our results of the seminiferous tubules diameter in cryptorchid patients treated and nontreated with hCG to the normal male patients studied by Aya et al. (18). We observed that the mean diameter in treated and nontreated patients of our study was smaller than that of the normal children studied by Aya et al. (18). In this study, normal 7 year old children present a mean diameter of 88.7 μm, while in our study, the mean diameter in patients with the same age was 75.79 μm for treated patients and 56.14 μm for nontreated patients.

In conclusion, the results of the present study suggest that hCG use as treatment for cryptorchidism, when employed in an early stage in life, could temporarily delay a progressive collagen increase. Relatively to tubular diameter, our results show that

there is no statistically significant change between treated and nontreated patients.

ACKNOWLEDGEMENTS

This work was supported by Rio de Janeiro Foundation for Research Support - FAPERJ, and National Council for Scientific and Technological Development - CNPq, Brazil.

CONFLICT OF INTEREST

None declared.

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Received: June 28, 2005
Accepted after revision: September 8, 2005

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EDITORIAL COMMENT

The most important finding of the present study has been the amelioration at collagen contents in testes subjected to hCG administration. On the other hand, the effects of hCG were previously assessed through parameters such as the number of spermatogonia, or Ad spermatogonia per tubular cross section (1,2). Therefore, the types of collagens subjected to alteration, and the means and mechanisms of this alteration require further clarification.

One of the mechanisms involved in the regulation of collagen production is G-protein linked signal transduction. Adenylyl cyclase over-expression through cAMP elevating agents promotes an antifibrotic phenotype in pulmonary fibroblasts (3). The lutropin/choriogonadotropin receptor belongs to the family of G protein-coupled receptors. Binding of hCG to the receptor stimulates adenylyl cyclase activity (4). Stimulation of adenylyl cyclase activity might have involved in the decrease of collagen content in testes subjected to hCG.

According to one of the current theories, undescended testis is associated with a decrease in sympathetic, but an increase in parasympathetic tonus. Since the neurotransmitters involved in autonomic nervous system also act through receptors coupled to G- proteins, and undescended testis is associated with less stimuli to activate adenylyl cyclase, the differences in signaling has been suggested to account for the alterations in an undescended testis (5,6).

The place of hormones in the process of descent and in the treatment of undescended testis that has been remained controversial, appears to remain controversial unless the definite understanding of the mechanism. The differences in collagen expressions may not only be important for the evaluation of the effects of hormones, but also may form another pathway that helps to unravel the mechanism of descent.

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EDITORIAL COMMENT

Human chorionic gonadotropin (hCG) has been used in cryptorchidism for decades. The main goal of the hormonal treatment is to avoid operation or to make operation easier. In addition, it has been presumed that hCG might have beneficial effects on testicular function. Previously it has been observed that hCG enhances the maturation of gonocytes and even increases the amount of them. The article presented in this issue shows that hCG treatment also prevents interstitial fibrosis at least in short term.

Surprisingly few studies, all with low number of patients, have been performed dealing with the long term effects of the hCG treatment. In some studies, it has been proposed that the beneficial effects of the hormone treatment on testicular function may last to the adulthood (1). However, there is also a certain controversy about the safety of the hormone treatment. For example in our own follow-up study (2) it turned out that the testes with preoperative hCG treatment were smaller in early adulthood than those without preoperative hormonal therapy. Histological analysis (3) suggested that hCG therapy initially increases the amount of germ cells, but after the testosterone level decreases along with the withdrawal of the therapy, part of the germ cells meet apoptotic cell death. Similar effect can be seen in hormone withdrawal therapy of hormone dependent cancers. Thus it seems to be important in histological analyses to look, what is the time interval between hCG treatment and sample collection. Is the initially positive response later turning to opposite? Also the patient age at treatment may be an important factor. In a study (4) hCG treatment had harmful long term consequences especially if it had been given to patients younger than three years of age. This is especially worrying, because patients should be treated at a young age to get optimal result in respect of fertility.

It seems that studies with bigger amounts of patients treated at a young age and with a long term follow-up are needed before we can decide for sure if hormonal therapy has beneficial or harmful effects on testicular function in adulthood. Meanwhile at least I am favoring surgical therapy without hormonal pretreatment.

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