Original Article

Extracellular matrix alterations in the Peyronie's disease

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GRAPHICAL ABSTRACT
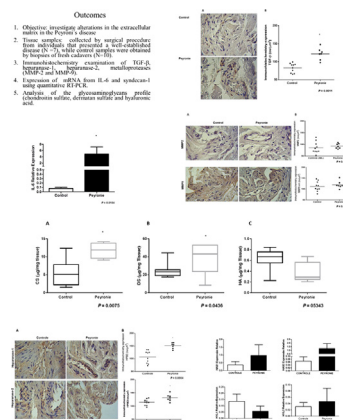
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ABSTRACT

Peyronie's disease is characterized by fibrous plaque formation of the tunica albuginea, causing penile deformity and fertility problems. The aim of the present study was to investigate alterations in the extracellular matrix in Peyronie's disease. The study used tissues collected by surgical procedure from individuals that presented a well-established disease, while control samples were obtained by biopsies of fresh cadavers. Immunohistochemistry analysis followed by digital quantification was performed to evaluate TGF-β, heparanases and metalloproteinases (MMPs). The profile of sulfated glycosaminoglycans, chondroitin sulfate and dermatan sulfate was determined by agarose gel electrophoresis, while hyaluronic acid quantification was obtained by an ELISA-like assay. The expression of mRNA was investigated for syndecan-1 proteoglycan (Syn-1), interleukine-6 (IL-6), hyaluronic acid synthases, and hyaluronidases. Pathologic features showed decreased apoptosis and blood vessel number in Peyronie's tissues. TGF-β and IL-6 were significantly enhanced in Peyronie's disease. There was an increased expression of heparanases, though no alteration was observed for MMPs. Hyaluronic acid as well as hyaluronic acid synthases, hyaluronidases, and dermatan sulfate were not changed, while the level of chondroitin sulfate

was significantly ($P = 0.008$, Mann-Whitney test) increased in Peyronie's samples. Heparanases and sulfated glycosaminoglycans seem to be involved in extracellular matrix alterations in Peyronie's disease.

Introduction

Peyronie's Disease (PD) was first described in 1743 [1], but is still poorly understood. It affects between 0.4% and 9% of men and has a highly adverse impact on the quality of life of patients and partners, because of the impairment of sexual performance and accompanying low self-esteem [2]. Ok PD's pathophysiology is not completely understood. Apparently, there is a disturbance of the healing mechanism of the tunica albuginea, which can be damaged by micro-traumas during sexual intercourse [3]. This disorder leads to the formation of a fibrous plaque, which results in a non-distensible area of tunica albuginea, which causes penile deformity during erection. Accumulating evidence suggests that the overexpression of transforming growth factor-β (TGF-β1) may be associated with the pathogenesis of PD [4].

Dupuytren's disease is a palmar fibromatosis leading to progressive digital flexion contracture [5] and appears to have the same physiopathology as PD. Several studies related to Dupuytren describe alterations in the extracellular matrix, an increased TGF- which affects the expression of major extracellular matrix (ECM) proteins, fibronectin, and collagen [5,6]. Thus, it is important to understand the possible alterations of ECM in patients with PD to clarify the pathogenesis of the disease.

Our aim was therefore to investigate the alterations in the extracellular matrix in Peyronie's disease in an effort to elucidate the molecular mechanisms involved in the development of the disease. Key molecules could be useful as potential targets for future therapies and thus benefit the treatment of patients.

Subject and methods

Patients and samples

The study was composed of tissues collected from individuals unaffected by Peyronie's disease, control group (N = 7) and samples from Peyronie's patients (N = 10). The Peyronie's patients included in this study had already been recommended for surgery and presented a well-established disease. Tissue samples in the control group were obtained from fresh cadavers. During the resection, samples of 1 cm were collected from the tunica albuginea of unaffected individuals and patients with Peyronie's disease. The samples were divided into three fragments, one fragment was stored in acetone for biochemical analysis (glycosaminoglycan quantification), the second fragment was stored in 10% buffered formalin for immunohistochemistry assays, and the third sample was stored in RNA Holder and used for molecular biology studies (mRNA expression analysis). *The study was approved by the Human Ethics Research Committee at Faculdade de Medicine do ABC (No. 242/2010).*

Pathological analysis

Tissue samples of albuginea (3 μm thick) previously fixed in formalin and incubated in paraffin were deparaffinized and rehydrated. Two staining were made: Hematoxylin - Eosin (HE) and Masson Trichome. Under the HE staining, microvessels, cells, and apoptotic events were evaluated. Under Masson Trichrome staining, fibrosis was assessed. This evaluation produced a semi-quantitative score: absent (zero); mild (+); moderate (++); and

intense (+++). Each sample was evaluated by three different pathologists.

Immunohistochemistry assay

The slices were incubated overnight at 4 °C with the primary antibodies anti-TGF-β, anti-heparanase-1, anti-heparanase-2, anti-MMP-2 and anti-MMP-9 (Santa Cruz Biotechnology®, CA, USA). Finally, the slides were incubated with a biotin-avidin-peroxidase complex and developed using 3,3′-diaminobenzidine as the chromogen (LSAB®, Dako Cytomation, Glostrup, Denmark) for 30 min. The slides were examined under a light microscope (Nikon Eclipse® TS100). Two independent observers scored 300 cells according to the presence of staining for each of the antibodies as mentioned above. The immunocytochemistry staining was analyzed by digital quantification following the methodology described by Matos et al. [7]. The values were expressed as optical units per square micrometer (ou/μm^2).

Real-time PCR

Quantitative Real-Time PCR (qPCR) was used to evaluate mRNA expression of interleukine-6 (IL-6), heparan sulfate proteoglycan syndecan-1 (Syn-1), hyaluronic acid synthases (HAS1, HAS2, and HAS3) and hyaluronidases (Hyl1 and Hyl2). Total tissue RNA was extracted using Trizol reagent (Life Technologies, CA, USA), according to manufacturer's instructions. cDNA samples were obtained with 1 μg of pure RNA for the reverse transcriptase PCR (RT-PCR), using the ImProm-II™ Reverse Transcription System (Promega, CA, USA) according to manufacturer's instructions. qPCR was performed according to manufacturer's instructions using 1 μg of cDNA and SYBR Green Master Mix (Life Technologies, CA, USA). The normalization of the expression of the target genes was determined using the geometric mean of the two endogenous genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, and the values of expression were corrected using $2^{-\Delta Ct}$.

Analysis of sulfated glycosaminoglycans

The tissues were homogenized in acetone, centrifuged for 15 min (3000g) and dried at 60 °C. The homogenized tissue was submitted to proteolysis with Alcalase (Novozyme) diluted 100 times. The proteolysis was carried out at 50 °C overnight, followed by trichloroacetic acid precipitation (TCA 90% in the presence of 5 M NaCl), to remove protein debris. The precipitation of sulfated glycosaminoglycans was performed using two volumes of methanol, at 20 °C, overnight. An aliquot of 5 μL of each sample was subjected to agarose gel electrophoresis, using 1,3-diaminopropane-acetate 0.05 M, pH 9.0. After electrophoresis and precipitation of sulfated glycosaminoglycans using cetyltrimethylammonium 0.1%, the gel was dried and stained with toluidine blue for identification and quantification of each glycosaminoglycan by densitometry using Epson Perfection V700 Photo equipment.

Hyaluronic acid quantification

The ELISA-like assay was performed for hyaluronic acid quantification as described by Martins and his co-authors [8]. A 96-well plate was coated with hyaluronic acid binding protein (link

protein), followed by addition of hyaluronic acid standard and conjugated link protein to biotin and europium labeled streptavidin. Subsequently, the samples containing hyaluronic acid were added promoting a dislodgement of standard linked hyaluronic acid, releasing consequently, europium in solution. The final fluorescence was quantified in a fluorometer, and the amount of fluorescence was directly proportional to the amount of hyaluronic acid in each sample.

Statistical analysis

The expression of mRNA and protein were determined by mean and standard deviation using nonparametric and unpaired tests. The glycosaminoglycans quantification was described by the median and Mann-Whitney test was used for comparison between two groups. For all analysis, a statistical significance level of 5% was adopted ($P < 0.05$), and Prism® software version 5.0 (GraphPad Prism Software Inc®, California, USA) was used.

Results

Pathological features

Histological analysis of penile tissue obtained from the control group and patients with Peyronie's disease demonstrated decreased number of blood vessels and apoptosis in Peyronie's tissues (Table 1). However, no differences were detected in the number of cells, nerves or fibers.

Table 1
Pathological Features from control and Peyronie's samples.

Patients	Total Cells	Fibrocytes	Other cells	Blood Vassels	Nerves	Fibers	Apoptosis	
AFM	53	13	40	10	2	(+++)	5	Control
ARC	27	8	18	Zero	Zero	(++)	3	
DESC	28	4	27	2	Zero	(++)	5	
ISS	57	16	39	4	6	(++)	9	
LSF	91	15	82	4	2	(++)	9	
M C O	109	35	79	12	8	(++)	16	
Average	61	16	48	5	3	(++)	7.8	
JAA	42	9	37	2	Zero	(++)	3	Peyronie
BCL	45	18	27	Zero	Zero	(++)	4	
JLV	41	13	19	Zero	Zero	(++)	6	
SMP	69	28	40	6	4	(+)	3	
JPN	94	17	61	Zero	4	(+++)	6	
JRCM	68	19	54	8	8	(++)	5	
PAOS	52	18	34	4	2	(+++)	4	
Average	59	17.4	39	2.5	2.6	(++)	4.4	

Each individual was indicated with initial letters of the name. The values expressed represent the number of cells, vessels and apoptotic events. The average numbers were obtained after the analysis of each sample by three different pathologists. The extracellular matrix fibers were determined by hematoxylin eosin staining and was quantified as mild (+), moderate (++) or intense (+++) labelling. Control, tissues collected from non-affected individuals; Peyronie, tissues obtained from patients with Peyronie disease.
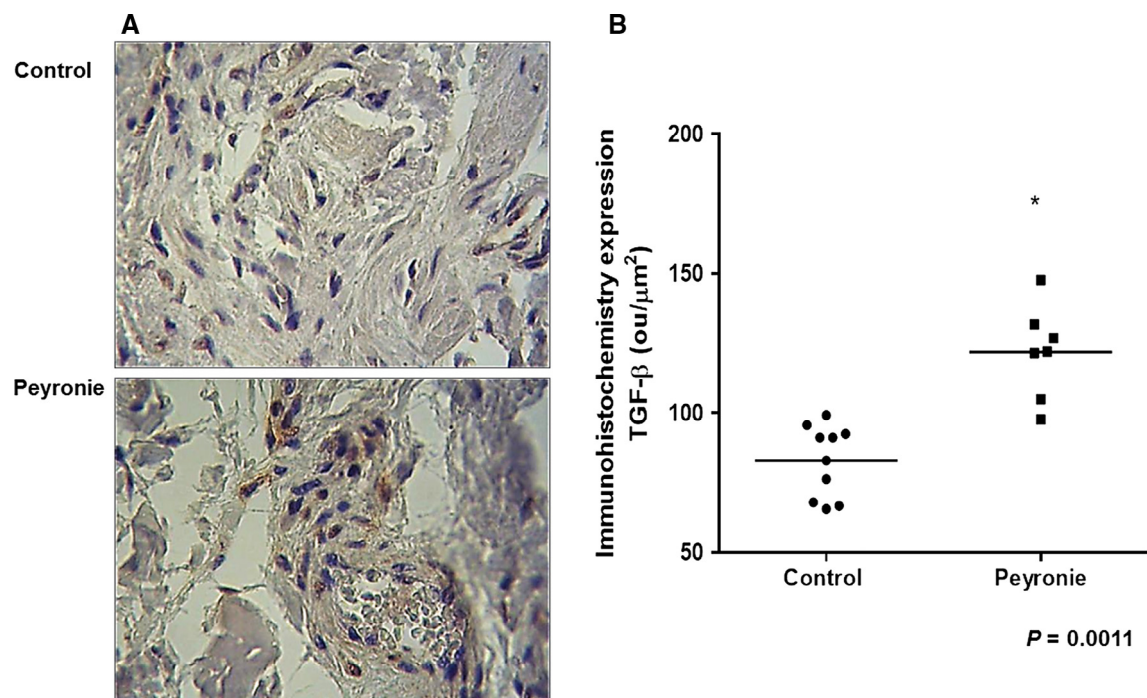

Fig. 1. Immunohistochemistry quantification of transforming growth fator-β (TGF-β). A, Immunohistochemistry reaction obtained from control and Peyronie's samples. B, The dots (circles and squares) represent the values obtained by digital quantification of immunohistochemistry reactions from each tissue of Peyronie's patients and unaffected individuals. The number of samples used was described in methods (N = 10 and N = 7), for control and Peyronie's group, respectively. The lines indicate mean value of TGF-β expression in each group (P^* = 0.0011; Nonparametric unpaired test).

Immunohistochemistry analysis

The assessment by immunohistochemistry analysis and digital quantification evidenced a significant increase in TGF-β expression in the Peyronie's samples (83.0 ou/μm^2) compared to control group (122.0 ou/μm^2 as shown in Fig. 1. The mRNA expression of interleukin-6 was higher in the tissues of Peyronie's patients than in the control group (Fig. 2). Increased levels of TGF-β is the major feature that determines the presence of Peyronie's disease. The rel-
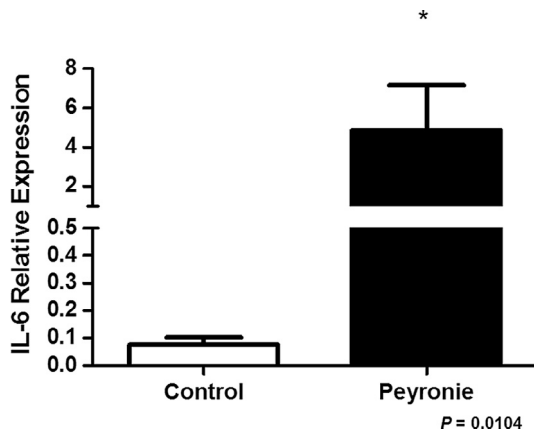
ative expression of IL-6 was higher in Peyronie's than in the control group, 4.86 ou/μm^2 and 0.076 ou/μm^2, respectively (Fig. 2).

To check for extracellular remodeling, we investigated some metalloproteases and glycosidases involved in such mechanism. There were no changes in the immunohistochemistry expression of metalloproteinase-2 (MMP2) and metalloproteinase-9 (MMP9) in Peyronie's and control group (Fig. 3). The mean of MMP2 expression was not altered; 85.95 ou/μm^2 and 93.04 ou/μm^2; for control and Peyronie's group, respectively (Fig. 3). There was also no difference between MMP9 expression in control (112.28 ou/μm^2) and Peyronie's group (119.71 ou/μm^2) as shown in Fig. 3. However, there was a significant difference in heparanase-1 and heparanase-2 expression. Both heparanases were significantly increased in the Peyronie's group compared to the control group (151.48 ou/μm^2 and 130.8 ou/μm^2) and (107.17 ou/μm^2 and 113.60 ou/μm^2), respectively (Fig. 4). The expression of heparan sulfate proteoglycan mRNA, syndecan-1 (Syn-1), was not altered, as shown in Fig. 5.

Glycosaminoglycan investigation

Fig. 6 shows that sulfated glycosaminoglycans, chondroitin sulfate (CS) was significantly increased in the tissues of patients affected by Peyronie's disease, compared to the control group, while no difference was observed for dermatan sulfate and non-sulfated glycosaminoglycan hyaluronic acid (HA). The median for CS was 5.49 μg/mg tissue in the control vs. 11.78 μg/mg tissue in Peyronie's group ($P = 0.008$). Dermatan sulfate was not altered in patients with Peyronie (43.13 μg/mg tissue) compared to control group (23.39 μg/mg tissue). On the other hand, there was no difference between the median values of HA in control (0.67 μg/mg of tissue) as well as Peyronie's (0.29 μg/mg tissue) group. No difference was observed in the expression of hyaluronic acid synthases (HAS1 and HAS2) and hyaluronidases (Hyl1 and Hyl2), when the
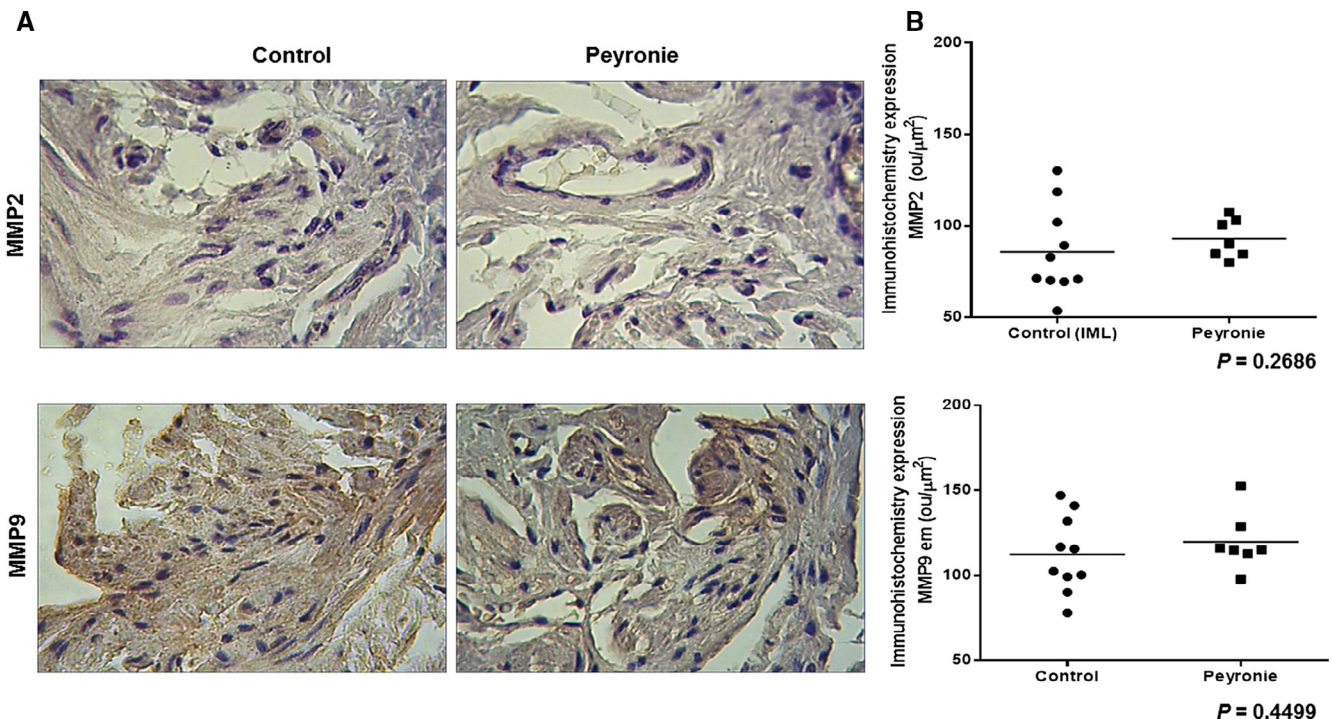

Fig. 2. Interleukin-6 (IL-6) expression. The IL-6 mRNA expression was determined by quantitative RT-PCR analysis as described in Methods. The normalization of the expression of the target genes was determined using the geometric mean of the two endogenous genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, and the values of expression were corrected using $2^{-\Delta Ct}$. The number of samples used was described in methods (N = 10 and N = 7), for control and Peyronie's group, respectively. Control, unaffected group; Peyronie's, patients affected by Peyronie's disease. The result was expressed as mean and standard deviation. Nonparametric unpaired test, $P^* = 0.0104$.

Fig. 3. Metalloprotease analysis. Panels A, Immunohistochemistry reactions were used to investigate Metalloprotease-2 (MMP2) and metalloprotease-9 (MMP9) expression. Panels B, the dots indicate the values obtained by digital quantification of MMP2 and MMP9 determined in the tissue of each control (unaffected individuals) and Peyronie's group. The number of samples used was described in methods (N = 10 and N = 7), for control and Peyronie's group, respectively. The lines represent mean value of MMPs expression. *P* values were obtained using Nonparametric unpaired test.
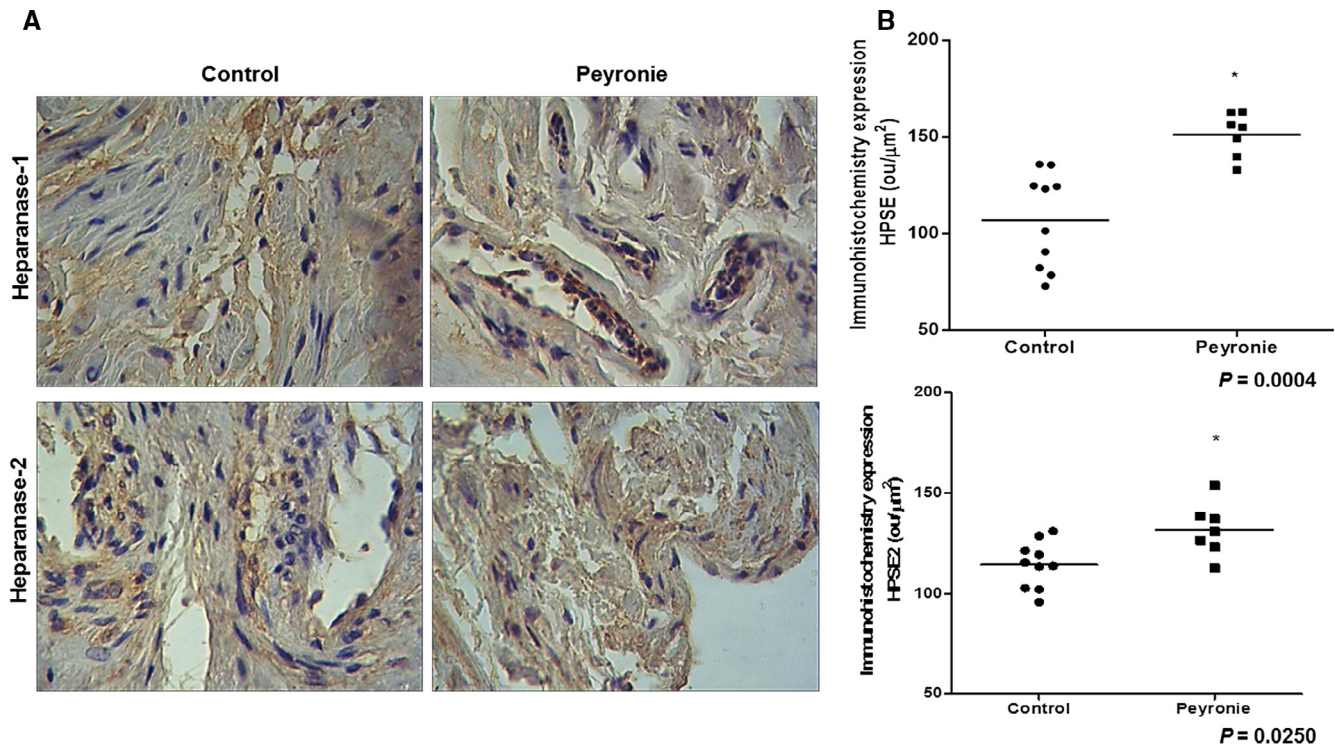
Fig. 4. Expression of heparanase isoforms. Heparanase-1 and heparanase-2 were investigated by immunohistochemistry reactions (Panels A), while the expression values were determined by digital quantification (Panels B). The number of samples used was described in methods (N = 10 and N = 7), for control and Peyronie's group, respectively. Each dot of the Scatchard plot specifies one sample obtained from control (unaffected individuals) and patients with Peyronie's disease, indicated by circles and squares, respectively. Both P values were obtained by Nonparametric unpaired test, $P^* = 0.0004$ and $P^* = 0.0250$, for control and Peyronie group, respectively.
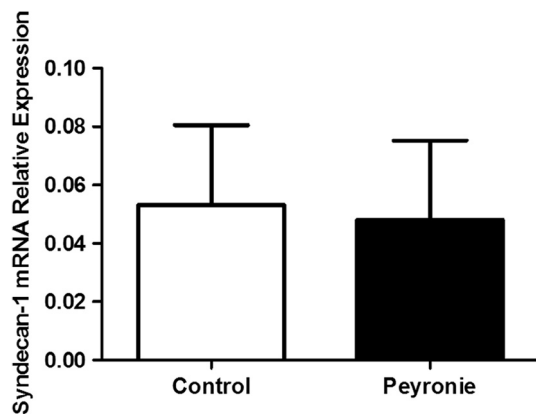

Fig. 5. Quantitative expression of syndecan-1. Quantitative RT-PCR was used to determine the expression of mRNA of syndecan-1, as previously described in Methods. The number of samples used was described in methods (N = 10 and N = 7), for control and Peyronie's group, respectively. The normalization of the expression of the target genes was determined using the geometric mean of the two endogenous genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. The values represent mean and standard deviation of syndecan-1 expression determined by $2^{-\Delta Ct}$.

samples obtained from unaffected and Peyronie's tissues were analyzed, as shown in Fig. 7. As well, there was a non-significant expression of mRNA hyaluronic acid synthase-3 (HAS3), in both the Peyronie's and control group, suggesting that HAS3 is not expressed in the albuginea tunica (data not shown).
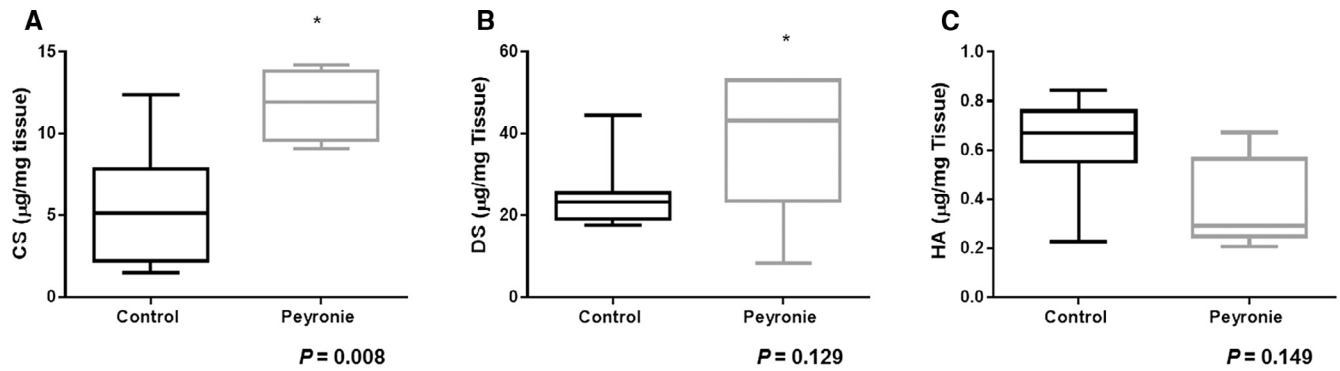
Discussion

A decreased number of blood vessels in Peyronie's samples compared to control samples indicated a less intense process of angiogenesis. Apoptosis was also reduced in the Peyronie's tissue samples. There were no differences in the number of cells, nerves or fibers comparing Peyronie's patients and non-affected tissues, possibly because the disease might be at a stage where such alterations were not expressive.

Increased levels of TGF-β proved the presence of Peyronie's disease in the analyzed samples of the patients investigated in the present study. A direct correlation was found between TGF-β level and the development and the severity of Peyronie's disease [4]. The increased level in IL-6 corroborates with the enhancement of TGF-β since both molecules are involved in the inflammatory process that occurs in Peyronie's disease.

Extracellular matrix remodeling involves the action of different enzymes. In the present study, heparanase-1 (HPSE) expression was significantly enhanced, while no difference was observed for metalloproteases (MMP2 and MMP9). These combined results indicate that, in Peyronie's disease, the glycosidase HPSE, which is involved in the degradation of heparan sulfate chains from proteoglycans, has a more pronounced role than metalloprotease activity in extracellular matrix remodeling. It is well known that oligosaccharides generated by the action of heparanase-1 can intensify the action of growth factors, cytokines, angiogenic and inflammatory factors [9].

The function of heparanase-2 (HPSE2) remains unknown. Some authors have described an increased expression of HPSE2 in tumor tissues [10,11]. HPSE2 can also modulate the catalytic activity of HPSE [12,13]. Therefore, HPSE2 might be involved in the regulation of heparanase-1 in Peyronie's disease. It is important to point out that increased expression of HPSE and HPSE2 is an entirely new result in the literature and such data open the possibility of further studies on this topic and may contribute to elucidate molecular mechanisms involved in the alterations in Peyronie's disease.

Fig. 6. Profile of glycosaminoglycans. A, chondroitin sulfate (CS) and B, dermatan sulfate (DS) were quantified by agarose gel electrophoresis. C, ELISA-like assay was used to determine the amount of hyaluronic acid (HA). The number of samples (N = 10 and N = 7), for control and Peyronie's group, respectively. Box-plot represents the median value of each group (central line) and ranges. The percentiles are shown in the attached table. There was a significant difference comparing control group (unaffected individuals) and Peyronie's (patients affected by Peyronie's disease) for CS, while no difference was observed for DS and HA. Mann-Whitney test was used to obtain P values.
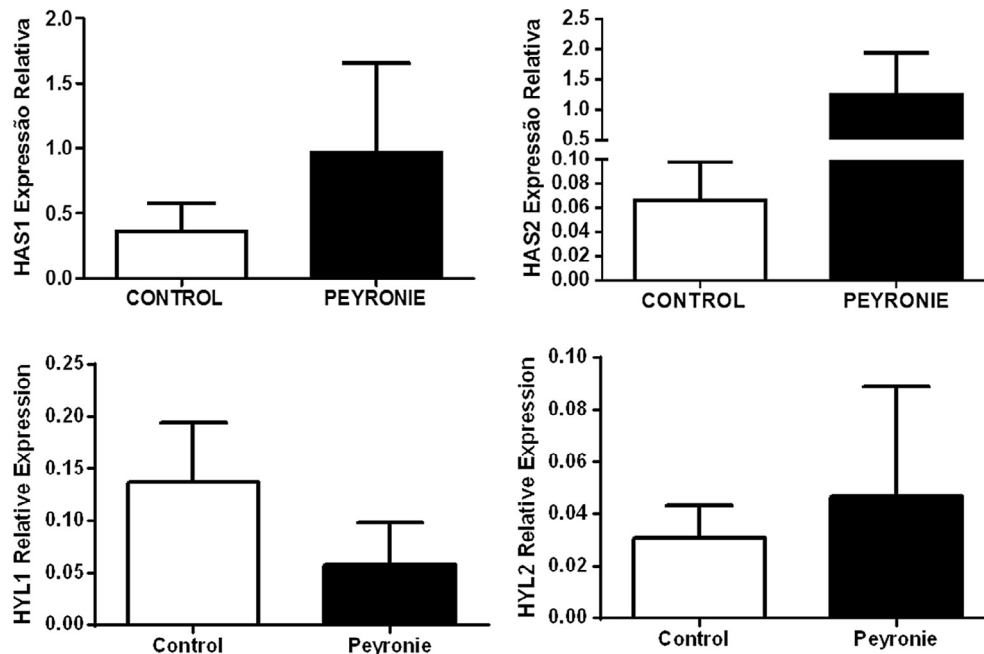

Fig. 7. Expression of hyaluronic acid synthases (HAS1, HAS2 and HAS3) and hyaluronidases (HYAL1 and HYAL 2). The expression of mRNA from hyaluronic acid synthases and hyaluronidases were obtained by quantitative RT-PCR. The number of samples used was described in methods (N = 10 and N = 7), for control and Peyronie's group, respectively. The normalization of the expression of the target genes was determined using the geometric mean of the two endogenous genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, and the values of expression were corrected using $2^{-\Delta Ct}$. The values are expressed as mean and standard deviation using nonparametric unpaired test.

The increased expression of heparanase-1 indicates that in tissues affected by Peyronie's there was a remodeling process of the extracellular matrix, and such events are not associated with metalloprotease (MMP2 and MMP9) expression.

Syndecan-1 is the most ubiquitous heparan sulfate proteoglycan (HSPG) present in the cytoplasmic membranes. The major function of HSPG is modulating the action of growth factors, and it is also involved in interactions between cell-cell and cell-extracellular matrix [14,15]. Some authors have demonstrated HSPG shedding with enhanced activity of heparanase-1 [16,17]. However, the present data showed that the expression of syndecan-1 was not altered in the tissues affected by Peyronie's disease.

Low molecular weight proteoglycans containing dermatan sulfate and chondroitin sulfate can modulate TGF-β positively, acting as a co-receptor, and enhance the response of this factor [5,18,19]. Consequently, the increase of chondroitin sulfate in Peyronie's samples reinforces the hypothesis that such compound is altered in albuginea tissues from patients with Peyronie's disease compared to non-affected tissues. It is not possible to determine if chondroitin sulfate is involved with the development of the Peyronie's disease or it is a result of the changes that lead to the plaque formation. Although we do not have experimental evidence, we can hypothesize that higher levels of chondroitin sulfate could intensify the effect of TGF-β.

It is well known that high molecular weight HA is involved in cell signaling pathways for adhesion, migration and cell proliferation as well as activation of tyrosine kinases. Nevertheless, low molecular weight HA seems to be involved in the inflammation process, inducing the production of cytokines and contributing to macrophage activation as well as angiogenesis [20–22]. Furthermore, high molecular weight HA was synthesized by HAS1 and HAS2, while HAS3 is involved in the synthesis of low molecular weight HA, and HA was degraded essentially by Hyl1 and Hyal2 [23,24]. No alterations observed in the expression of the enzymes involved in the synthesis and degradation of HA validate the result, which showed no difference in the quantification of this glycosaminoglycan in both Peyronie's and control tissues.

These combined data suggest that hyaluronic acid is possibly not involved in extracellular matrix alterations in the development of Peyronie's disease and also elucidate that HAS3 might not be expressed in the albuginea tissue.

A recent article in the literature found that intralesional injection of hyaluronic acid, in the early stage of the Peyronie's disease, improved plaque size, penile curvature and sexual satisfaction [25]. Moreover, another study using hyaluronic acid as treatment after laparoscopic pelvic surgery showed a significant reduction in bowel adhesion to abdominal wall [26].

Although the amount of hyaluronic acid was not altered in Peyronie's disease, the treatment with exogenous molecule, especially high molecular weight hyaluronic acid (HMW-HA), might be effective in decreasing scar formation (tissue regeneration) and blocking the side effects of inflammation as previously described as a biological function of HMW-HA [20].

Conclusions

Increased expression of heparanases as well as enhanced amounts of CS suggest that such molecules are involved in the molecular alterations of Peyronie's disease. Higher levels of CS may act as a co-receptor, thus enhancing the response of TGF-β mediated by proteoglycans such as decorin and biglycan. Heparanase-1 may be related to an intensification of the inflammatory process, essential for plaque formation, while heparanase-2 could be important in modulating the activity of heparanase-1, since both heparanases have affinity for heparan sulfate. These novel data demonstrated extracellular matrix changes in Peyronie's disease. Such findings may contribute to the elucidation of key molecules that are altered in the albuginea tissue of patients with Peyronie's disease and may be useful as target molecules for future therapies.

Conflict of interest

The authors have declared no conflict of interest.

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