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Stereological and biochemical analysis of muscular and connective tissue components in the penile corpus cavernosum adjacent to the fibrous plaque of Peyronie's disease

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OBJECTIVE

To investigate the structural organization of the connective tissue in the corpus cavernosum (CC) adjacent to the fibrous plaque in Peyronie's disease (PD) using stereological and biochemical techniques, as most studies on PD have focused on the analysis of the fibrous plaque that forms in the tunica albuginea (TA). Because this fibrotic reaction is mediated by various inflammatory soluble factors, adjacent connective tissues might also be affected and this secondary effect might explain, for example, the erectile dysfunction that occurs in PD.

PATIENTS AND METHODS

During surgery biopsies were taken from the CC adjacent to the fibrous plaque and from the plaque itself in seven patients with PD (mean age 48.3 years). All the patients had normal erections. Control samples were similarly located samples from 'normal' penises obtained during autopsy of five men (mean age 52.3 years). Tissue samples were stained with Weigert's stain (elastic fibres), Van Gieson's stain (connective tissue), and Sirius red (collagen). Stereological analysis was done using a 42-point grid to determine volumetric densities (Vv). Total collagen content was estimated as micrograms of hydroxyproline per milligram dry CC.

RESULTS

The Vv of elastic fibres was significantly reduced in PD by 17.3% compared with controls, at a mean (SD) of 19.49 (3.27)% vs 23.56 (1.87)% ($P < 0.05$). While in PD the Vv of smooth muscle at 34.46 (2.06)% and connective tissue at 35.39 (6.15)% were not significantly different from those of controls at 38.38 (3.17)% and 38.02 (5.03)%, respectively. The Vv of elastic fibres in the fibrous plaque was decreased by 38.3% compared with the normal TA, at 20.25 (5.49)% vs 32.81 (4.75)% ($P < 0.02$). The mean (SD) collagen concentration in the CC from controls was 77.94 (24.26) μg/mg and in the patients with PD was 66.57 (19.39) μg/mg, which did not differ significantly. Sirius red-stained sections under polarized light showed that, in the normal CC, collagen-associated colours were homogeneously distributed. However, in the PD samples, stained collagen had a disrupted orientation and had a more heterogeneous birefringence, implying looser collagen bundles.

CONCLUSIONS

The quantitative analyses indicated that collagen in the CC close to the fibrous plaque was not affected, although its organization was noticeably altered. The CC elastic fibres were reduced though, and there was a similar change in the fibrous plaque of the TA. These results suggest that, although occurring primarily in the TA, the PD fibrous plaque may induce changes in the adjacent CC.

KEYWORDS

penis, Peyronie's disease, extracellular matrix, elastic system fibres, collagen, smooth muscle

INTRODUCTION

Peyronie's disease (PD) is thought to be a connective tissue disorder of the penile tunica albuginea (TA). It occurs more frequently in men aged 40–60 years [1,2] with a prevalence of 0.38–23% depending on the population under study. Although in the past this disease was usually considered rare, more recent studies have shown that the incidence of PD is much higher than previously thought [3,4].

PD can be regarded as a response to recurring microvascular trauma, which leads to exaggerated healing of the TA [5]. This response initiates as a perivascular inflammatory infiltrate in the TA and culminates with the formation of a fibrotic plaque consisting mostly of scar tissue [6]. Collagen and elastic system fibres are highly disorganized in this plaque, an alteration that adversely affects the extensibility and compliance of the TA, thereby leading to penile deformity [7,8]. The plaque is usually located on the dorsal surface of the penis. Lateral, ventral, as well as 'hourglass' deformities can also occur. The latter is more rare, and results in even greater difficulty for penetration [5].

The aetiology of PD is still unclear. Most studies have shown that trauma during penetration is the probable stimulus that triggers the fibrotic response, which itself may be modulated by genetic predisposition. Other hypotheses to explain the onset of PD

include a failure in fibrin removal, collagen alterations, and loss of tissue elasticity due to normal ageing, which places a stress on the TA [1]. Recently, it was reported that in patients with diabetes mellitus, penile curvature is more severe [2]. Several histological studies have shown that in plaques from patients with PD there is an increase in the expression of TGF-β1, an important mediator in inflammatory processes [5,9–11]. Additionally, because PD is associated with Dupuytren contracture (15–25%), auricular fibrosis, plantar fibromatosis and scleroderma, an autoimmune factor might also be involved [7]. Other possible causative factors of PD include vascular abnormalities and psychological disorders. These might act as potential mechanisms for the development of erectile dysfunction (ED) in men with PD [12,13].

In the initial stages of the disease, most patients with PD do not report difficulties in obtaining or maintaining an erection. However, in later stages, penetration becomes difficult not only because of the deformity but also due to pain. Clinical studies have shown an association between ED and PD, although this is still not well established [3,4,14]. One of the hypotheses to explain ED in some patients with PD, stresses the importance of damage to structural components of the TA, with progressive reduction in the amount of elastic fibres and a disruption of collagen bands [8].

Most PD investigations have focused on the analysis of the primary lesion, i.e. on structural alterations of the TA and its fibrous plaque. However, it is possible that other regions of the penis are also affected, especially those that are close to the plaque, such as the adjacent corpus cavernosum (CC). If such an ancillary lesion exists, it might explain, at least in part, the ED that is normally associated with PD.

In the present study, this issue was addressed by analysing, using morphological and biochemical methods, the connective tissue of the penile CC adjacent to the fibrous plaque from patients with PD.

PATIENTS AND METHODS

The Ethical Committee on Human Research of the State University of Rio de Janeiro approved this study protocol and all patients signed informed consents.

Biopsies from the fibrous plaque in the TA and from the immediately adjacent CC were obtained from seven patients, with a mean (range) age of 48.3 (40–68) years, during corrective surgery for PD. The criteria for prescribing surgery to these patients were the presence of penile curvature of >45 ° associated with significant impairment of vaginal penetration, and a period of at least 6 months of penile curvature stability. During the preoperative evaluation, all patients reported spontaneous erections without the need of oral drugs. Also, drug-induced erection tests, using prostaglandin, were carried out in all patients to assess the degree of curvature and penile rigidity, which was adequate after administration of 10 μg of the drug. The surgical procedure consisted of an 'H' incision of the plaque associated with a penile crura graft [15]. The control samples consisted of equivalent TA and CC (macroscopically normal samples) obtained during autopsy of five age-matched men (mean 52.3 years) who had died in accidents.

The tissue specimens were fixed in 10% buffered formalin and routinely processed for paraffin embedding. Sections of 5 μm were obtained and stained with: (i) haematoxylin-eosin to assess the integrity of the tissue; (ii) Weigert's resorcin-fuchsin with previous oxidation to stain elastic fibre system fibres; (iii) Van Gieson's stain to label connective tissue and smooth muscle cells; and (iv) Picrosirius red under polarized light to detect differences in overall connective tissue organization. The specificity of the Weigert's method was confirmed by immunolabelling with an anti-elastin antibody (monoclonal, E 4013, Sigma, Saint Louis, MO, USA), and that of the smooth muscle staining by immunolabelling with an anti-smooth muscle α-actin (Zymed Laboratories, 08–0106 predilute antibody). Appropriate positive and negative controls for the immunostaining were done before labelling the penile samples.

Morphological data was quantified using stereological methods. For each individual and for each histological staining technique, 10 sections of TA and CC were obtained, and for each section, 10 fields were analysed. All images were photographed with a digital camera directly coupled to the microscope at ×200. The volumetric density (Vv) of histological structures was then evaluated by superimposing an M-42 test system on the digital images following techniques that have been described in detail elsewhere [16].

For biochemical analysis, the CC tissue samples taken immediately after excision during surgery or autopsy were 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 CC tissue was obtained and weighed.

The concentration of total collagen in the CC tissue was determined by a colorimetric hydroxyproline assay. Thus, 5–14 mg of dry, defatted CC were hydrolysed in 6 M HCL for 18 h at 118 °C as previously described [17]. The assay was then carried out in the neutralized hydrolysates using a chloramin T method [18]. Results were expressed as micrograms of hydroxyproline per milligram of dry, defatted CC.

The statistical procedures of Sokal and Rohlf [19] were followed. A two-tailed Wilcoxon two-sample test was used for comparison of stereological and biochemical variables between controls and patients with PD. All results are given as the mean (SD), with $P < 0.05$ considered to indicate statistical significance.

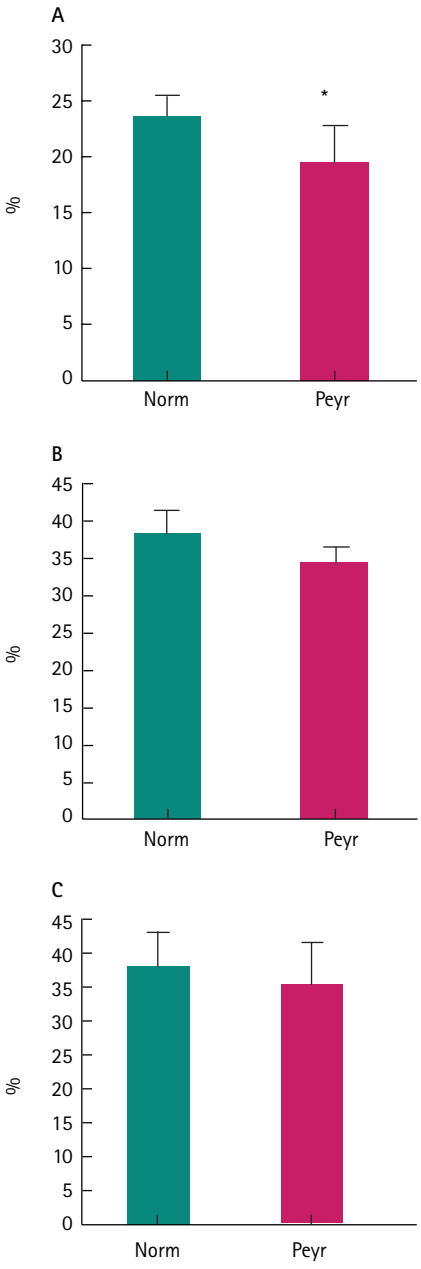
RESULTS

The stereological quantification in the CC showed that the Vv of elastic system fibres was significantly reduced by 17.3% in PD compared with controls, at a mean (SD) of 19.49 (3.27)% vs 23.56 (1.87)% ($P < 0.05$; Figs 1A and 2A,B). By contrast, in PD the Vvs of smooth muscle at 34.46 (2.06)% and connective tissue at 35.39 (6.15)% were not significantly different from those of controls at 38.38 (3.17)% and 38.02 (5.03)%, respectively (Figs 1B,C and 3A,B).

To investigate whether the penile tissue primarily affected by PD was similarly altered, the relative content of elastic system fibres in the TA was also determined. As in the CC, the Vv of elastic system fibres in the PD fibrous plaque was decreased by 38.3% compared with the normal TA, at 20.25 (5.49)% vs 32.81 (4.75)% ($P < 0.02$; (Figs 4 and 5A,B).

The quantitative analyses therefore indicated that the CC connective tissue close to the fibrous plaque was unchanged in patients

*FIG. 1. The Vv of elastic system fibres (**A**), smooth muscle (**B**), and connective tissue (**C**) in the CC adjacent to the TA of five normal men (Norm) and adjacent to the fibrous plaque of seven cases of PD (Peyr). Bars represent the mean (SD). For each stereological variable, results for Peyr were compared with the corresponding Norm using the Wilcoxon two-sample test, and significant differences (P < 0.05) are indicated by an asterisk.*



with PD. However, histological preparations using Sirius red staining observed under polarized light, revealed additional details about the structural organization of collagen,

*FIG. 2. Photomicrograph of elastic system fibres (arrow) in the CC in controls (**A**) and in the PD group (**B**). Weigert's resorcin-fuchsin, ×400.*

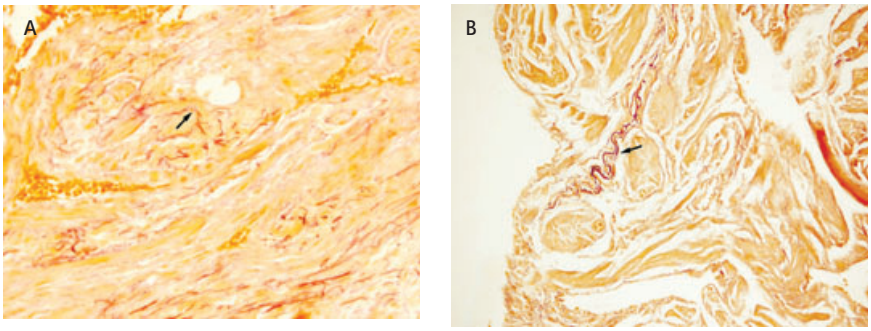


*FIG. 3. Photomicrograph of collagen in the CC in controls (**A**) and in the PD group (**B**). Picrosirius red, ×200.*

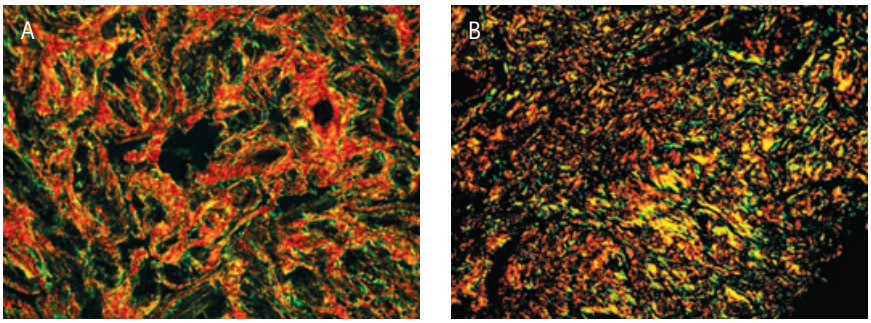
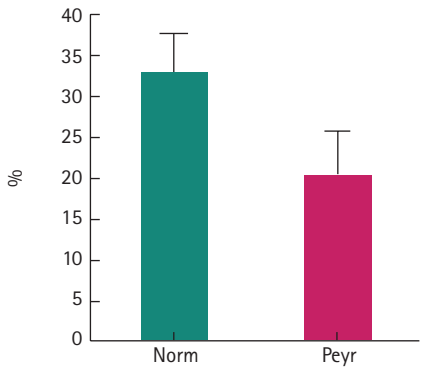


FIG. 4. The Vv of elastic system fibres in the TA from five normal men (Norm) and in the fibrous plaque from seven cases of PD (Peyr). Bars represent the mean (SD). The Wilcoxon two-sample test indicated that the two means are significantly different (P < 0.02).



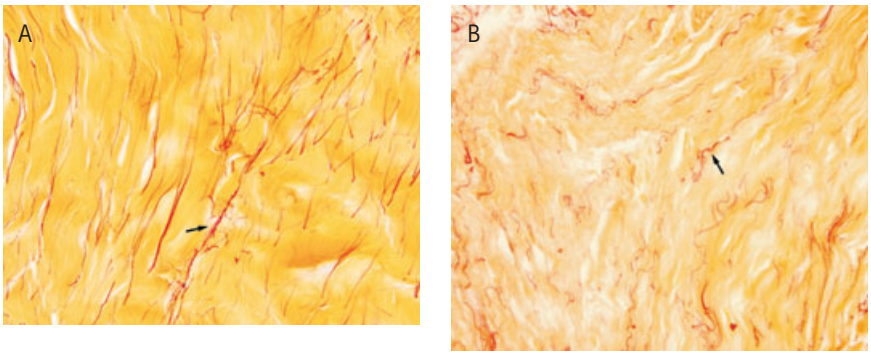
which showed disease-related modifications in this component of the extracellular matrix. Accordingly, in the normal tissue, collagen-associated colours were homogeneously distributed in the trabeculae and suggested that the bundles were more tightly packed (Figs 3A and 6). Conversely, in the PD samples, stained collagen had a noticeably disrupted orientation and had a more heterogeneous birefringence, which altogether imply a looser organization of the collagen bundles (Fig. 3B).

The determination of connective tissue content by stereological methods is also an approximate quantification of collagen, as this protein is the most abundant component of that tissue. Thus, to confirm that collagen content was unchanged in the PD CC despite being structurally disrupted, we biochemically determined total collagen concentration. The results showed that this concentration in the CC tissue from controls (77.94 (24.26) μg/mg) and PD (66.57 (19.39) μg/mg) did not differ significantly (Fig. 6), which agrees with the morphological data.

DISCUSSION

Many theories have been proposed about the aetiology of PD. According to the most widely accepted, the disease is caused by an excessive healing in the TA from repeated trauma to the penis, together with a genetic predisposition [20]. This injury might affect the erectile tissue, and there is evidence showing an association between PD and ED

*FIG. 5. Photomicrograph of elastic system fibres (arrow) in the TA in controls (**A**) and in the PD group (**B**). Ωειγερτ σ ρεσορχιν–φυχησιν, ×400.*



[3,14,21,22]. However, these studies did not investigate the underlying mechanisms that would account for the ED. Because the CC is the main structure involved in erection, components of this tissue, such as smooth muscle cells, the extracellular matrix, blood vessels, and nerve endings might be altered and thereby affect erection. In the present study, we focused our analysis on smooth muscle cells and the extracellular matrix of the CC, which are important components involved in normal erection and in ED [23,24]. The present results showed that these components are modified in the CC close to the fibrous plaque, which therefore supports an association between PD and ED.

In its earlier stages, PD does not affect sexual function, and the present data were indeed obtained from patients with PD that had a normal erection. However, the present results suggest that these individuals may eventually develop ED as the CC already showed significant alterations.

COLLAGEN

In a study using three patients with PD (aged 39–67 years), the authors reported an increased collagen content in the CC [25]. However, the patients already had ED. The present collagen results, using both morphological and biochemical methods, did not show a significant difference between the PD and the normal CC. However, the results of the Picrosirius staining imply alterations in the overall organization of collagen bundles in PD [26].

ELASTIC FIBRE SYSTEM

Sattar *et al.* [27] described the importance of elastic fibres for obtaining and sustaining erection. Loss of tissue elasticity can make the penis less resistant to dilation during erection, which results in lower cavernosal pressure and thereby may contribute to the onset of ED. Although the patients with PD in the present study were potent, our results showed that their CC elastic fibres were significantly reduced. Therefore, this reduction may be one of the earliest alterations in the CC of patients with PD.

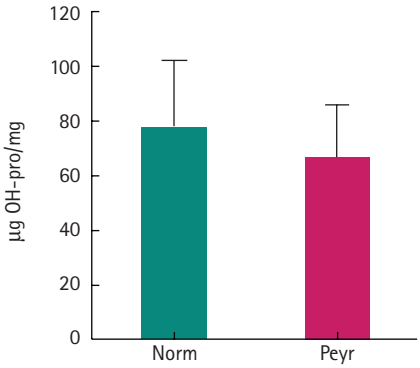
A qualitative analysis of the CC, carried out in individuals with ED from various causes [28], showed a content of elastic system fibres similar to that found in the present study. A similar result was obtained by Iacono *et al.* [29], which showed a decrease in the amount of elastic fibres in the TA and CC of patients with ED also due to various causes. Lopez and Jarow [12] evaluated veno-oclusive dysfunction and found it to be the main causative factor associated with ED in patients with PD. It is possible that this obstruction could be related to a decrease in the content of elastic fibres.

SMOOTH MUSCLE

The CC smooth muscle was not changed in PD. Again, these results are in agreement with those reported by Costa *et al.* [28], who studied individuals with severe ED. The same observation was made by Jevitch *et al.* [30] in an investigation using individuals who were potent and individuals with ED. According to this latter study, the most important modification in smooth muscle was not a variation in cell number, but changes in ultrastructural features.

The present results imply that PD is not restricted to the TA, as it somehow affects the underlying erectile tissue. Our results also indicate that, of the extracellular matrix components, elastic system fibres are one of the first to undergo modifications. Thus, it may be concluded that the high incidence of ED among patients with PD is due to simultaneous and progressive alterations in the CC. Although the present results refer only to the CC adjacent to the fibrous plaque, it is possible that the factors that induced this supposedly early alteration will eventually affect deeper regions of the tissue, thereby leading to ED.

FIG. 6. The concentration of total collagen in the CC adjacent to the TA of five normal men (Norm) and the CC adjacent to the fibrous plaque of seven cases of PD (Peyr). Tissue samples were submitted to acid hydrolysis, followed by a hydroxyproline (OH-pro) assay to estimate collagen concentration, which is expressed as μg OH-pro per mg dry CC. Bars represent mean (SD). The Wilcoxon two-sample test indicated that the two means are not significantly different.



CONFLICT OF INTEREST

None declared.

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Abbreviations: **PD**, Peyronie's disease; **TA**, tunica albuginea; **ED**, erectile dysfunction; **CC**, corpus cavernosum; **Vv**, volumetric density.