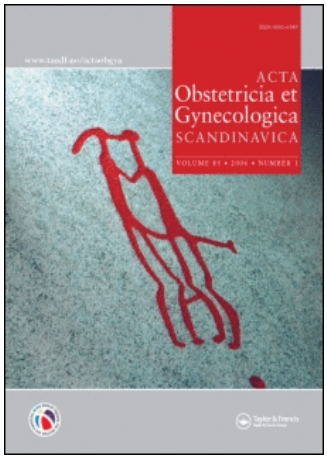

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ORIGINAL ARTICLE

Connective tissue alterations in women with pelvic organ prolapse and urinary incontinence

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Abstract

Background. Alterations in collagen synthesis and metabolism have previously been reported in patients with pelvic organ prolapse (POP) and/or urodynamic stress incontinence (USI). Since urinary incontinence does not always associate with POP, the objective of this study was to examine connective tissues from patients with USI plus POP, and patients with prolapse only. *Methods.* Biopsies from the uterosacral ligaments were obtained during the operation from POP patients ($n = 28$), and from continent women (control group, $n = 12$) who underwent surgery for other benign reasons. POP patients were classified following urodynamic tests and symptom questionnaire with respect to the presence ($n = 14$) or absence ($n = 14$) of USI. N-terminal propeptides of collagen (PINP and PIIINP), TGF-β and leptin were measured in plasma. Hydroxyproline and glycosaminoglycan (GAGs) concentrations and total hexosaminidase activity were measured in tissue samples. Histological sections were prepared using Masson's trichrome technique, and digitised solutions were used for imaging provided by Soft Imaging System GmBh. Statistical evaluations were made by the Kruskal-Wallis test. *Results.* A significant decrease in hydroxyproline content was found in USI+POP women in comparison to controls ($p < 0.05$). In contrast, histopathological examination revealed an increased density of collagen in USI+POP patients. Hexosaminidase activity was decreased in both groups with POP, but no change in the amount of GAGs was observed. Markers of collagen synthesis (PINP, PIIINP), and factors related to the collagen synthesis (TGF-β, leptin) remained unaltered. *Conclusion.* Our biochemical and morphological findings suggest a different organisation of collagen fibres in tissues of patients with USI+POP, when compared with both the controls and the POP patients.

Key words: *Collagen, hexosaminidase, urinary incontinence, glycosaminoglycans*

Abbreviations: *GAGs: glycosaminoglycans, PINP: N-terminal propeptides of collagen type I, PIIINP: N-terminal propeptides of collagen type III, POP: pelvic organ prolapse, TGF-β: transforming growth factor-beta, t-Hx: hexosaminidases, USI: urodynamic stress incontinence*

Introduction

Although the association of pelvic organ prolapse (POP) with urinary incontinence is frequent, it has been established by clinical observations that cases with prolapse do not always suffer from urinary incontinence. Various etiologic factors may contribute to the development of both POP and incontinence. Among these, abnormalities in the connective tissue of the pelvic floor are of prominent importance, and several researches indicate that women with either urodynamic stress incontinence (USI) or prolapse exhibit connective tissue alterations (1–3). However, the previous findings relating to the structure of the pelvic connective tissue are controversial (4–10). In these studies, the most widely used method is collagen quantitation by measuring hydroxyproline concentration in the tissue. Measurements of the N-terminal propeptides of collagen type I (PINP) and III (PIIINP) in blood are also used as markers of collagen formation. Although

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a reduction in the amount of collagen in stress incontinence has been reported previously by several researchers (3,11,12), no significant changes in the levels of mRNA for procollagen types I and III have been observed (8). Collagen cross-links have also been examined in incontinent subjects, and have been found either diminished (13), or unaltered with respect to the controls (3).

Glycosaminoglycans (GAGs) closely associate with collagen and elastin in the connective tissue. They are degraded by the lysosomal glycosidases and glycoside sulphatases, among which hexosaminidases (Hx) were reported to be the dominant enzymes released by the fibroblasts into the extracellular milieu (14). Despite their importance in the integrity and maintenance of the connective tissue, GAGs and Hx have not gained much interest in studies related to pelvic floor disorders.

Recent studies have shown that transforming growth factor-beta (TGF-β) is unique among cytokines due to its fibrogenetic potency. TGF-β_1 stimulates the synthesis and maturity of type I collagen. The ability of TGF-β_1 to stimulate proliferation and extracellular matrix component synthesis has previously been shown in cultured fibroblasts (15). Overexpression of TGF-β_1 reduces collagen degradation by inhibiting the expression of collagenases and increasing the production of the tissue inhibitor metalloproteinase (16).

Leptin, the ob gene product known to be involved with collagen synthesis, increases human alpha 1 (I) collagen mRNA and type I collagen production (17). It also increases TGF-β type II receptor mRNA, and augments the effect of TGF-β_1 on collagen production (18). Leptin has been shown to induce matrix metalloproteinase-2 expression and activity without altering collagen synthesis, thus suggesting that leptin has the potential to prevent extracellular matrix accumulation (19). Leptin accelerates the healing of colonic anastomoses in rats by increasing hydroxyproline concentrations and tissue proliferation (20). To our knowledge, there is no study on the circulating leptin and TGF-β levels in humans with pelvic floor disorders.

The objectives of our study were to see if there are alterations in the amount of collagen, GAGs, and in the activity of Hx in the uterosacral ligament of patients with USI and/or POP, and to investigate whether the histological characteristics of the tissue correlate with the biochemical findings. Attempts were also made to determine the levels of leptin, PINP, PIIINP and TGF-β in the plasma of all subjects studied.

Material and methods

Subjects

Forty women were included in this study according to the criteria mentioned below. They were divided into 3 groups according to the findings at clinical examination, symptom questionnaire and urodynamic evaluation. All patients were examined by the same physician in supine lithotomy position with maximum straining. The first group consisted of 12 continent women without prolapse in whom a hysterectomy was performed abdominally for benign uterine diseases. Fourteen patients with genital prolapse, but without USI, were included in the POP group, and 14 women with both incontinence and prolapse were included in the USI+POP group. The subjects in this group had symptoms of stress incontinence, as assessed by the Bristol Lower Urinary Tract Symptom Questionnaire (21). Subjects in the second and third groups, who had been matched for the stage of prolapse, had a vaginal hysterectomy due to symptomatic prolapse. The grade of prolapse in these cases was 2 according to the modified Baden-Walker Half Way System (22). All subjects were postmenopausal (no menstrual period for at least 12 months or longer) in order to minimise the interfering effects of estrogens. Women on hormone replacement therapy (HRT) or with pelvic malignancies were excluded from the study. The patients included in this study were matched with respect to age (median: 52; range: 45–64), BMI (median: 30; range: 26–35), and parity (3 births). The local ethics committee approved the study protocol. Each patient was informed and all gave their consent for the study. All subjects underwent a hysterectomy in the Department of Obstetrics and Gynecology by the same gynaecologist.

Histological examinations

During surgery, uterosacral ligaments were removed, collected and prepared for histological examination. Tissue samples were obtained at the level of the cervical insertion of the ligament. All biopsy specimens were fixed in 10% formalin for 24 h, embedded in paraffin, 5 μm sections were mounted onto glass slides, and dehydrated. Images of tissue sections were analysed using light microscopy (Leitz Wetzlar, Germany), a camera, and an image analysis program, after staining with Masson's trichrome technique (23). Digitised solutions were used for imaging provided by Soft Imaging System GmBh (Germany). In each slide, the green and red stained areas represented collagen and smooth muscle,

respectively. Each specific coloured area was calculated as number of pixels, and expressed as percentage of the entire image pixel amount.

Biochemical examinations

GAGs were measured in tissue samples using the spectrophotometric method based on metachromatic dyes (dimethylmethylene blue = DMB, Serva Fembiochemica, Heidelberg, Germany) after papain digestion of interfering proteins as follows: tissue samples (5 mg/mL) were incubated overnight at 60°C in 20 mM sodium-phosphate buffer, pH 6.8, containing 1 mM EDTA, 2 mM dithiothreitol (Fluka, Germany) and 2 mg/mL papain (Sigma, Germany). Iodoacetic acid was then added to obtain a 10-mM final concentration (1:1 v/v), and the volume was made up to 5 mL by the addition of 50 mM Tris/HCl, pH 8.0. Some 100 μL of sample was mixed with 2.5 mL DMB in a polystyrene tube, and A_{525} was read. The assay was calibrated by use of standards containing 1–5 μg chondroitin sulphate C (24).

For hydroxyproline measurements, tissues were hydrolysed in HCl for 16 h at 110°C (100 mg/2 mL). The HCl was then evaporated with nitrogen and the remainder was mixed in 10 mL bidistilled H_2O. Hydroxyproline standards were prepared in 0.001 N HCl. Tubes containing 0.5 mL of hydrolysate (or standard) were mixed with water (1.3 mL), potassium borate buffer (1 mL) and chloramine-T (0.3 mL), and incubated for 20 min at room temperature. Na-thiosulphate (1 mL), KCl (1.5 g) and toluene (2.5 mL) were added to each tube, maintained in a water-bath at 100°C for 20 min, and then brought to room temperature. Some 2.5 mL of toluene was added to the tubes and thoroughly mixed. The tubes were kept at +4°C for 20 min. Subsequently, 1 mL of the toluene extract was transferred into separate tubes, Ehrlich reagent (0.4 mL) was added and incubated for 30 min. Absorbances were recorded at 565 nm against reagent blank (25).

Homogenates (1:10 w/v) were prepared in citrate-phosphate buffer (pH = 4.4) using a ultrasonicator (VIRTIS-virsonic); and used for the measurement of total hexosaminidase (t-Hx) activity. Briefly, an aliquot (0.1 mL) of homogenate was added to 0.9 mL of 0.04 M citrate-phosphate buffer (pH 4.4), and allowed to stand for 10 min in an ice-bath. Some 50 μL of diluted homogenate was pipetted into tubes containing 100 μL of substrate (1.0 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide in 0.04 M citrate-phosphate buffer, pH 4.4). Incubation was carried out for 60 min at 37°C in a shaking water bath. The samples were then chilled on ice, 5 mL of 0.17 M glycine-carbonate buffer (pH 9.9) was added. Fluorescence was measured by Jasco FP-750 fluorometer (365 nm exitation, 450 nm emission). Substrate cleavage was determined by the comparison of fluorescence against a standard curve prepared using 4-methylumbelliferone dissolved in 0.17 M glycine-carbonate buffer over a concentration range of 0–10 nmoles. Standards of 4-methylumbelliferone are included in each assay (26). Protein concentrations of the homogenates were measured and enzyme activity was expressed as nmol/mg protein (27).

Sera were separated from venous blood samples drawn before surgery and kept at −80°C until studied. Each biochemical measurement was carried out within the same run. TGF-β_1 was measured by the ELISA kit (Bender MedSystems, Vienna, Austria, cat. no: BMS249/2, lot no: 9675006); the limit of detection was 0.04 ng/mL. Leptin was measured by the ELISA kit (BioVendor Laboratory Medicine, Brno, Czech Republic, cat no: RD191001100), the limit of detection was 0.5 ng/mL. Both ELISA determinations were carried out using EL × 800 microplate reader (Biotec Instruments, NY) by a software quantitation programme (KC4 DOS, NY, USA). PINP (cat no: 67034; detection limit = 2 ng/mL) and PIIINP (cat. no: 68570; detection limit = 0.3 ng/mL) were determined by the radioimmunoassay kits obtained from Orion Diagnostica (Espoo, Finland), using gamma counter (RIASTAR, Packard Canberra, Netherlands).

Statistical evaluations were performed using the Kruskal-Wallis test (SPSS 10.0 for windows statistical software, Chicago, IL, USA). Spearman ranking procedure was used for the correlation analyses. When 0.05 was given for the required significance level α, the power of our study was found to be 0.84.

Results

Tissue analyses

The morphological analyses by light microscopy showed that uterosacral ligaments were mainly composed of collagen fibres and smooth muscle cells (Figure 1). Histological preparations from women with prolapse had similar appearance of collagen staining with those from controls (Figure 2). In the subjects with POP + USI, the staining of collagenous connective tissue was markedly increased with respect to control and POP subjects (Figure 3). Following an image analysis program, the percentages of red-stained areas (smooth muscle) and green-stained areas (collagen) were calculated (Figure 4). The

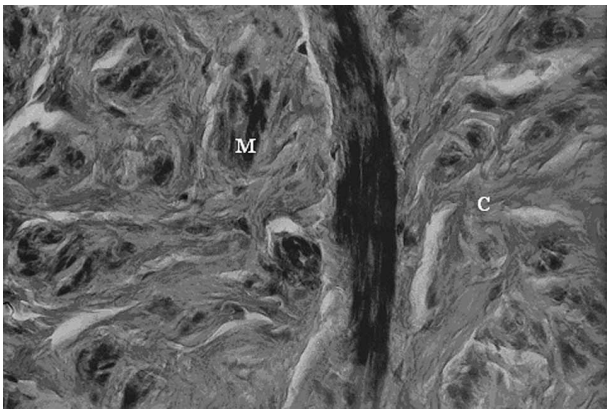

Figure 1. Microscopic appearance of tissue specimen from a patient without prolapse or USI by Masson's trichrome staining (×120). M, smooth muscle; C, collagen.

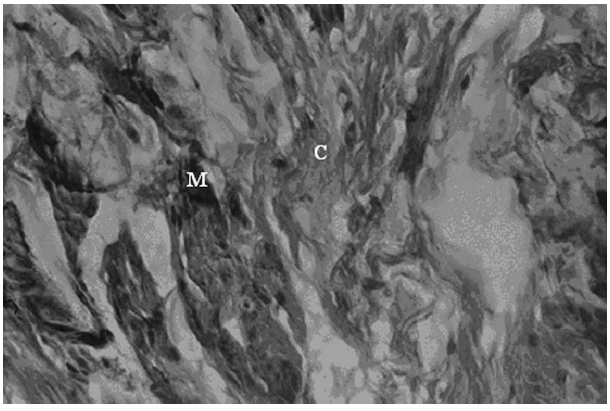

Figure 2. Microscopic appearance of the uterosacral ligament from a patient with POP. In this section, the staining pattern is similar to that demonstrated in Figure 1. Masson's trichrome staining (×120).

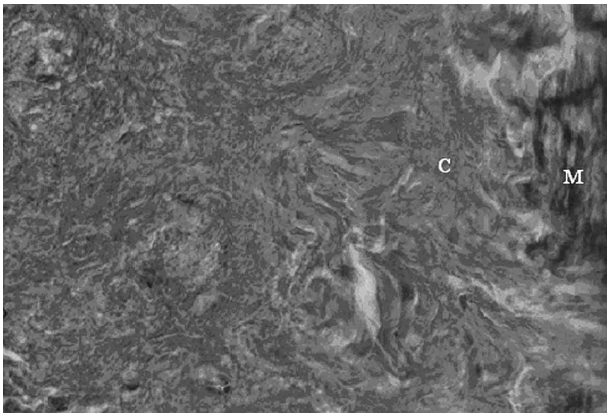

Figure 3. Microscopic appearance of the uterosacral ligament from a patient with POP+USI. Collagen (C) content is markedly increased. Masson's trichrome staining (×120).

percentages of the smooth muscle areas were slightly lower in the POP+USI group (54 versus 63.6% of controls and 59% of POP group), whereas green areas

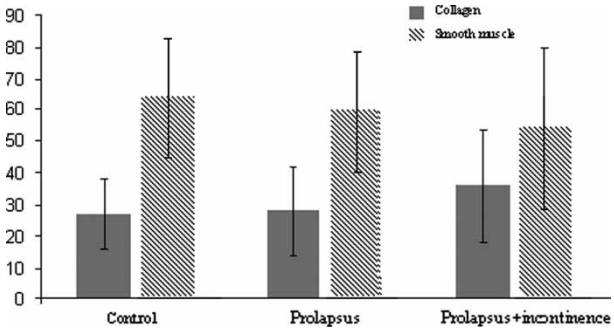

Figure 4. Percentages of collagen fibres and smooth muscle cells in histological sections of uterosacral ligaments after staining with Masson's trichrome technique.

representing the collagen fibres were increased (36%) when compared to both control (27%) and POP (28%) groups (Figure 4). The differences between the percentages in each group did not reach a statistical significance.

The results obtained from the biochemical analyses are shown in Table I. POP+USI group revealed significantly lower values for hydroxyproline than those obtained from the other study groups ($p < 0.05$). In POP+USI group, the hydroxyproline concentration of the tissue and the percentage of the green-stained areas (collagen) was correlated negatively ($r = -0.503$, $p = 0.079$).

In 2 groups of patients with prolapse, significant decrements were observed in the activity of total hexosaminidase compared to controls, regardless of the presence of incontinence. The mean concentrations of GAGs were similar in the tissue specimens of the patients in the 3 groups (Table I).

Blood analyses

The parameters studied in sera of the subjects (TGF-β_1, leptin, PINP, PIIINP) did not reveal any significant difference among the groups. TGF-β_1 levels were slightly lower in patients with urinary incontinence than those in the other groups (Table I).

Discussion

In recent years, a number of studies have examined the relationship between collagen and different types of pelvic floor disorders, such as prolapse and urinary incontinence. Several researchers have identified a reduction in collagen content in the pelvic support structures in these disorders (4,6,10). Although there are differences between the study populations, a decrease ranging between 20 and 40% is consistent in most studies. On the other

Table I. Mean values (±SD) of the parameters measured in tissue samples and sera of subjects.

Parameters	Control ($n=12$)	POP ($n=14$)	POP+USI ($n=14$)
OH-prolin (μmol/g tissue)	169±46.1	145±30.8	114±48.7*
t-Hx (nmol/mg tissue protein)	46.1±20.8	23.3±8.8*	25.7±12.3*
GASs (ng/mg tissue)	1641±926	1725±725	1651±464
TGF-β_1 (ng/mL) serum	107±39.01	120±42.9	85±27.8
Leptin (ng/mL) serum	31.7±18.1	21.7±8.6	30.7±13.2
PINP (μg/L)	61.3±28.4	49.9±27.6	59.0±39.8
PIIINP (μg/L) serum	6.45±1.83	6.66±2.65	6.48±1.72

*$p<0.05$ when compared with the control.
OH-prolin, t-Hx, and GAGs were studied in tissue, and the remaining variables in sera.

hand, collagen content was reported to be 30% higher in the stress urinary incontinence and POP subgroups; and the diameters of the collagen fibrils were 30% larger than the controls (5). In another study, the structural characteristics of collagen have been found altered (28). These changes were thought to increase the rigidity of extracellular matrix, thereby causing impairment in the mechanical function of connective tissue. Data from a previous study demonstrated an increased collagenolytic activity in women with urinary incontinence, that is restricted to uncrosslinked collagen (9), which may indicate a derangement in maturation of collagen fibres in these subjects, such as impaired cross-link formation. On the other hand, collagenolytic activity has been found unaltered despite the reduction of total collagen in the pubocervical fascia (3). Although tissue collagen content was low in some cases, no alteration in the amount of mRNA for collagen types I and III was observed between the continent and incontinent group (8). It was suggested that the difference in the quantity of protein could be a consequence of altered collagen metabolism. The discrepancy among the findings of the different studies may be explained by the fact that each study uses a different patient population, and each sample may be from different pelvic tissues. The uterosacral ligaments, in particular, provide the anatomical support of the uterus. The cervical and intermediate portions of the uterosacral ligament support more than 17 kg of weight before failure. Therefore, their structural alterations have been thought important in the pathogenesis of prolapse (29). In the present study, the biochemical and histomorphological assays were carried out in the uterosacral ligament. The hydroxyproline content of this tissue was significantly decreased in patients with POP+USI. In contrast to the biochemical result, collagen staining covered more space in the tissue preparations of POP+USI patients than in those of the patients with prolapse only, as shown by the quantity analysis of sections using Soft Imaging System. This discrepancy between the histological appearance of collagen fibres and the biochemical data related to the low hydroxyproline content is the main finding of our study. Similarly, tissue collagen content has been found decreased in cases with urinary incontinence, but no alteration in its expression has been observed (8). It is known that hydroxyproline takes place in the primary structure of collagen following post-translational hydroxylation reaction of prolyl residues, and is responsible for the intermolecular hydrogen bonding that stabilises the triple helix structure of this protein. If the amount of hydroxyproline decreases due to impairment in the post-translational hydroxylation reactions, the interhelical hydrogen bonding would lessen, thereby leading to enlargement of collagen fibril diameter. Our observations led us to suggest that the changes are primarily related to the quality rather than quantity of collagen in USI+POP subjects. The finding of the unaltered PINP and PIIINP levels in sera of these subjects is also in agreement with our hypothesis. The altered structure of elastic fibres has previously been observed in the periurethral connective tissue of women with urinary incontinence, and are believed to contribute to the occurrence of USI (30).

GAGs and lysosomal Hx were measured as structural and functional components of the extracellular matrix. Total hexosaminidase activity was found diminished in all subjects with prolapse irrespective of incontinence, whereas no difference in the GAG content was observed in the tissues studied. In a previous study, no significant alteration was seen in proteoglycan concentration (5). It has been reported that various factors may influence the release of different lysosomal components (31). Therefore, the diminished hexosaminidase activity observed in our study may not directly be related to the integrity or damage of connective tissue.

Conclusion

According to our observations, the changes in the connective tissue in patients with urinary incontinence and prolapse are more prominent than in subjects with prolapse only. These changes seem to be primarily related to the quality of collagen rather than its quantity. It is not yet possible to claim that a change in collagen structure or quantity solely causes USI and POP, for the underlying mechanisms of these disorders are multifactorial and progressive. Since all studies have examined patients after they have developed clinical symptoms, future prospective studies that follow up women before and after a disorder has developed should be considered.

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