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ORIGINAL ARTICLE

Uterine Prolapse: Immunohistochemical Study of the Pelvic Ligaments

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Summary
Introduction. Uterine prolapse is a pathological condition when pelvic support system loses elasticity and the uterus descends down into the vagina, leading to pain and even protrusion of tissue from the vagina.
Aim of the study. Compare morphology of pelvic ligaments in women with uterine prolapse and without it.
Materials and methods. Biopsies of *lig. teres uteri* were taken during the reconstructive surgery from 7 women with uterine prolapse and the control group of 3 women. Tissues were stained with hematoxilin and eosin, periodic acid – Schiff method and with antibodies of bFGF, FGFR 1, VEGF, PGP 9.5, Collagen III and IV, MMP-9, microscoped at 400X magnification and evaluated semiquantitatively. Data were analysed using non-parametric statistics – Mann – Whitney U test.
Results. VEGF was statistically significantly increased (U = 3.5, p = 0.022, two-tailed Mann – Whitney U test) in the prolapse group, in comparison to the control group. Other parameters did not display any statistically significant difference when comparing the two groups, however, amount of GAGs stained with periodic acid – Schiff method showed a notable tendency to decrease in the prolapse group in comparison to the control group.
Conclusions. Increased number of VEGF positive endothelium indicates hypoxia and stimulation of angiogenesis in female pelvic ligaments with uterine prolapse. Tendency of GAGs to decrease in the pelvic ligaments of females with uterine prolapse suggests qualitative degradation of tissue.
Key words: uterine prolapse, immunohistochemistry, vasoendothelial growth factor; glycosamynoglycans.

INTRODUCTION

Pelvic organ prolapse is a descent of the uterus down into the vagina or a descent of vagina itself. The condition is graded depending on the level of the descent of the uterus: to the upper vagina (1st degree), at the vaginal opening (*s. introitus*) (2nd degree) and *procedentia* (the uterus and the cervix are both prolapsed out of the *introitus*) (3rd degree). Vaginal prolapse may be 2nd or 3rd degree. Symptoms of the 1st degree prolapse are minimal, however 2nd and 3rd degree prolapses present with pain, pressure and a sensation of "organs falling out". Vaginal mucous may be chronically inflamed, secondarily infected and ulcerated. Urinary incontinence may also follow. Severe symptoms require surgery, for example, hysterectomy with a repair of pelvic support structures. One study shows that prevalence of symptomatic uterine prolapse is 3.6% within women aged 51-60 years, and that up to 15% of hysterectomies are administered due to this condition (5).

Up to this day pathogenesis of the uterine prolapse is not fully understood. Risk group consists of women in menopausal period of life and women who have experienced delivery more than once (2, 5, 7). Several studies (6, 7, 9, 10) state that this could be explained by the deformation of the pelvic support system, especially, *lig. latum uteri*, *lig. teres uteri*, because of the mechanical stress that these ligaments are exposed to during delivery and pregnancy. Hormone deficiency during menopause is also considered a major etiological factor (7).

Ligaments are a type of dense regular connective tissue, which mostly consists of the extracellular matrix (ECM). The ECM incorporates, primarily, collagen, elastic fibers and glycoproteins. Collagen type I-III are inextensible fibers responsible for the tensile strength of the tissue. Collagen type IV, however, forms a non-fibrous structure as support for a more flexible basal lamina (7). These structures as well as matrix metalloproteinases (MMP) are mainly produced by fibroblasts. MMPs are responsible for proteolytic degradation of collagens, which is a crucial factor for tissue remodeling, for example during the healing process after tissue damage induced by delivery. A clot forms around the damaged area, cells within the clot release growth factors and other molecules. This leads to an invasion by neutrophils and macrophages, which begin phagocytosis of necrotic tissue. This process is followed with yet another release of growth factors that stimulate fibroblast proliferation. Granulation tissue is formed by fibroblasts. This tissue consists of irregular collagen, glycosaminoglycans, it is highly vascularised. Slowly ECM becomes more organized. There is a decrease in cellular content and an increase in collagen type I, which cross-links it to healthy tissue. Repaired tissue is always mechanically weaker than undamaged ligaments (11).

Majority of researches focus on studying three directions in order to explain the pathogenesis of the uterine prolapse: changes in morphology of the connective tissue in the uterine supportive system (6, 7, 9, 11), the

roles of growth factors in this process (3, 8, 10), changes in innervations and vascularization of the ligaments after the prolapse (7, 8, 10).

Several studies focus on comparing glycoproteins and collagen in healthy women and women with prolapse. Scientific reports are conflicting on this matter, but the majority tends to note the decreased ratio of I/III collagen in women with the uterine prolapse, thus the reduction of type I collagen is compensated by the increase in type III collagen (10). However, another study states that the genital prolapse has an association with a moderate reduction of collagen type III (7). Women with a uterine prolapse have an increased expression of MMP-9 (11). Increase in collagen type III together with the expression of MMP-9 is a feature of tissue that is remodeling (7). Concentration of glycosaminoglycans (GAGs), a mucopolysaccharide that is bound to a core protein to form proteoglycans, seems to be decreased in the parametrium of postmenopausal women (the uterine prolapse risk group) (13). However, no significant change in the amount of GAGs in pelvic ligaments of women with uterine prolapse was observed, when comparing to the control group (1).

A review article (10) and several other studies (3, 8, 13) were dedicated to the roles of growth factors in ligament healing. A significant factor in stimulation of angiogenesis and cell migration and proliferation is the Basic Fibroblast Growth Factor (bFGF). *In vivo* studies have shown that application of bFGF to an injured ligament correlates with the increase of collagen type III expression and cellular proliferation. Application of bFGF reduces expression of type I procollagen, and that bFGF has a significant impact only on the formation of granulation tissue, afterwards it effects the healing tissue less potently (13). The bFGF mediates its effects through an interaction with cell surface receptors, for example, fibroblast growth factor receptor 1 (FGFR1). Unfortunately, there were no scientific studies found, concerning the expression of these receptors in pelvic ligaments.

Vascular Endothelial Growth Factor (VEGF) is most active during the proliferative and remodeling phases. It is actively expressed during such stimuli as hypoxia and other growth factors, leading to angiogenesis when blood vessels are produced by sprouting from existing vasculature. Cells around the injured tissue express VEGF, which has an effect on endothelial cells, by stimulating proliferation, differentiation and migration (8). In response to VEGF endothelial cells express MMP-9, which degrades the basal lamina, allowing endothelial cells to migrate and align further into injured tissue, forming anastomosis with other vessels (11). VEGF also increases microvascular permeability, thus stimulating inflammation (10).

Protein gene product (PGP 9.5) is a hydrolase izoenzyme and is frequently used as a neural marker. Its antibody usually stains peripheral nerves and ganglia. This technique was used in one study to evaluate the nerve density in the uterine ligaments in women with prolapse. The nerve density was diminished in women with the uterine prolapse in relation to the control group (10).

AIM OF THE STUDY

Purpose of this study was to focus on the immunohistochemical detection of growth factors, their receptors, matrix degrading enzymes, neuropeptide-containing innervation and tissue ischemia and quality proteins in the pelvic ligaments of females with uterine prolapse, and their comparison to pelvic ligaments of females without uterine prolapse. This will allow us to understand the pathophysiological processes involved in uterine prolapse more clearly.

MATERIALS AND METHODS

Biopsies of *lig. teres uteri* were gained during reconstructive surgeries of 6 women with 3rd degree uterine prolapse and 1 woman with a 2nd degree vaginal prolapse, thus the prolapse group consists of 7 women (N_1). Control group consisted of 3 women (N_2). Biopsies of *lig. teres uteri* were taken during surgical procedures unrelated to pelvic organ prolapse, including 2 cases of uterine leiomyoma and 1 case of an ovarian cyst (Table 1).

Ligaments were stained with hematoxilin and eosin and immunhistochemically with antibodies of basic Fibroblast growth factor (bFGF, 1:200 solution, Abcam, England), Fibroblast growth factor's receptor 1 (FGFR1, 1:100 solution, Abcam, England), Vasoendothelial growth factor (VEGF, 1:50 solution, Santa Cruz, USA), Protein gene product 9.5 (PGP 9.5, 1:600 solution, DakoCytomation, Denmark), Collagen III (Col III, 1:50 solution, Quartett, Germany), Collagen IV (Col IV, 1:30 solution, Invitrogen, USA) and with matrix metalloproteinase 9 (MMP-9, 1:100 solution, R&D systems, Germany). Materials were also stained by periodic acid – Schiff method (PAS kit 04-130802, Bio-optica, Italy) in order to localize glycosaminoglycans and glycoproteins.

Specimens were microscoped and photographed at 200X, 250X, 400X magnification, and evalued semiquantitatively. Depending on the realtive quantity of positively stained structures, specimens were rated as having a few positive structures (+), moderate quantity of positive structures (++), numerous quantity of positive structures (+++) and abundant amount of positive structures (++++).

Data analysis was conducted using Statistical Package for the Social Sciences (SPSS) program version 18.0. Results of semi-quantitative evaluation were transformed into numerical form, where + is equal to 1, ++ is equal to 2, +++ is equal to 3, ++++ is equal to 4 and a negative result is equal to 0. The prolapse group was compared to the control group using non-parametric statistics, specifically Mann-Whitney U test. Two-tailed P values of ≤ 0.05 were considered as statistically significant.

RESULTS

Specimens in the prolapse group had signs of acute and chronic inflammation. No significant abnormalities of

tissue were acknowledged in the control group. More specifically prolapse group patients 1, 2 and 3 had sclerotic blood vessels. Patient 4, 6 and 7 had marked proliferation of fibrous connective tissue. Patient 5 and 7 had signs of edema, dilated veins and an infiltration of macrophages. Control patient 1 had dense fibrous connective tissue with no signs of infiltration.

VEGF was expressed by the endothelium in both groups (Table 1, Figure 1 and 2). VEGF was statistically significantly increased (U = 3.5, p = 0.022, two-tailed Mann – Whitney U test) in the prolapse group, where there was a moderate quantity of positive structures (Table 2), in comparison to the control group, where results ranged from weakly to moderately positive.

Number of bFGF, FGFR1, PGP 9.5, Collagen III and IV, MMP – 9 antibodies, and structures strained with periodic acid – Schiff method did not confirm any statistically significant difference between the prolapse group and the control group (Table 2). However, there was a notable tendency.

Quantity of FGFR1 positive lemmocytes and fibroblasts was increased in the prolapse group (moderate to numerous quantity of positive structures), in comparison to the control group (moderate quantity of positive structures), (U = 6.0, p = 0.199, two-tailed Mann – Whitney U test).

Quantity of collagen IV positive structures in the perineurium and the basement membrane of the endothelium was increased in the prolapse group (moderate quantity of positive structures), in comparison to the control group (a few to moderate quantity of positive structures), (U = 4.0, p = 0.129, two-tailed Mann – Whitney U test).

Periodic acid – Schiff method allowed to identify glycosaminoglycans (GAGs) in the connective tissue of the ligaments (Figure 3 and 4). Amount of GAGs was decreased in the prolapse group (numerous quantity of positive structures), in comparison to the control group (numerous to abundant quantity of positive structures), (U = 4.5, p = 0.156, two-tailed Mann – Whitney U test).

Number of PGP 9.5, collagen III, bFGF, MMP-9 positive structures had neither a notable tendency, nor statistically significant difference when comparing the prolapse group to the control group.

DISCUSSION

The study has demonstrated a statistically significant increase of VEGF, which was identified in the endothelium in the pelvic support ligaments (*lig. teres uteri*), in patients with uterine prolapse. There were no other studies found that had researched the relative quantity of VEGF in the pelvic support ligaments during pelvic organ prolapse, but several studies show that VEGF is a marker of hypoxia and angiogenesis in connective tissue (11). In other words amount of VEGF is usually increased in regions where blood supply was stopped or decreased, for example due to trauma or mechanical damage, in order to restore blood flow by producing blood vessels' collaterals.

VEGF usually mediates expression of MMP-9, which degrades the basal lamina of blood vessels, thus allowing migration of the endothelial cells (3), followed by proliferation of type III collagen (10). Neither increased number of MMP-9 positive structures, nor type III collagen positive structures in comparison to the control groups was acknowledged in this study. It is crucial to highlight that some studies have stated that type III collagen can be also decreased during pelvic organ prolapse (4). Therefore no correlation between uterine prolapse and the amount of type III collagen was identified.

Even though no statistically significant decrease in the number of glycosaminoglycans can be seen in the prolapse group, the results (Table 1) clearly show that 3 out of 7 patients in the prolapse group had a few to moderate amount of positive periodic acid – Schiff reactive structures, whereas all patients in the control group had a numerous to abundant amount of positive structures. This allows us to conclude that there is a notable tendency for GAG to decrease in patients with uterine prolapse, and that by increasing the number of patients that participate in this study in both - the control group and the prolapse group – the difference may be proved statistically significant. This is a sign that mechanical durability of ligaments in patients with uterine prolapse is also decreased.

CONCLUSIONS

1. Number of VEGF positive endothelial cells is increased in *lig. teres uteri* in patients with uterine prolapse, suggesting notable presence of tissue hypoxia and stimulation of angiogenesis.
2. The amount of GAGs has a notable tendency to decrease in *lig. teres uteri* in patients with uterine prolapse, suggesting qualitative degradation of tissue.

Table 1. Summary of the semi-quantitative results of the immunohistochemical and periodic acid – Schiff methods per each patient separetly and comparison of both groups

Immunhistochemical study:

Uterine prolapse group									Control group			
	1	2	3	4	5	6	7	Σ	1	2	3	Σ
Age	66	69	72	73	60	76	68		45	70	43	
Pathology	pelvic organ prolapse								Leimyoma	Ovarian cyst	Leiomyoma	
FGFR1	+++	++	+++	++	++	++	+++	**++/+++**	++	++	++	**++**
bFGF	0/+	0/+	+	+	0/+	0/+	+	**0/+**	+	0/+	0/+	**0/+**
VEFG	++	++	++	++	++	++	++	**++**	++	+	+	**+/++**
PGP 9.5	+	++	+	++/ +++	0/+	++	++	**+/++**	++	++	++	**++**
collagen III	+++	++++	+++	+++	+++	+++	++++	**+++/ ++++**	+++	++++	+++/ ++++	**+++/ ++++**
collagen IV	++	++	+++	+/++	0/+	++/ +++	+/++	**++**	+	+/++	+/++	**+/++**
MMP 9	0/+	+	0/+	++	++	++	0/+	**+/++**	++	+	+	**+/++**
Periodic acid - Schiff method:												
GAG	+++	++++	++	++++	+++/ ++++	+/++	++	**+++**	++++	+++/ ++++	++++	**+++/ ++++**

(+ - a few positive structures, ++ - moderate quantity of positive structures, +++ - numerous quantity of positive structures, ++++ - abundant quantity of positive structures).

Table 2. Data analysis results using non-parametric statistics (Mann – Whitney U test). VEGF displays statistically significant difference (p<0.05).

Mann - Whitney U test (two-tailed):

	Mean Ranks		**P value**	**U score**
	Prolapse group	Control group	≤0.05	
Patients	N_1=7	N_2=3		
VEGF	6.50	3.17	**0.022**	3.5
FGFR1	6.14	4.0	0.199	6.0
bFGF	5.64	5.17	0.789	9.5
PGP 9.5	5.07	6.50	0.439	7.5
collagen III	5.07	6.50	0.434	7.5
collagen IV	6.43	3.33	0.129	4.0
MMP 9	5.21	6.17	0.629	8.5
GAG	4.64	7.50	0.156	4.5

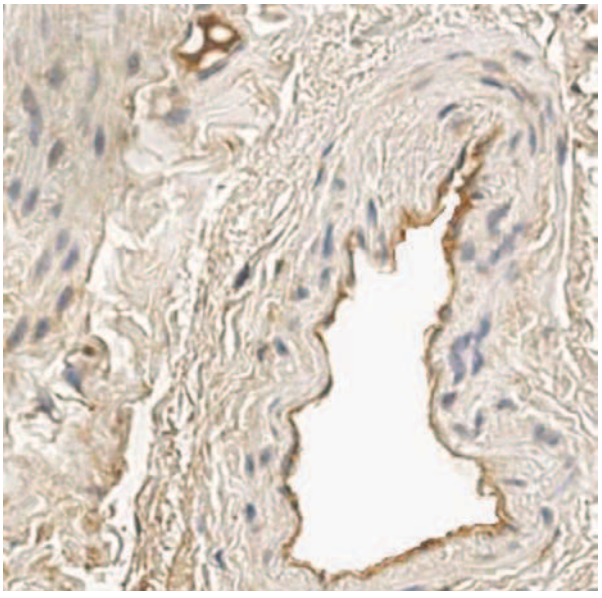

Fig. 1. VEGF in the endothelium, patient 1 with uterine prolapse, x400

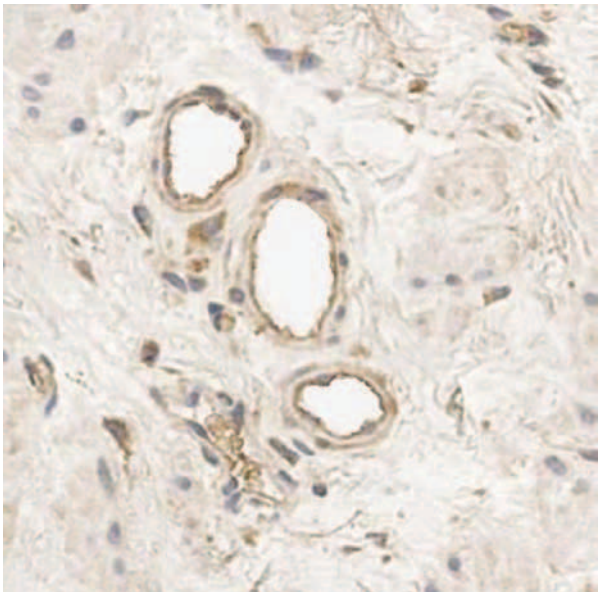

Fig. 2. VEGF in the endothelium of venules, control patient 3, x400

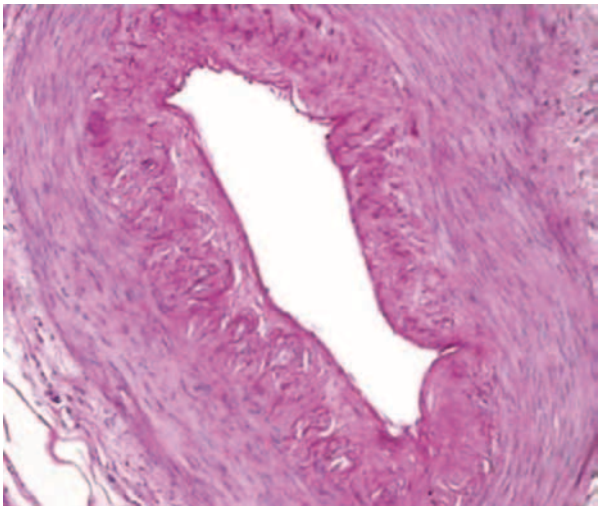

Fig. 3. Periodic acid – Schiff reaction. GAGs in the artety of patient 4 with uterine prolapse, x200.

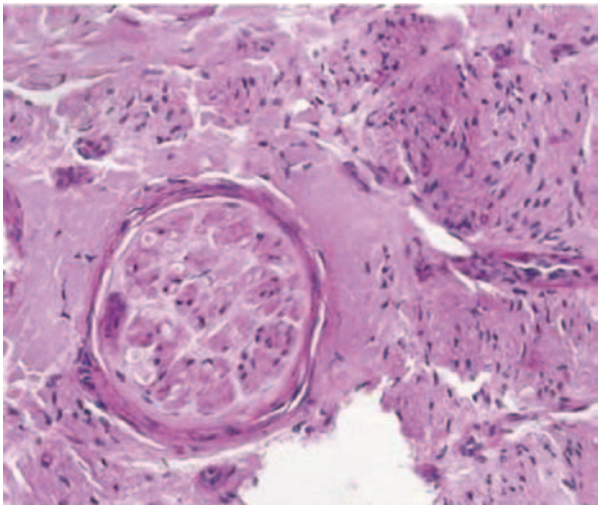

Fig. 4. Periodic acid – Schiff reaction. GAGs in the perineurium of patient 5 with uterine prolapse, x400

Conflict of interest: None

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