SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the background of female pelvic organ prolapse and stress urinary incontinence

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The Examination takes place at the Library of the Department of Obstetrics and Gynecology, Faculty of Medicine, University of Debrecen, March 20, 2019. 12:00

Head of the Defense Committee:	Gabriella Szűcs, MD, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of the Department of Obstetrics and Gynecology, Faculty of Medicine, University of Debrecen, March 20, 2019. 14:00

1. INTRODUCTION AND AIMS

Aging of population has been increasing rapidly worldwide in well developed countries. According to the data of the Hungarian Central Statistical Office there was 130 elder citizen for 100 children in Hungary on 1st January 2018. Prevalence of pelvic floor disease is increasing by aging of the population. Pelvic floor disease is a common term for lower urinary tract disease, pelvic organ prolapse (POP), fecal incontinence and functional disorders related to the pelvic floor such as chronic pelvic pain or sexual dysfunction. Lifetime prevalence of pelvic floor disease in the female population is above fifty percent. Patients need for high quality of life and the severe negative effect of pelvic floor disease on quality of life results in increasing number of visits at urogynecology care units.

The aim of our studies was to investigate the background of pelvic organ prolapse and urinary stress incontinence.

Heat exposure on the vaginal wall by low frequency energy based methods by laser or radio frequency radiation are key methods in the treatment of urinary stress incontinence. We hypostatize that heat exposure of vaginal smooth muscle cells leads to alternation of their extracellular matrix composition. To support this hypothesis we investigated the proliferation of vaginal smooth muscle cells, and their collagen and elastin production while exposed to heat (65 °C).

Our study on pelvic floor prolapses was based on the possible association between the uni- or bilateral avulsion of the levator muscle and the POPQ status. We hypostatize that multi compartement prolapse and higher POPQ score is associated with wider levator urethra gap (LUG) and bilateral levator muscle avulsion.

2. MATERIALS AND METHODS

2.1. Effect of heat exposure on collagen and elastin production of vaginal smooth muscle cells

2.1.1. Isolation and characterization of vaginal smooth muscle cells

We collected vaginal wall samples from 4 healthy patients who had TAH for other non-malignant gynecological condition and were not affected by prolapse and/or stress urinary incontinence. Exclusion criteria were immunological or connective tissue disorders, endometriosis, hormone replacement therapy or vaginal pessary use. Informed consent was given by every patient. After hystercetomy, under sterile conditions full-thickness pieces of the anterior vaginal wall (approximately 0.5 × 0.5 cm) were taken from the vaginal cuff at the anterior midline portion, than primary smooth muscle cultures were made. Specimens were preserved in cold DMEM/F-12 (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS), 1 mM glutamine, 0.075% Na2HCO3, 100 µg/mL streptomycin and penicillin. After maximum 4 h from harvesting, the tissue was mechanically crushed in cold cultured media. The tissue fragments were washed three times with phosphate-buffered saline and then put onto fibronectin-coated plastic dishes (Thermo Fischer Scientific, Rochester, Unites States of America). After the primary outgrowth, clones with a morphology resembling the smooth muscle phenotype were patch cloned and propagated in culture medium.

Eight-well fibronectin coated plastic dishes were used for characterization of the cells (5,000 cells per well). Next cells were washed twice with PBS solution at 80% confluence state, than fixed in 4% PBA at 37°C for 30 minutes. For verification of the intracytoplasmatic distribution of F-actin fibres Tryton X100 (0,1%) and Rhodamine Phalloidin solution were used at 37 °C for 45 minutes. The cells were moved to DAPI containing medium to staining of nucleoid acids (Vectashield, Vector Laboratories, Burlingame, CA) and after glycerin dip confocal microscope was performed. Immuncytochemic staining was also done. Endogen peroxidase activity was blocked by a commixture of hydrogen peroxide and methanol, cells were sequentially treated with primary mouse antibody, biotinylated anti-mouse immunoglobulin, and streptavidin-biotin-peroxidase coplex (LSABTM +/HRP kit, Dako, Carpinteria, CA). Diamino-benzidine was used as chromogen in the presence of hydrogen peroxide. After the reactions, cultures were

stained with hematoxylin-eosin. Every step was carried out at room temperature (22 °C). To verify the smooth muscle cells anti smooth muscle cell actin AB were used (monoclonar mice AB, 1:250, 30 min incubation, clone 1A4, catalogue number #0851, Dako, Carpinteria, CA). The expression of caldesmon (a calmodulin binding protein) were obtained with monoclonal anti-mice AB, 1:100 for 30 minutes (clone h-CD, catalogue number #M3557, Dako, Carpinteria, CA). Cytochemic staining was performed in citrate puffer. Mice serum was used as negative control.

2.1.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Proliferation assay (MTT) was performed from the 4. to 6. passages (five thousand cells / well) that were cultured in ninety-six-well culture plates in a total volume of 200 µL DMEM/F-12 with 10% FBS (Costar, Cambridge, MA, USA). The cell culture was exposed to 65 ° C for 30 or 60 seconds. The new culture media was heated to 65 ° C and placed onto ninety-six-well plates, then placed into incubator set at 65 ° C for 30 or 60 seconds. By the MTT assay kit cell proliferation was assessed at 48 hours after the heat exposure (American Type Culture Collection, Catalog #30-1010K, Manassas, VA, USA). Experiments of both treated and control cells were performed in six replicate wells. At 48 hours the relative number of viable cells was determined by incubating the cells with 1 mg/mL of MTT for four hours. The live cells utilized MTT, resulting in the accumulation of formazan crystals, which were then solubilized with acid isopropanol for one hour. Optical density was measured at 570nm.

2.1.3. Fastin Elastin Assay

In a total volume of 200 µL or 2 mL DMEM/F-12 with 10% FBS smooth muscle cells were grown to near confluence from the 4. to 6. passages (5,000 cells / well) in ninety-six- or six-well culture plates (Costar, Cambridge, MA, USA). Cells in ninety-six-well plates with serum-free DMEM/F-12 were exposed to 65 ° C for 30 or 60 seconds. The culture media was heated to 65° C and placed onto the ninety-six-well culture plates, then it was positioned in a cell culture incubator set for 30 or 60 seconds at 65 ° C. In six replicate wells all treatment and control groups were examined. 48 hours after the initiation of treatment supernatants and cell lysates were collected. Heat exposure has a more noticeable effect on tropoelastin production at 48

hours, as preliminary studies shown. Fastin Elastin Assay (FEA) kit used as the recommendation of manufacturer (Biocolor Ltd., Carrickfergus, UK) for treating supernatants and cell lysates. The FEA is a quantitative dye-binding method for the analysis of extracted elastins. The dye label worked by 5, 10, 15, 20-tetraphenyl-21, 23-porphine tetra-suffocated. The dye reagent binds to the basic-nonpolar amino acid sequences from elastins of mammalian. The Recovered dye-bound elastin and the standard was measured at 513 nm. Quadruplicate measurements were performed. The measured elastin protein amounts were normalized to corresponding cell numbers.

2.1.4. Sircol Collagen Assay

The Sircol Assay is used for the analysis of acid and pepsin-soluble collagens (Manufacturer: Biocolor Ltd., Carrickfergus, UK). These assays can assess the rate of newly synthesized collagen during rapid development and growth periods. Sircol Assay is able to monitor collagen production of in vitro cell cultures and in vitro ECM collagens, soluble in cold acid or pepsin, recovered from newly formed ECM deposited onto cell culture-treated plastic surfaces. Smooth muscle cells were cultured in ninety six- or six-well culture plates (Costar, Cambridge, MA, USA) from the fourth to sixth passages (5,000 cells per well) in a total volume of 200 µL or 2 mL DMEM/F-12 with 10% FBS to near confluence. Cells were exposed to 65 ° C for 30 or 60 s in 96-well plates with serum-free DMEM/F-12. Fresh culture media was placed into the 96-well culture plates and preheated to 65 ° C. Plates were incubated at 65 ° C for 30 or 60 s. Both treatment and control cells were examined in six replicate wells. 48 h after the initiation of treatment cell lysates and supernatants were collected. Collagen production was examined by Sircol Collagen assay, according to manufacturer's instructions. After spent medium removal, cold acetic acid (0.5 M) with pepsin (0.1 mg/mL) was added to culture plates. After incubation with Sirius red dye, the absorbance of the extract was measured at 555 nm with a spectrophotometer.

2.1.5. Statistical analysis

Analysis of variance (ANOVA) was performed for multiple comparisons. P value lesser than 0.05 was set as level of significance. SigmaStat software (SPSS Inc., Chicago, IL, USA) was used for calculations.

2.2. Association between pelvic organ prolapse types and levator-urethra gap as measured by 3D transperineal ultrasound.

2.2.1. Study sample

We performed a retrospective cohort study on 98 women. Women with symptomatic pelvic organ prolapse were included in the study. Patients with incomplete charts and poor ultrasound image quality were excluded. The Institutional Ethical Board approved the study protocol. Demographics, obstetrical and surgical history, POP-Q status and LUG measurements were obtained from each subject.

2.2.2. Assessment of Pelvic Organ Prolapse

Assessment of pelvic organ prolapse was performed by the standardized POP-Q examination. We considered POP present if patients complained of a “bulge,” “lump,” or “something coming down” or “falling out” through the vagina and the documented POP-Q revealed stage II or worse anterior or posterior prolapse or apical descent greater than 50 percent of total vaginal length (Point C > - [total vaginal length/2]). For the study, we categorized prolapses as mild (stage II) or severe (stages III-IV) based on POP-Q staging, on the number of vaginal compartments involved (1 vs. 2 and 1 vs. 3) and on the affected vaginal compartment [Type 1 (anterior only), Type 2 (posterior only), Type 3 (combined anterior and posterior) and Type 4 (any prolapse with apical compartment involvement)]. The primary outcome was the levator avulsion that is categorized into 3 levels: no damage present, unilateral avulsion, and bilateral avulsion.

2.2.3. 3D Transperineal Tomographic Ultrasound for LUG measurement

Ultrasound transducer (transabdominal or transvaginal - GE Healthcare, Voluson E8, Austria) was positioned at the vaginal introitus and the external urethral orifice, without applying pressure and with alignment of the axis of the probe to the patient's long body axis in lithotomy position. Volume datasets were obtained at rest, saved and underwent post processing at a later date by 4D View software version 5.0 (GE Medical Systems). LUG measurements were performed as has been published before. For further analysis axial planes at the level of minimal hiatal dimensions were identified in the mid-sagittal plane. Slices at this plane were obtained by tomographic ultrasound imaging, both 2.5 mm and 5 mm above. Calipers were set in the center of the urethra and on the most medial aspect of the muscle insertion on the inferior pubic ramus. LUG measurements were performed bilaterally and recorded. In our study we used volumes at rest, that has been deemed near- equivalent to the original tomographic imaging for levator avulsion stipulated assessment of volumes obtained on maximal contraction.

2.2.4. Statistical analysis

For all variables of interest descriptive statistics were calculated. For continuous variables means and standard deviations were calculated. For categorical variables frequency and percentage were calculated. Student's t-test was performed to analyze the mean values between the two groups. Generalized logit models were used for evaluation of the association between prolapse stage and type and the probability of having levator damage. SAS 9.4 (SAS Institute Inc, Cary, NC) software was used for all statistical analyses. Level of significance was set at the level of alpha=0.05.

3. RESULTS

3.1. The effects of heat exposure on vaginal smooth muscle cells elastin and collagen production.

No effect of heat exposure was observed (65 ºC for 30 or 60 seconds at 48 hours) on vaginal SMC proliferation (relative cell number, mean ± SD, 0.34±0.01 vs. 0.34±0.01 vs. 0.35±0.01, P=NS).

Significant increase was found in the level of cell culture surface deposited elastin production after exposed to heat at 48 hours [mean ± SD, 30 seconds 155 ± 5% of control (P<0.01) and 60 seconds 516 ± 40% of control (P<0.01).

However we observed significantly lower level of tropoelastin released into culture media after 60 seconds of heat exposure compared to controls at 48 hours [mean ± SD , 30 seconds 102 ± 5% of control (P=NS) and 60 seconds 70 ± 2% of control (P=0.04).

Significant increase was observed at 48 hours by heat in cell-culture surface deposited collagen production [mean ± SD, 30 seconds 170 ± 6% of control (P<0.01) and 60 seconds 123 ± 6% of control (P<0.01).

We found no difference in collagen released into the media after heatexposure [mean ± SD, 30 seconds 120 ± 20% of control (P=NS) and 60 seconds 100 ± 20% of control (P=NS)].

3.2. Association between pelvic organ prolapse types and levator-urethra gap as measured by 3D transperineal ultrasound

Ninety-eight symptomatic patients (median age 63 (± SD, ± 13), gravida 3 (± SD, ± 1), Para 3 (± SD, ± 1) were enrolled. The average BMI was 30 (± SD, ± 7). The median POP stage of prolapse was 2, there was anti-prolapse surgery or hysterectomy in the history of 40 patients. POPQ examination was obtained. 56 women had a prolapse affecting only a single vaginal compartment. While others had multi compartment prolapse, in 23 patients affecting two and in 19 patients affecting all three vaginal compartments. Anterior compartment prolapse was the most common. 34 women had prolapse involving only the anterior compartment and 19 women had only posterior compartment prolapse. 13 women had combined form affecting both anterior and posterior region. LUG was found to be significantly larger in women with

multi compartment POP compared to single compartment POP (28.9±4.1 mm vs. 22.7±4.1 mm, P<0.01). In women with severe POP (POP-Q Stage 3-4) LUG was significantly larger compared to mild POP (POP-Q Stage 2) (28.8±4.7 mm vs. 23.3±4.5 mm, P<0.01). Severe prolapse patients (stage III-IV) were 32.3 times more likely to have bilateral levator avulsion than patients with mild prolapse (stage II).

Women with POP involving two vaginal compartments were 6.6 times more likely to have bilateral damage than those who had single compartment POP. Alll three vaginal compartment involvement was 75.8 times more likely to be associated with bilateral levator avulsion than single compartment POP.

As mentioned in the Materials and Methods we have categorized prolapse based on the specific vaginal compartment affected [Type 1 (anterior only), Type 2 (posterior only), Type 3 (combined anterior and posterior) and Type 4 (any prolapse with apical compartment involvement)]. 4 POP was found to be 8 times more likely to have unilateral damage than type 2 and to have bilateral damage 57 times more likely than type 2. POPs involving the apical compartment were 57 times more likely to have bilateral levator avulsion than prolapses affecting the posterior compartment only.

4. DISCUSSION

Stress urinary incontinence (SUI) is one of the most common types of urinary incontinence. Transient increase in intraabdominal pressure and a weakening of support that is naturally provided by the urethra, bladder and pelvic structures are the main causes of the disease. For the maintenance of urinary continence support from collagen, elastic connective tissue, and muscles is essential that may degenerate with age and under various pathological conditions. Urethral support can be improved by energy-based tightening treatments. In our study we found that heat exposure has no effect on vaginal SMC proliferation, but positively affects collagen and elastin production.

Non-ablative low-energy radiofrequency (RF) methods have been described as possible treatment for SUI. This treatment modality also received FDA (Renessa/Lyrette, Novasys Medical Inc., Newark, CA) approval. Small regions of periurethral collagen gets denatured by the low energy heat exposure of the proximal urethra that leads to alternatiom in tissue compliance, improving its function. During the procedure, periurethral tissue is heated to 65 ºC. Other methods have also been described for vaginal wall remodeling with CO2 laser, Er:YAG laser and RF. All these trigger a photo-thermal effect inside the vaginal wall as deep as 0.5 mm.

No effect on vaginal SMC proliferation was found after heat exposure to 65 ºC for 30 or 60 seconds. No significant difference was found between controls and heat-exposed cells by an MTT assay at 48 hours after heat exposure. This suggest that heating of the tissue to 65 ºC for a minute or less does not have a negative effect. Cells besides SMC such as nerve tissue, may be far more sensitive to heat.

We also analyzed collagen and elastin synthesis of SMC as a response to heat exposure. Both cell surface deposited elastin and collagen and elastin and collagen released into the culture media was analyzed. Cell-culture deposited elastin increased significantly and gradually by heat exposure. 60 seconds of heat exposure resulted in increased elastin production by more than 5 folds (normalized by cell number). On the contrary similar elevation of tropoelastin production was not observed into the media after heat exposure, it decreased by 30%. This may be explained as the cells were depositing elastin on the surface rather than releasing it into the media.

Regarding to collagen production, significantly higher levels were found in cell lysates of after heat exposure. Both 30 and 60 seconds of treatment resulted in significantly increased level of collagen, the increase was found to be lower after longer treatment. Secreted collagen levels were not affected by heat exposure compared to controls.

We are the first group to the best of our knowledge to investigate the effects of heat exposure on vaginal SMC. Our hypothesis was that heat exposure of the vaginal wall may have positive remodeling effects on the tissue that is represented in increasing elastin and collagen production and alternation of the connective tissue structure. Heat treatment mainly effects the cell surface deposited elastin and collagen and has no effect on the secreted levels. This supports the hypothesis that newly composed ECM molecules remain in the vaginal wall.

Limitations to our study lies in the in vitro nature of experiments and the sample size. The nondynamic environment and the simplified model might only slightly reproduce the actual complex in vivo environment. Measurements were only performed on tropoelastin (as an indirect measure of mature insoluble elastin) and we did not examine the different matrix metalloproteinase expressions through the degradation process. Mature form of elastin and collagen are assembled from tropoelastin and tropocollagen that are secreted into the extracellular matrix. Newly formed tropoelastin and tropocollagen may be collected from the media of tissue cultures while mature forms are found in the tissue itself. Only one temperature setting was evaluated with two exposure times, as it mimics an FDA-approved treatment modality.

In conclusion, changes in the extracellular matrix composition of the vaginal/periurethral tissues after heat exposure treatments may contribute to the mechanism of action of many innovative devices (RF and laser) that are used for vaginal wall alternation to gain better urethral support. Long-term effects of these treatments are still not known. Future in vitro and in vivo studies are necessary to understand the mechanisms of action and to discover potential side-effects of novel treatments.

Similarly to urinary incontinence, pelvic organ prolapse is also very common and results in a huge impact on quality of life. The life-long prevalence of POP that needs surgery is 11 %, and there is a 30% chance for having a relapse. The high rate of recurrence makes the introduction of novel therapeutic modalities necessary, which modalities can lower the chance of post-surgery recurrence.

In our second study we analyzed the measurement of LUG by 3D transperineal tomographic ultrasound as an indicator of levator ani avulsion and pelvic organ prolapse severity and types. Women with severe prolapse were more likely to have bilateral levator avulsion than women with mild prolapse. In addition, patients with POP involving all three vaginal compartments were much more likely to have bilateral levator avulsion than single compartment POPs. Apical compartment prolapses were also much more likely to have bilateral levator avulsion than prolapses only affecting the posterior compartment. We hypothesize that patients with severe, multi compartmental prolapses or apical compartment prolapses are most likely to have significant bilateral avulsion. These are the most difficult prolapses to manage and have the highest recurrence ratio. Underlying levator avulsions that are not being repaired at the time of surgery might be responsible for the high failure rate. During increased abdominal pressure a wide opening on the levator plate exposes the surgical repair site to more unopposed forces that may also lead to increased failure rates. Previously, others have shown that women with levator avulsion were twice as likely to have stage 2 or higher pelvic organ prolapse compared to those without as there is an increased risk of cystocele and uterine prolapse. Pubovisceral avulsions diagnosed by MR imaging or perineal ultrasonography, are associated with higher POP stages and also with recurrence after surgery. If the POP affects the posterior compartment only, the recurrence rate after surgery appears to be the lowest (~11 %). Recurrence rates are the highest in the anterior compartment (up to 52 %) in patients with levator avulsion. We found the lowest rate of levator avulsion in only-posterior compartment POP patinets. Perhaps the low posterior recurrence rate can be be partly explained by the low percentage of levator avulsions in this group.

Limitations of this study are the small sample size as we may be missing other associations between prolapse type and LUG/avulsion. LUG measurements were performed at rest, while the POP-Q measurements were performed at maximum Valsalva. LUG could be measured at rest as published by Dietz et al. (rather than at the time of volumes obtained during pelvic floor contraction as originally described) and it does not affect the ability to assess levator defects. An important limitation of this study is the lack of a reproducibility as well. Lastly, we only enrolled symptomatic POP patients and excluded symptomatic women with no POP on pelvic exam, and asymptomatic women with no prolapse (control group). Larger populations are recommended to confirm these association between POP and LUG in future studies.

In conclusion bilateral levator ani avulsion diagnosed by at rest LUG measurement seems to be associated with multi compartment, severe pelvic organ prolapse.

Pelvic floor diseases, mostly urinary incontinence and pelvic organ prolapse cause high burden on patient care physicians in the aging societies of well developed countries. Keen interest on good quality of life by patients puts a challenge on researchers to develop new diagnostic and treatment approaches. This thesis aims to add new findings on the field of urognecology in the presented topics.

5. SUMMARY OF MAJOR RESULTS AND SCIENTIFIC NOVELTIES

To the best of our knowledge, we are the first group to investigate the effects of heat exposure on vaginal SMC.

1. We found that heat exposure to 65 ºC for 30 or 60 seconds does not affect vaginal SMC proliferation
2. Our findings suggest that heating tissue to 65 ºC for a minute or less does not appear to have a negative effect.
3. Analysis of both the cell-culture surface deposited elastin and collagen, as well as the elastin and collagen released into the culture media demonstrated that cell-culture deposited elastin was significantly increased when exposed to heat.
4. Regarding collagen production, significantly higher levels were found in cell lysates of vaginal SMC after heat exposure.

Our findings support the hypothesis that heat exposure of the vaginal wall may have positive remodeling effects on the tissue, increasing elastin and collagen production and altering the connective tissue structure.

We investigated the LUG in patients with Pelvic Organ Prolapse

5. We have found that women with severe prolapse were more likely than women with mild prolapse to have bilateral levator avulsion.
6. In addition, women with POP involving all three vaginal compartments were much more likely than single compartment POP to have bilateral levator avulsion.
7. Prolapses involving the apical compartment were also much more likely than prolapses only affecting the posterior compartment to have bilateral levator avulsion.

In summary, bilateral levator ani avulsion as diagnosed by LUG measurement at rest is associated with multi compartment, severe prolapse.

6. PUBLICATIONS

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Candidate: Bence Kozma
Neptun ID: ZCPGUU
Doctoral School: Doctoral School of Neurosciences

List of publications related to the dissertation

1. **Kozma, B.**, Larson, K., Scott, L., Cunningham, T. D., Abuhamad, A., Póka, R., Takács, P.: Association between pelvic organ prolapse types and levator-urethra gap as measured by 3D transperineal ultrasound.
   J. Ultrasound Med. [Epub ahead of print], 1-6, 2018.
   DOI: http://dx.doi.org/10.1002/jum.14644
   IF: 1.53 (2017)
2. **Kozma, B.**, Candiotti, K., Póka, R., Takács, P.: The Effects of Heat Exposure on Vaginal Smooth Muscle Cells: elastin and Collagen Production.
   Gynecol.Obstet.Invest. 83 (3), 247-251, 2018.
   DOI: http://dx.doi.org/10.1159/000486785
   IF: 1.183 (2017)

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List of other publications

3. **Kozma, B.**, Majoros, A., Pytel, Á., Póka, R., Takács, P.: A percutan nervus tibialis stimuláció szerepe egyes kismedencei kórképek kezelésében.
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   DOI: http://dx.doi.org/10.5005/jp-journals-10009-1025

Total IF of journals (all publications): 5,5
Total IF of journals (publications related to the dissertation): 2,713

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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