RESEARCH ARTICLE

pISSN: 0126-074X | eISSN: 2338-6223
https://doi.org/10.15395/mkb.v56.3694
Majalah Kedokteran Bandung. 2024;56(4):257–262

Majalah Kedokteran Bandung (MKB)

Received: December 6, 2023
Accepted: Juni 26, 2024
Available online: December 31, 2024

Differences in Elastin and Collagen Levels in the Levator Ani Muscle of Primiparous and Multiparous Normal Postpartum Women

Annisa Astika Rada,[1] Rahajeng,[1] Pande Made Dwijayasa,[2] Didik Agus Gunawan[3]

[1]Department of Obstetrics and Gynecology, Faculty of Medicine, Brawijaya University, Malang, Indonesia
[2]Department of Obstetrics and Gynecology, Faculty of Medicine, Brawijaya University, Dr Iskak General Hospital, Tulungagung, Indonesia
[3]Department of Obstetrics and Gynecology, Faculty of Medicine, Brawijaya University, Ngudi Waluyo General Hospital, Blitar, Indonesia

Abstract

The levator ani, comprising the pubococcygeus, puborectalis, and iliococcygeus muscles, is crucial for supporting pelvic organs, maintaining continence, and ensuring pelvic stability. Weaknesses in these muscles or ligaments can lead to pelvic organ prolapse (POP), a condition where the pelvic organs descend and protrude through the vaginal introitus. Collagen and elastin, the key constituents of the extracellular matrix in the levator ani muscle, play a significant role in maintaining its structural integrity and can be influenced by parity. This study aimed to determine the differences in elastin and collagen levels of the levator ani muscle of primiparous and multiparous patients. This was an observational analytical study on 18 postpartum female patients consisting of 9 primiparous and 9 multiparous in January- March 2023. Samples were levator ani muscle biopsies from perineal lacerations of at least grade II from the inpatient Obgyn Department of the Faculty of Medicine, Universitas Brawijaya. Examination was done using the immunohistochemical method. Results showed that the percentage of histological area secreting elastin and collagen was higher in the primiparous group than multiparous, thus the levels were higher ($p<0.001$; $p=0.001$; $p<0.05$). In conclusion, elastin and collagen levels were lower in multiparous women compared to primiparous women. Future studies can evaluate factors affecting the decline in elastin and collagen in multiparous women using a larger sample size.

Keywords: Levator ani, multiparous, pelvic organ prolapse, postpartum, primiparous

Introduction

The levator ani is a critical muscle group within the pelvic floor, comprising the pubococcygeus, puborectalis, and iliococcygeus muscles. Its primary role is to provide support to the pelvic organs, maintain continence, and assist in pelvic stability.[1] During childbirth, the levator ani undergoes significant strain as it facilitates the passage of the fetus through the birth canal. The stretching and potential injury to these muscles can lead to pelvic floor dysfunction, impacting bladder and bowel control, and contributing to pelvic organ prolapse.[1] The incidence of POP is strongly associated with advancing age. The exact prevalence of pelvic organ prolapse is unknown, but an analysis of hospital procedure codes in the United States revealed that approximately 200,000 surgeries are performed annually for POP treatment.[2] According to a 2008 cross-sectional study involving 1961 women, POP occurred in 9.7% of women aged 20–39 and 49.7% of women aged >80 years.[2,3]

Levator ani influenced by intrinsic and extrinsic factors. Intrinsic factors include collagen, genetics, race, aging processes, and menopausal conditions. On the other hand, extrinsic factors contributing to POP encompass pregnancy and childbirth, a history of hysterectomy, parity, hormone replacement therapy, increased body mass index, constipation, as well as diseases or occupations associated with prolonged increased intra-abdominal pressure.[4] Among these factors, pregnancy and childbirth are identified as having the most significant impact.[5,6] The higher the number of childbirths, the greater the likelihood of prolapse occurrence. During pregnancy, the endometrium undergoes rapid growth and differentiation,

Corresponding Author:
Annisa Astika Rada
Department of Obstetrics and Gynecology, Faculty of Medicine, Brawijaya University, Malang, Indonesia
Email: annisaastikarada@gmail.com

leading to remodeling and damage to the extracellular matrix.[7] Consequently, alterations in the composition of the extracellular matrix can result in changes in mechanical properties, potentially leading to POP.[1]

The key to the stability of the connective tissue structure forming the foundation of the pelvis lies in extracellular matrix components such as collagen, fibronectin, and elastin, as well as integrins. The extracellular matrix cycle is influenced by the activity of Matrix Metalloproteinases (MMP). MMP is a component responsible for degrading extracellular matrix components such as collagen and elastin. MMP and TIMP-1 play a crucial role in the process of pelvic organ prolapse in postpartum patients due to molecular and biochemical changes in the sacrouterine ligaments, vaginal walls, and levator ani.[7]

Kolagen serves as a constituent of the extracellular matrix, providing high tensile strength that allows tissues to dynamically withstand loads. It is the most abundant fibrous protein in the extracellular matrix of the vagina.[1] Collagen I influences tissue stiffness, while collagen III is associated with tissue elasticity.[8] The levator ani muscles are a crucial part of the pelvic floor structure, playing a significant role in determining support for pelvic organs.[9] Elastin, on the other hand, is a connective tissue protein found in the extracellular matrix, contributing to tissue resilience and recoil. It enables tissues to maintain their shape and long-term deformability.[1]

The history of primiparous and multiparous deliveries is associated with differing risks of POP, which is thought to result from variations in elastin and collagen levels. However, the influence of elastin and collagen on the levator ani muscles has not been extensively studied. Therefore, this study aims to investigate the differences in elastin and collagen levels in the levator ani muscles between primiparous and multiparous patients.

Methods

This study used an analytical observational design with a cross-sectional approach. Samples were collected using purposive sampling from biopsies of the levator ani muscle in patients who experienced minimal Grade II perineal lacerations during uncomplicated postpartum deliveries. Postpartum levator ani muscle tissue was utilized for analysis. The biopsy samples underwent anatomical pathology examination using immunohistochemical techniques to evaluate the expression of collagen and elastin proteins. Elastin and collagen levels were then compared based on the patients' delivery history (primiparous vs. multiparous). This study received ethical approval from the Ethics Committee for Health Research at Regional General Hospital dr. Saiful Anwar Malang (approval number 400/065/K.3/102.7/2023).

A biopsy procedure of the Levator ani muscle tissue was performed, where a biopsy sample with dimensions of 5x5x5 mm was obtained using Metzenbaum scissors. The biopsy was specifically targeted at the pubococcygeal muscle. The biopsy was executed laterally (20–25 mm) from the urethrovesical junction and at the midpoint between the urethra and arcus tendinus. Biopsies were only conducted on patients with grade 1 perineal tears to minimize bias. Grade 1 perineal tears involve only the perineal skin and do not affect the underlying muscles, including the pubococcygeus. This approach ensures that the muscle tissue sampled is not influenced by the additional trauma and healing processes associated with more severe tears, providing a more accurate representation of the baseline differences in elastin and collagen levels in the levator ani muscle of primiparous and multiparous women.The collected sample was placed into a tube containing 10% Neutral Buffered Formalin Solution. The tube was labeled with specimen details (name, registration number, and sample number), and the sample was then stored at a temperature of 20–25°C.

The excised biopsy tissue was placed in a formalin buffer solution (10% formalin in Phosphate Buffer Saline at pH 7.0). The tissue was immersed in the fixation solution for 18–24 hours. Subsequently, the tissue was placed in distilled water or running water for 1 hour to remove the fixation solution. The tissue was then sequentially immersed in ethanol of increasing concentrations: 50%, 70%, 80%, (0%, and 3 times absolute alcohol, each for 1 hour. Following that, tissue clarification was carried out by immersing it in an alcohol-xylene solution for 1 hour, followed by 2 immersions in pure xylene solution for 1 hour each. Once clarified, tissue sections were placed in molten paraffin in the Tissue Tek TEC 5 Embedding tissue console for 4–8 hours. The tissue sections were attached to cassettes, left in molds, and positioned on the Tissue Tek TEC 5 Cryo console until the paraffin solidified, and the paraffin block could be removed from the mold. The tissue in the

paraffin block was cut into 4-micrometer-thick sections using the Accu cut SRM TM 200 Rotary Microtome. Paraffin ribbons with approximately 4 tissue sections were placed in a water bath set at a temperature of 40–45 °C. The fully stretched paraffin ribbons were taken using labeled glass slides and placed on a hot plate set at a temperature of 35–45 °C, left for approximately 5 hours or overnight.

Deparaffinization was carried out by immersing the tissue slides twice in xylene solution for 5 minutes each. The slides were then rehydrated using absolute ethanol, 90%, 80%, and 70% ethanol for two minutes each, followed by rinsing with running water for one minute. The slides were placed in deparaffinization solution (xylene 1 and xylene 2) for 5 minutes, then in rehydration solution (absolute alcohol, 90%, 80%, 70%) and running water, each for 5 minutes.

The slides were stained using the Verhoeff Van Gieson staining kit following the provided instructions. The slides were immersed in the working solution (Hematoxylin solution, Ferric Chloride solution, and Lugol Iodine solution) for 15 minutes, then rinsed with running water. At this stage, the tissue would appear dark purple, tending towards black in some tissue areas. The slides were dipped in the differentiation solution 10-20 times, then rinsed with running water. At this stage, elastin tissue would remain black, cell nuclei would appear purple, while other parts would remain transparent. The slides were immersed in Sodium Thiosulfate solution for 1 minute, then rinsed with running water. At this point, elastin and cell nuclei would appear the same as in the previous step, black and purple, while collagen tissue would be colorless, and other components like muscle cells, blood cells, and other tissues would appear yellow.

Next, the slides were immersed in Van Gieson solution for 2-5 minutes, then rinsed twice with 95% ethanol and once with absolute ethanol. At this stage, collagen cells would appear pink, while other cells and tissues would maintain the same appearance as in the previous step. The slides were air-dried, and once dry, a cover glass was applied after adding Entellan. Microscopic examination was conducted at each staining step to ensure appropriate coloration without excessive staining.

The staining process using the Verhoeff Van Gieson staining kit (ab150667) from Abcam, Boston, allows for the differentiation of various tissue types through the expressed colors. The followings are the colors and corresponding cell or tissue types:- Black Color: Elastin tissue, - Red Color: Collagen tissue, - Purple Color: Cell nuclei, - Yellow Color: Muscle cells, blood cells, and other cells.

Elastin and collagen percentages were obtained using a rapid digital analysis method. Each specimen was observed using an Olympus BX 53 Microscope with a 40x objective lens. Ten random fields of view were captured from each specimen. The resulting photos were saved in JPEG format. Each specimen photo underwent analysis for two tissue types: elastin tissue (black color) and collagen tissue (pink color). The analysis was conducted using ImageJ 1.53t software.

The analysis process began by opening the photo file to be analyzed using ImageJ 1.53t software. The color separation in the photo winas then performed using the color deconvolution feature with the FastRed FastBlue DAB vector. The resulting color images were blue and black in image 1, red in image 2, and yellow in image 3. Image 1 was used for elastin analysis, and image 2 was used for collagen analysis. The analysis process involved selecting the image to be analyzed (image 1 or image 2) and blocking the desired red color using the threshold feature (capturing the portion corresponding to the desired elastin or collagen). This process was done by comparing the thresholded portion with the original photo to ensure that the counted portion was the desired one.

Once the desired portion was confirmed, the next step was to measure the percentage using the measurement (percentage) feature. The obtained data represented the percentage of the blocked portion using the threshold against the field of view. The analyzed image photos were saved in JPEG format. The percentage data obtained from both elastin and collagen tissues were organized and further subjected to statistical data analysis.

The prerequisite for conducting parametric tests is that the data must follow a normal and homogeneous distribution. Normality is assessed using the Shapiro-Wilk test, with a p-value>0.05 indicating a normal distribution. Homogeneity is assessed using Levene's test, with a p-value>0.05 indicating homogeneity. If the assumptions for parametric tests are not met, non-parametric tests are used. In this study, the dependent variables are the expression levels of elastin and collagen.

Differential analysis is performed using an independent t-test to compare the mean values of elastin and collagen expression between the

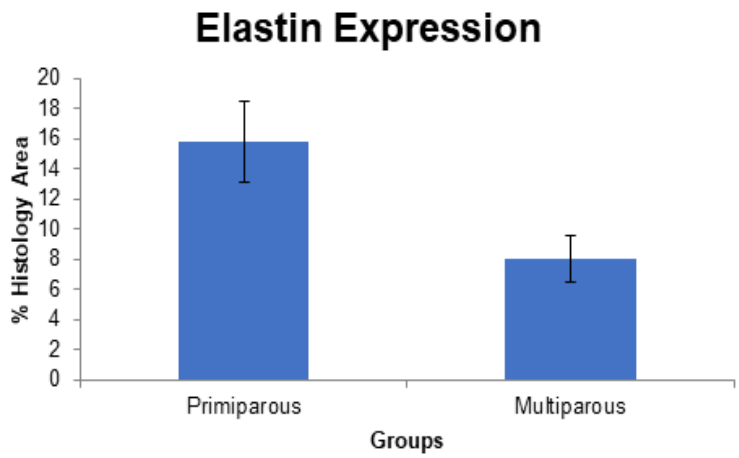

Figure 1 Difference in Elastin Levels in the Levator Ani Muscle After Normal Delivery in Primiparous and Multiparous Women

groups. The expression levels of elastin and collagen are compared between the primiparous and multiparous groups. If the assumptions for parametric tests are violated, the Mann-Whitney U test is employed instead. Statistical significance is determined by rejecting the null hypothesis (H_0), with a p-value<0.05.

Result

Based on the results of the normality test for both groups, the Shapiro-Wilk test was used, as the sample size was fewer than 50 subjects. The Shapiro-Wilk test results showed a p-value > 0.05, indicating that the data from both groups follow a normal distribution.

The differences in elastin levels in the levator ani muscle following normal delivery between primiparous and multiparous women are presented in Figure 1.

Based on Figure 1, a difference in elastin levels in the levator ani muscles following normal delivery is observed between primiparous and multiparous women. The figure shows that the percentage of histological area expressing elastin is higher in the primiparous group compared to the multiparous group. Statistical analysis using an independent t-test revealed a significant difference (p=0.001; p<0.05).

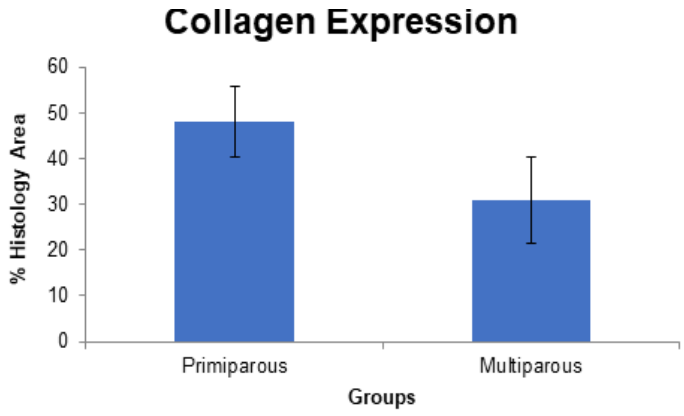

Figure 2 Difference in Collagen Levels in the Levator Ani Muscle After Normal Delivery in Primiparous and Multiparous Women

The analysis of differences in collagen levels in the levator ani muscle following normal delivery between primiparous and multiparous women is presented in Figure 2.

Based on Figure 2, a difference in collagen levels in the levator ani muscles following normal delivery is observed between primiparous and multiparous women. The figure shows that the percentage of histological area expressing collagen is higher in the primiparous group compared to the multiparous group. Statistical analysis using an independent t-test revealed a significant difference (p=0.000; p 0.05).

Discussion

In this study, it is demonstrated that the elastin levels in the levator ani muscle after normal delivery are higher in primiparous women compared to multiparous women. This is attributed to the trauma experienced by the levator ani muscle during childbirth. Research conducted by Guler *et al*. indicates that elastin production in primiparous animals is higher than in animals with injuries at four and eight weeks post-injury, supported by a decrease in smooth muscle connections in the muscularis vagina. Trauma resulting from childbirth will enhance remodeling in the extracellular matrix (ECM), leading to a decrease in fibroblasts. This decrease in fibroblasts subsequently reduces the elastin levels.[12]

The findings of this study are supported by previous research. The earlier study aimed to evaluate the elastin levels in primiparous and multiparous rats. The results of that research indicated that the elastin levels in primiparous Sprague-Dawley rats were higher than in multiparous rats. Elastin levels increased over time from two days postpartum to two weeks postpartum.[13] However, the results of this study differ from previous research. The earlier study suggested that primiparous rats had higher elastin expression compared to multiparous rats.[14] The disparity in the findings of these studies is hypothesized to be due to differences in the age of the rats. Variations in rat age lead to differences in estrogen hormone levels, influencing elastin levels.[15]

This study indicates that the collagen levels in the levator ani muscle after normal delivery are higher in primiparous women compared to multiparous women. Type I and III collagen are dominant extracellular tissues in the matrix component and significantly contribute to the biomechanical properties of the vagina. Imbalances between collagen I and collagen III and a decrease in smooth muscle fractions have been observed in prolapsed vaginal tissues.[16]

Pregnancy induces changes in the pelvic tissues and reproductive organs of women to support fetal growth and facilitate childbirth through the birth canal. These changes involve alterations in the extracellular matrix (ECM), a structure composed of collagen, elastic fibers, and smooth muscle. Collagen, being the most abundant protein in the human body's ECM, plays a crucial role in providing structure and strength to the pelvic muscle and reproductive tract tissues.[17]

The findings of this study align with previous research. The earlier study aimed to understand the role of the extracellular matrix in the remodeling of myometrial tissue during pregnancy in rats. The results of that research demonstrated an increase in collagen expression during pregnancy, followed by a decrease after delivery. This indicates that collagen expression is influenced by estrogen and progesterone hormones.[18]

The results of this study differ from research conducted on other experimental animals. A study by Emmerson[19] indicated a reduction in collagen in primiparous ewes compared to nulliparous ewes. Additionally, there was no significant difference in collagen levels between primiparous and multiparous ewes. The vagina was also weaker and less rigid in multiparous ewes compared to nulliparous and primiparous ewes, suggesting a loss of muscle mass rather than elastic fibers.[19]

This study indicates a decrease in elastin and collagen levels in the levator ani muscle after normal delivery in multiparous women compared to primiparous women. However, several limitations must be noted. Firstly, the sample size was limited to 18 individuals, comprising both primiparous and multiparous women. Secondly, the study did not consider other factors that may influence elastin and estrogen levels, such as comorbidities, hormone levels, and patient age. Therefore, further research is needed to evaluate the impact of these factors.

References

1. Handa VL, Roem J, Blomquist JL, Dietz HP, Muñoz A. Pelvic organ prolapse as a function of levator ani avulsion, hiatus size,

and strength. Am J Obstet Gynecol. 2019 Jul;221(1):41.e1-41.e7. doi: 10.1016/j.ajog.2019.03.004
2. Aboseif C, Liu P. Pelvic Organ Prolapse. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan. Updated 2022 May 1.
3. Chen CJ, Thompson H. Uterine Prolapse. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Updated 2022 May 8.
4. Pangastuti N, Sari DCR, Santoso BI, Agustiningsih D, Emilia O. Gambaran faktor risiko prolaps organ panggul pasca persalinan vaginal di Daerah Istimewa Yogyakarta. MKB. 2018;50(2):102–8.
5. Qureshi SS, Gupta JK. Pelvic organ prolapse: prevalence and risk factors. Am J PharmTech Res. 2015;15(6):122–8.
6. Shi JW, Lai ZZ, Yang HL, Yang SL, Wang CJ, Ao D, et al. Collagen at the maternal-fetal interface in human pregnancy. Int J Biol Sci. 2020;16(12):2220–34.
7. Rahajeng R. The increased of MMP-9 and MMP-2 with the decreased of TIMP-1 on the uterosacral ligament after childbirth. Pan Afr Med J. 2018;30:283.
8. Gong R, Xia Z. Collagen changes in pelvic support tissues in women with pelvic organ prolapse. Eur J Obstet Gynecol Reprod Biol. 2019;234:185–9.
9. Tang JH, Zhong C, Wen W, Wu R, Liu Y, Du LF. Quantifying levator ani muscle elasticity under normal and prolapse conditions by shear wave elastography: a preliminary study. J Ultrasound Med. 2020;39(7):1379–88. doi:10.1002/jum.15232
10. Chi N, Lozo S, Rathnayake RAC, Botros-Brey S, Ma Y, Damaser M, Wang RR. Distinctive structure, composition and biomechanics of collagen fibrils in vaginal wall connective tissues associated with pelvic organ prolapse. Acta Biomaterialia. 2022;152:335–44. doi:10.1016/j.actbio.2022.08.059
11. Weintraub AY, Glinter H, Marcus-Braun N. Narrative review of the epidemiology, diagnosis and pathophysiology of pelvic organ prolapse. Int Braz J Urol. 2020;46(1):5–14. doi:10.1590/S1677-5538.IBJU.2018.0581
12. Guler Z, Roovers JP. Role of fibroblasts and myofibroblasts on the pathogenesis and treatment of pelvic organ Prolapse. Biomolecules. 2022;12(1):94.
13. Downing KT, Billah M, Raparia E, Shah A, Silverstein MC, Ahmad A, Boutis GS. The role of mode of delivery on elastic fiber architecture and vaginal vault elasticity: a rodent model study. J Mech Behav Biomed Mater. 2014;29:190–8.
14. Setyaningrum T, Listiawan MY, Tjokroprawiro BA, Santoso BI, Prakoeswa CR, Widjiati W. Role of elastin expression in thickening the postpartum vaginal wall in virgin and postpartum rat models. World's Vet J. 2021;11(2):228–34.
15. Szymański JK, Słabuszewska-Jóźwiak A, Jakiel G. Vaginal aging-what we know and what we do not know. Int J Environ Res Public Health. 2021;18(9):4935.
16. Mao M, Li Y, Zhang Y, Kang J, Zhu L. Tissue composition and biomechanical property changes in the vaginal wall of ovariectomized young rats. BioMed Res Int. 2019;2019:8921284.
17. Dhital B, Gul-E-Noor F, Downing KT, Hirsch S, Boutis GS. Pregnancy-induced dynamical and structural changes of reproductive tract collagen. Biophys J. 2016;111(1):57–68.
18. Ouellette A, Mahendroo M, Nallasamy S. Collagen and elastic fiber remodeling in the pregnant mouse myometrium. Biol Reprod. 2022;107(3):741–51.
19. Emmerson S, Young N, Rosamilia A, Parkinson L, Edwards SL, Vashi AV, et al. Ovine multiparity is associated with diminished vaginal muscularis, increased elastic fibres and vaginal wall weakness: implication for pelvic organ prolapse. Sci Rep. 2017;7:45709.