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Low Expression of Lysyl Oxidase-Like 1 (LOXL1) on the Sacrouterine Ligament as a Risk Factor of Stage III-IV Uterine Prolapse

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Abstract: ***The stage III-IV uterine prolapse commonly presents as discomfort lumps that pop out of the genitals, accompanied by sexual impairment, urinary disorders, rectal emptying disorders, vaginal discharge, back pain, and other diseases that may impact women's quality of life. The etiology of stage III-IV uterine prolapse is still not clear. The sacrouterine ligament is a structure implicated in the pathogenesis of uterine prolapse where Lysyl Oxidase-Like 1 (LOXL1) is an essential part of the sacrouterine ligament. This study aims to prove the low LOXL1 expression in the sacrouterine ligament as a risk factor for stage III-IV uterine prolapse. This observational case-control study involves 44 women who underwent a complete hysterectomy at Sanglah General Hospital, Denpasar. The case group was the patient with stage III-IV uterine prolapse, while the control group was a non-uterine prolapse patient. The Chi-Square test was used to assess the odds ratio of increasing LOXL1 expression to the incidence of stage III-IV uterine prolapse. Low LOXL1 expression has a 5.95-fold higher risk of developing stage III-IV uterine prolapse (OR = 5.95; 95% CI = 1.23-4.33; p = 0.006) than high LOXL1 expression with a cut-off value. The point is 1,772 based on the Receiver Operating Characteristic (ROC) curve. Low LOXL1 expression in the sacrouterine ligament is a risk factor for stage III-IV uterine prolapse.***

Keywords: LOLX1, Pelvic organ prolapse, Sacrouterine ligament, Uterine Prolapse

1. Introduction

Uterine prolapse is a part of pelvic organ prolapse (POP) disorders. Its prevalence increases with age. One study reported a 10% increase in the risk of POP with each decade of increasing patient age, and the incidence was between 43-76%. Approximately 41% occurred at the age of 50-79 years which includes 34% incidence of anterior vaginal wall prolapse (cystocele), posterior vaginal wall prolapse (rectocele) 19%, and 14% of the uterine prolapse.[1]

Data in the United States shows the surgery required for uterine prolapse is 2.7-3.3 in 1,000 women, and in the UK is 2 in 1,000 women per year.[1] The incidence of uterine prolapse in Indonesia is still uncertain. Several previous studies in Indonesia reported a variable incidence of uterine prolapse ranging from 11.38% to 26.4%, which is quite high. Primary prevention efforts are very crucial in uterine prolapse because about 30% of cases need a pelvic floor reoperation every year.[2] Therefore, the need for a paradigm shift in handling uterine prolapse from a passive curative paradigm to an active preventive paradigm.

The exact etiology of uterine prolapse is unknown. Many factors are thought to cause uterine prolapse in which vaginal delivery is the most known risk for uterine prolapse. The trauma during vaginal delivery causes damage and weakens the levator ani muscle. To maintain the normal position of the uterus, the sacrouterine ligament becomes tense as it takes over the main function of the levator ani. The combination of a persistent increase in pressure with other risk factors will cause decreased strength of the sacrouterine ligament. The decrease in strength depends on changes in the metabolic molecules in the main structure of the sacrouterine ligament.[3]

The sacrouterine ligament consists of cells and extracellular matrix, such as collagen, elastin, proteoglycans, and glycoproteins. Collagen, the main component, contributes 70% of the entire extracellular matrix.[4] Several theories suggest that the integrity of this connective tissue depends on lysyl oxidase (LOX), an extracellular enzyme associated with the extracellular matrix of collagen and elastin.[5] LOX is a copper-dependent monoamine oxide secreted by fibrogenic cells including fibroblasts and smooth muscle cells. The mammalian genome has up to five members of the LOX family, the lysyl oxidase-like (LOXL), lysyl oxidase-like 1 (LOXL1), lysyl oxidase-like 2 (LOXL2), lysyl oxidase-like 3 (LOXL3), and lysyl oxidase-like 4 (LOXL4).[6] LOXL1 plays a role in the conversion of immature elastin and collagen forms into tropoelastin and tropocollagen. The previous studies found that the lack of LOXL1 protein causes the formation of normal elastic fibers in the postpartum uterine tract which can lead to uterine prolapse, enlargement of the lung air spaces, vascular abnormalities with the accumulation of tropoelastin at the same time. Unlike the other LOX prototypes, LOXL1 localizes specifically to the elastogenesis site. Thus, LOXL1 is an important aspect of elastin fibers that may play a role as a risk factor of uterine prolapse.[6,7]

2. Method

This observational nested case-control study was conducted at the Sanglah General Hospital Denpasar from June to December 2019. The uterine samples obtained from total

hysterectomy were analyzed at the Laboratory of the Faculty of Veterinary Medicine Udayana University. This research has received ethical eligibility from the Research Ethical Committee of Udayana University Faculty of Medicine/Sanglah Hospital Denpasar with ethical clearance number 2667/UNI14.2.2VII.14/LP/2019.

The inclusion criteria were patients who had undergone a total hysterectomy at Sanglah General Hospital in Denpasar during the study period, aged between 35 - 70 years, and willing to participate in this study. The exclusion criteria were patients with malignancies and had a history of hormonal therapy within 1 year before the study period. All subjects had signed informed consent. This study used a consecutive sampling method.

The stage III-IV uterine prolapse is determined based on clinical staging using the POP-Q system in patients undergoing total hysterectomy. Stage III uterine prolapse is a descent of the uterine cervix across the hymen by more than 1 cm, but not more than (TVL-2) cm, whereas stage IV uterine prolapse is a descent of the uterine cervix more than (TVL-2) cm, or complete uterine eversion is known. based on clinical examination and/or patient's medical record. The sacrouterine ligament is the connective tissue of the pelvic floor that attaches the uterine cervix to the sacrum bone. The tissue sample was taken from the part of the sacrouterine ligament which was still attached to the uterine cervix after a total hysterectomy was performed at Sanglah General Hospital Denpasar.

LOLX1 expression in tissue samples was determined using immunohistochemical staining. Cells expressing LOLX1 will be brown-yellow. The cells were then quantified using digital analysis methods. A preparation with a magnification of 400 times was photographed with an Optilab camera. Each preparation was photographed 5 times using the JPEG format. Calculating the number of LOXL1 expressions on the sacrouterine ligament using Adobe Photoshop CS3 and Image J software. The LOXL1 network that appears brown is selected using the Magic Wand function by Adobe Photoshop CS3 and the color channels are separated through the RGB stack function in Image J. Then a threshold value is created, Then run the measure function so that you get the percentage of brown pixels from the total pixels automatically.

The LOXL1 expression is calculated based on the intensity of staining and the percentage of pixels that has positive staining, as follows: 0 = no cells were stained brown-yellow; 1 = cells were stained with weak brown-yellow; 2 = cells were stained with medium brown-yellow; 3 = cells were stained with strong yellow-brown. Immunohistochemical scoring using formula: [0 x (% unpainted pixels) + 1 x (% poorly painted pixels) + 2 x (% medium-painted pixels) + 3 x (% strong-painted pixels)]. From this calculation, the CPI score was ranging between 0-300.

A normality test was done in all numerical data. The association between stage III-IV uterine incidence and subjects characteristic, i.e age and BMI, was analyzed using an independent T-test. Meanwhile, the association between the parity and stage III-IV uterine prolapse incidence was analyzed using a Mann-Whitney test. Also, a chi-square test was used to compare the workload and LOLX1 expression between the case and control group. The result was considered significant if the *p*-value <0,05. All data analysis was performed using the Statistical Package for the Social Sciences (SPSS) for windows version 21.0 software.

3. Result

There were 44 patients involved in this study, which consist of 22 patients with stage III-IV uterine prolapse and 22 patients without uterine prolapse. The age and BMI variables were normally distributed and presented using mean ± standard deviation (SD), while parity variables were not normally distributed and presented using median and interquartile range (IQR). There were no significant differences in age, parity, BMI and work load between the case and control groups in this study (Table 1).

Table 1: The Characteristics of Subjects

Variable	Groups		*p*
	Case (n=22)	Control (n=22)	
Age (years) mean ±SD	51.32±4.46	49.23±2.96	0.08[a]
Parity (child) median (IQR)	3.14 (1.08)	2.77 (0.92)	0.24[b]
BMI (kg/m^2) means±SD	21.89±1.93	23.00±2.14	0.08[a]
Workload			
Light work n (%)	14 (63.6)	10 (45.5)	0.226[c]
Heavy work n (%)	8 (36.4)	12 (54.5)	

[a]Independent T-test; [b]Mann-Whitney test; [c]Chi-square test

Immunohistochemical staining was used to show the LOLX1 expression on the sacrouterine ligament. LOLX1 expression was shown as a brown-yellow stained cell in the sacrouterine ligamentous tissue (Figure 1).

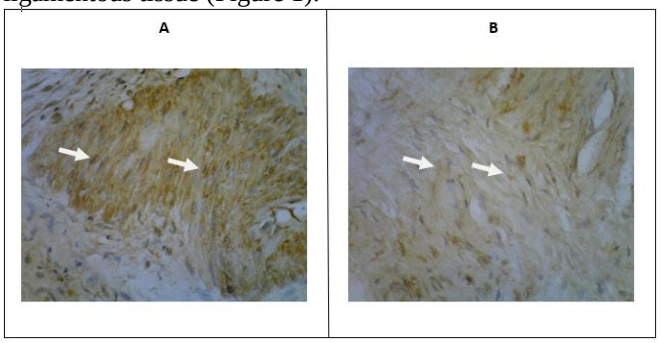

Figure 1: The LOLX1 Expression on The Sacrouterine Ligament. (A) High expression of LOLX1 in the control group shown by high intensity of brown-stained cells (white arrow); (B) Low expression of LOLX1 in the case group shown by low intensity of brown-stained cells (white arrow).

The Chi-Square test was used to determine the association of low LOXL1 expression and the risk of developing stage III-IV uterine prolapse. The cut-off point of LOLX1 expression was determined using Receiver Operating Characteristic (ROC) curve. The LOLX1 expression was classified as high intensity (>1.772) and low intensity (<1.772). It was found that low LOXL1 expression was a significant risk factor for stage III-IV uterine prolapse (OR = 5.95; 95% CI = 1.23-4.33; p = 0.006).

Table 2: The Association Between LOLX1 Expression and Stage III-IV Uterine Prolapse

LOLX1 Expression	Groups		OR	95%CI	p
	Case	Control			
Low	14	5	5.95	1.23-4.33	0.006
High	8	17			

4. Discussion

Pelvic floor muscles and pelvic organ tissues will tend to experience a decrease in strength with increasing age. Increasing age will result in pelvic organ denervation which causes pelvic organ dysfunction. Sacrouterine ligament resistance also decreased significantly in menopausal women, this can increase the progression of uterine prolapse.[8-10] A study done by Swift et al. found that stage I and II pelvic organ prolapse was more common in young women. Meanwhile, pelvic organ prolapse stage III and IV was 2.6% more at the age >40 years and the prevalence increased to 21% in women aged >70 years.[11] Similarly, another study conducted by Siri in 2013 in Makassar also reported that uterine prolapse was most commonly found at over 45 years of age, amounting to 91.4%.[12] The mean age of the case group (51.32±4.46 years) in this study was slightly higher than the control group (49.23±2.96 years), even though there was no significant association (p>0.05). Thus, this was consistent with the previous studies where the reported incidence of uterine prolapse increases with increasing age.[13]

Trauma during childbirth causes anatomical and physiological changes to the pudendal nerve, anal sphincter, and pelvic organ supporting fascia. Further, this will cause the pelvic floor structure changes that may cause uterine prolapse. During labor, neuromuscular damage may occur. When the pelvic floor muscles are relaxed or injured, the genital hiatus opens and the pelvic organs must be maintained by the supporting ligaments. The ligaments can only maintain this heavy load for a short period. Then, the connective tissue will stretch and failure will occur if the pelvic floor muscles do not close the pelvic floor at the right time. This will result in uterine prolapse.[4] However, there was no significant difference between the parity of women in the case group (3.14) and the control group (2.77) in this study (p> 0.05). This was similar to a study conducted by Aznal et al. in Malaysia. Aznal et al. reported that there was no significant difference between the parity of premenopausal women without POP, postmenopausal women without POP, and postmenopausal women with POP (p=0.35).[14]

The mechanism of uterine prolapse in obese women is due to an increase in intra-abdominal pressure that causes weakness in the innervation of pelvic floor muscles and fascia that support the pelvic floor muscles.[15] In this study, the mean BMI of women in the case group (21.89±1.93) was higher than the mean BMI of women in the control group (23.00±2.14). However, the difference in BMI was not significant (p> 0.05). Similar results were found in the study of Ernawati and Bachtiar in 2018, the results of statistical analysis showed that the BMI had no significant relationship with the incidence of POP the (p=1,000).[16] Besides, another study conducted by Nygaard also stated that there was no significant association between BMI and POP with a value of p = 0.64.[17]

Heavy workloads are reported to cause a decrease in levator ani muscle strength. Heavy work that is thought to have a risk of causing or exacerbating POP is work that uses repeated increases in intra-abdominal pressure.[18,19] The type of work itself was not statistically significant because there was no significant difference found between the case and control groups in this study (p=0.226). Nevertheless, the majority of subjects in the control group did light work (63.6%), while the majority of the case group did heavy work (54.5%). This was consistent with the study of Forner et al. Their study found that the proportion of participants with uterine prolapse symptoms in the group with heavy weight lifting activities (7.1%) was significantly lower than the inactive group (21.3%), and groups with light weight lifting activities (19.4%; p <0.001). There was no significant difference in the prevalence of POP symptoms between inactive and mild, moderate, and severe groups (p> 0.05).[20]

The sacrouterine ligament is a condensation of the endopelvic fascia which is the main support that keeps the uterus in place.[21] Ligaments are layers of connective tissue that mostly consist of collagen tissue with high tensile strength. The cells in the ligaments are mostly made up of fibroblasts. Collagen makes up 70-80% of the ligament fibers which mostly type I collagen and a small portion of type III, V, and VI collagen. The sacrouterine ligament is composed of cells (fibroblasts or fibrous connective tissue, chondrocytes or cartilages, osteoblasts, and osteocytes or bones), an extracellular matrix consisting of fibers (collagen and elastin), glycoproteins (Fibronectin, Tenascin, Link protein, Fibromodulin, Osteopontin) and proteoglycans (Aggrekan, Versikan, Biglykan, Dekorin, Perlekan).[4]

In this study, there was a significant difference in the level of LOXL1 expression in the sacrouterine ligament between the case and control group (p = 0.006). The majority of subjects in the case group had a low LOXL1 expression level compared to the control group. In contrast, in the control group, the majority had a high LOXL1 expression. Low LOXL1 expression was proved to increase the risk of uterine prolapse by 5.95 times (OR = 5.95, 95% CI = 1.23-4.33, p = 0.006). These results are consistent with the study conducted by Alarab et al regarding LOXL1 expression in patients with POP in premenopausal women. Alarab et al found that decreased LOX enzyme expression may cause disruption of the stability of the pelvic tissue and causes uterine prolapse.[22] Kleanthis et al also assessed the distribution and expression of LOX in 44 postmenopausal women, including 21 patients with uterine prolapse and 23 patients without prolapse. There was a low LOX expression in women with uterine prolapse and high expression in women without prolapse (p <0.005). This study concluded that there was a strong relationship between LOX enzyme expression and the incidence of pelvic organ prolapse.[6]

The sacrouterine ligament is the main ligament that supports the cervix and upper vagina. LOX is an enzyme that plays an important role in the biogenesis of the connective tissue matrix by forming extracellular matrix protein cross-links,

which are cross-links to collagen and elastin. The main component of elastic fibers is an amorphous polymer in the form of a monomer, which forms cross-links within the extracellular gap with the help of LOXL1 to form a polymer. The deposition of the elastin polymer is an important aspect of maintaining elastic fibers which are a key component of the sacrouterine ligament and depend on LOXL1 which functions as both a cross-linking enzyme and a support element to ensure elastin deposition.[7]

In older postpartum uterine tissue, the defect of elastin polymerization is much more pronounced than in younger uterine tissue, suggesting that LOXL1 requirements for redeposition of the elastin polymer increase through the reproductive cycle. Deposition of the new elastin polymer can continue in wild-type adult tissue but stops with the loss of LOXL1. The selective role of LOXL1 in elastin is also supported by measurements of desmosine and hydroxyproline, which represent the cross-linkages of elastin and collagen. Desmosine levels were significantly lower in some LOXL1 tissues compared to the corresponding wild tissue types, whereas hydroxyproline levels remained unchanged.[7]

Neupane et al also found that mice that lose LOXL1 do not deposit normal elastic fibers into their genitourinary tract and had severe postpartum uterine prolapse and lower urinary tract dysfunction with lower bladder capacity and urinary pressure.[23] The previous studies found decreased LOXL1 expression in the premenopausal, postmenopausal, or mixed population of women with stage III-IV uterine prolapse, suggesting a suboptimal elastin polymerization mechanism to cause uterine prolapse.[5,22] This study also found a lower level of LOXL1 mRNA indicating a possible epigenetic mechanism.[5]

5. Conclusion

Uterine prolapse is the descent of the uterus into, even out of the vaginal canal, caused by the weakening of the pelvic floor muscles, ligaments and fascia that support the uterus. This study proved that low LOXL1 expression in the sacrouterine ligament is a risk factor for stage III-IV uterine prolapse. Further research is needed regarding LOXL1 expression with stage III-IV uterine prolapse so that it can be used as a reference for predictors of the progression of stage III-IV uterine prolapse.

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