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RESEARCH ARTICLE

EXPRESSION OF INTERLEUKIN-8 IN PARAFFIN BLOCK OF SACROUTERINE TISSUE IN PELVIC ORGAN PROLAPSE PATIENTS VS NON PELVIC ORGAN PROLAPSE PATIENTS

M. I. P. Lubis[1], M. R. Z. Tala[2], E. Ardiansyah[2]

[1]Department of Obstetrics and Gynaecology, University of North Sumatera General Hospital - Medan, Indonesia
[2]Division of Urogynaecology, Department of Obstetrics and University of North Sumatera General Hospital - Medan, Indonesia

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ABSTRACT

This study aimed to determine the differences in immunohistochemical expression of Interleukin-8 (IL-8) in women with pelvic organ prolapse and without pelvic organ prolapse. This research was an analytical study of 2 unpaired study subjects with cross sectional design by conducting immunohistochemistry examination of interleukin 8 (IL-8) in paraffin blocks of sacrouterine ligament tissue in women with and without pelvic organ prolapse at Haji Adam Malik General Hospital Medan in January to February 2017. Most of the subjects in pelvic organ prolapse group were more than > 60 years old, mutipara, and had menopause. Most of them having pelvic organ prolapse grade 3. From the results of data analysis, a significant relationship was found between menopausal status with IL-8 expression based on Allred scores. There were also a significant relationship found between parity and IL-8 expression based on Allred scores with statistically significant differences between IL-8 expression based on Allred scores in pelvic organ prolapse group versus non pelvic organ prolapse.

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INTRODUCTION

Uterine prolapse is the condition of uterus descent through the pelvic floor or genital hiatus due to weakening of the pelvic floor muscles, especially the levator ani muscle, pelvic ligaments, and pelvic fascia supporting the uterus. It cause the uterus descends into the vagina and may come out of the vagina.[1] Pelvic Organ Prolapse (POP) occurs in almost half of all women. Although nearly half of women who had given birth were found to have POP through a physical examination, only 5-20% were symptomatic.[2,3,4] POP prevalence increases by about 40% every decade of a woman age.[8]More severedegree of POP is found in older women, i.e., 1st degree of 28% -32.3%, second degree of 35% -65.5%, and third degree in 2-6%.[5] Etiologyof POP is multi-factorial. Risk factors that have been studied include pregnancy, vaginal delivery, menopause, estrogen deficiency, increased long-term intra-abdominal pressure (constipation, lifting heavy items, chronic obstructive pulmonary disease, straining), race, body mass index (BMI), genetic anatomical factors, biochemical and supporting tissue metabolism factors, and surgical history (Burch hysterectomy and colposuspension).[5, 6,7,8,9,10] In healthy women, the uterus is supported by ligaments of connective tissue consisting of extracellular matrix (ECM).

***Corresponding author: M. I. P. Lubis,**
Department of Obstetrics and Gynaecology, University of North Sumatera General Hospital - Medan, Indonesia

This requires a balance between Matrix Metalloproteinase (MMP) and specific regulators that play a role in maintaining connective tissue homeostasis in normal tissues. ECM derives its strength from fibril proteins (collagen I, III, V and elastin) and is produced and maintained by fibroblastic cells, namely fibroblasts and myofibroblasts. Fibroblastic cells remodel the matrix around them and maintain tissue homeostasis by producing anabolic molecules and catabolic enzymes such as MMP. Production and remodeling affect the composition and mechanical properties and integrity of the surrounding tissue which depends on the balance between ECM synthesis and degradation. In uterine prolapse, imbalances and increased metabolism of collagen and elastin are found.[11,12] In uterine prolapse, collagen and elastin fibers were disorganized, MMP activity were increased, and TIMP activity were decreased. In the MMP family, the components of Matrix Metalloproteinase-2 (MMP-2 / gelatinase A, 72 kDa), Matrix Metalloproteinase-9 (MMP-9 / gelatinase B, 92 kDa), collagen IV, and gelatin, are major structural components of basal membrane. At the post-translation level, all MMPs are under the control of the Tissue Inhibitor of Metalloproteinase (TIMP). The Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) specifically inhibits MMP-9 while the Tissue Inhibitor of Metalloproteinase-2 (TIMP-2) inhibits MMP-2. However, some studies do not prove the same. In this case, there may be other cells responsible for excess in vivo enzymatic activity. [1,13.] Many factors play a role in maintaining the balance of the

degradation process and matrix synthesis of these macromolecules, but in vivo efforts to control this mechanism are not yet known. Biochemical factors in the form of cytokines and cellular immune systems are involved in the inflammatory process. This process affects collagen metabolism in connective tissue. Cytokines such as interleukin (IL) and tumor necrosis factor-α (TNFα) stimulate the synthesis of MMP enzymes by cellular immune cells (macrophages, lymphocytes, monocytes, T cells) and induce MMP-2, MMP-9 and TIMP-1inhibition.[14] Several studies have found an association between Interleukin-8 (IL-8) and MMP-2, MMP-9, and TIMP-1. The effect of IL-8 on cellular protease levels has not been specifically investigated in uterine organ support, but in other systems, the effects of IL8 have been shown to depend on MMP and TIMP activity. Inflammation was found to have an important role in increasing MMP levels and decreasing TIMP levels including IL-8.[15]

IL-8 has the most influence with MMP-9. IL-8 is a potent neutrophil activator and most of MMP-9 is produced by neutrophils themselves. Moreover, IL-8 has been shown to induce the release of MMP-9 from neutrophils, although the mechanism that regulates the release of MMP-9 remains poorly understood. Inoue *et al*. (2000) found that MMP-9 activity correlated with the expression of IL-8 in cancer cells. Chakrabakti *et al*. (2005) showed IL-8 mediated the release of MMP-9 due to stimulation of specific CXCR2 in the flow of Src-kinase and protein kinase.[16,17,18] At the transcription level, the TIMP-1 gene is induced in large numbers in response to several proinflammatory cytokines. TIMP-1 will complete the effects of destruction of MMP-2 and MMP-9 and reduce the protective effect of the extracellular matrix. In TIMP, Moreau *et al*. (1999) found that IL-8 (2.5 ng / mL) triggered inhibition of TIMP-1. They examined the effects of these particles on MMP-1, MMP-3, MMP-9, TIMP-1, and TIMP-2 levels in human monocyte macrophage culture media. [19,20] However, no studies have directly assessed the association of IL-8 with the incidence of uterine prolapse. Given the influence of IL-8 which affects many connective tissue matrix degradators that will contribute to the incidence of uterine prolapse, it is hoped that measuring levels of one biochemical mediator, IL-8, can be an important marker in the incidence of uterine prolapse.

Study

This research is an analytical study of 2 unpaired research subjects with cross sectional design by conducting immunohistochemistry examination of interleukin 8 (IL-8) in paraffin blocks of sacrouterine ligament tissue in women with and without pelvic organ prolapse at Haji Adam Malik General Hospital Medan in January to February 2017. The case group consisted of paraffin of sacrouterine ligament tissue obtained from the total vaginal hysterectomy on the indication of grade III and IV pelvic organ prolapse diagnosed via POP-Q system. While the control group consisted of total abdominal hysterectomy on the indication of non pelvic organ prolapse. Patients in both control and case groups must have a minimum age of 40 years, without a history of respiratory morbidity or diabetes, giving birth only through vaginal delivery and never undergo any pelvic surgery. After obtaining approval from the ethics commission in conducting the research, the research began by collecting data from the department of anatomical pathology at Haji Adam Malik Hospital in Medan along with the patient's medical record data. Paraffin tissue blocks were then cut to 4 μm then fixed above the glass object. Mouse monoclonal immunohistochemistry IL-8 antibodies were then conducted. The preparation was heated on a hot plate at 60^{o}C for 10 minutes then left for 10 minutes at room temperature. Xylol was given two times for 5 minutes each, fixed with 100% alcohol two times for 5 minutes followed by 80% alcohol once for 3-5 minutes. Then the preparation was being soaked in 0.5% H_2O_2 in methanol for 30 minutes. Then *decloaking chamber* was conducted, and the preparation was left until it reached room temperature, then it was being washed using PBS two to three times. Pap pen was given to the tissue around and the tissue was dripped by the NHS. Next, a monoclonal mouse IL-8 human antibody was given to the preparation and it was immersed one night. The preparation then being washed using PBS three to five times, dripped with secondary antibodies for 30 minutes, washed again using PBS three to five times and then washed using DAB. Next, the preparation was washed using running water. After that, counter staining was conducted using with Hematoxyllin-Meyer staining, then the preparation was washed using running water, given LiCO3 (Lithium carbonat) and washed again using running water. After that, the preparation was fixated using fixing 96% alcohol once for 5 minutes and using 100% alcohol three times for 5 minute long each. Xylol was given 2 times for 5 minutes long each. Then the mounting process was done. Interpretation was carried out by two Pathologists. Histopathological examination was performed using a 400x magnification light microscope. Then the IL-8 immunohistochemical expression data were analyzed for each study group. Data analysis and statistical tests were conducted in a computerized manner. To analyze the difference in accuracy of the two observers, the kappa value being calculated; if the validity was > 75%, no significant differences were found between the two observer observations. The relationship between variables was conducted using Chi square statistical test with 95% confidence level and $p < 0.05$ was considered as meaningful.

RESULTS

The case and control group consisted of 20 people each. After tabulating the data analysis by two observers, it was found that the kappa test was 90.2% which showed a high degree of conformity in assessing IL-8 expression, the results of an immunohistochemical examination from one observer could be used in this study, in this case we used observer 1.

Table 1. Characteristics of Research Subjects

Characteristics	Case		Control	
	N	%	n	%
Age (years)				
40 – 49	1	5	11	55
50 – 59	8	40	9	45
≥ 60	11	55	0	0
Parity				
0	0	0	3	15
1	0	0	6	30
2	0	0	4	20
≥ 3	20	100	7	35
Menopausal Status				
NotMenopause	0	0	12	60
Menopause	20	100	8	40

The case group was dominated by age of more than 60 years (55%) and age of 40-49 years in control group (55%).

According to the number of parity, in case and control groups we found parity of more than ≥3 (100% and 35% respectively). All of the subjects in case group had already experienced menopause (100%) while in the control group the majority had not had yet menopause (60%). (Table 1) Based on the degree of POP, most cases of POP were in grade III (65%) and others were in grade IV (35%). (Table 2) Table 3 shows that menopause women have an Allred IL-8 expression score of mostly positive.

Table 2. Distribution of Research Subjects Based on POP Degree

POP Degree	Case	
	n	%
3rd Degree	13	65
4th Degree	7	35
Total	20	100

Table 3. Association between Menopausal Status and IL-8 ExpressionBased on Allred Score

Menopausal Status	IL-8 Expression based on Allred Score				*p**
	Negative		Positive		
	n	%	n	%	
Menopause	8	40	20	100	*0,001*
Not menopause	12	60	0	0	
Total	20	100	20	100	

**Chi Square*

Statistically there was a significant relationship between menopausal status and Allred IL-8 expression with p value of<0.05. Table 4 shows that women with parity of ≥3 had mostly positive Allred score of IL-8 expression. Statistically there was a significant relationship between parity and Allred IL-8 expression with p value of <0.05. Table 5 shows that the all subjects with positive IL-8 expressions experienced POP with a statistically significant relationship between IL-8 expression and POP incidence (p <0.05).

Table 4. Association between Parity and IL-8 Expression Based on Allred Score

Parity	IL-8 Expression based on Allred Score				*p**
	Negative		Positive		
	n	%	n	%	
0-2	13	65	0	0	*0,001*
≥3	7	35	20	100	
Total	20	100	20	100	

*Chi Square

Tabel 5. Difference in IL-8 Expression in Women with and Without POP Based on Allred Score

IL-8 Expression based on Allred Score	Study Group				*p**
	Case		Control		
	n	%	n	%	
Negative	0	0	20	100	*0,001*
Positive	20	100	0	0	
Total	20	100	20	100	

**Chi Square*

DISCUSSION

Age: This study found that the case group was dominated by age ≥60 years (55%) with parity ≥3 (100%). In Indonesia alone, genital prolapse is more common among women who have ever given birth, menopausal old women and women with quite heavy work.[2] The older the woman, the more tone will be decreased. In US, data were obtained that the age was associated with the incidence of pelvic organ prolapse in women over 50 years.[21] The Oxford Family Planning Cohort Study of 17,000 women, showed that those with two history of baby deliveries had an eight times increased risk folding in hospital for POP compared to nullipara.[14]

Menopausal Status: All of subjects in case group had experienced menopause (100%). In women who have menopause, in addition to the lack of estrogen (hypoestrogenism) produced by the ovary and due to the aging factors which is causing pelvic floor muscles such as the pelvic diaphragm, urogenital diaphragm and ligament and fascia to undrego atrophy and is weaken, so that eventually the vaginal atrophy occurs. This situation will cause the muscles and fascia to not perform their function properly as an organ support, causing genital prolapse.[14]

IL-8 Expression and POP Incidence: This study showed that all subjects with positive IL-8 expression experienced POP where there was a statistically significant relationship between IL-8 expression and POP incidence (p <0.05). The effect of IL8 on cellular protease levels has not been specifically studied in uterine organ support, but in other systems, the effects of IL8 have been shown to depend on MMP and TIMP activity. Inflammation was found to have an important role in increasing MMP levels. MMP-2 and MMP-9 were found to increase, due to decreased regulation of α-SMA and / or PDGF-BB, and greater expression of IL-1β, IL-6, IL-8, CXCL1 and CCL2.15.[22] Neutrophils are the source of MMP-9 in the body. Unlike most cell types, which only express MMP-9 under proinflammatory stimulation, neutrophils produce MMP-9 and store it in their tertiary granules. After stimulation, neutrophils release MMP-9 quickly from the granule. Some mediators, including Formil-Met-Leu-Phe (fMLP), tumor necrosis factor (TNF) and IL-8 [CXC chemokine ligand 8 (CXCL8)], have been shown to induce the release of MMP-9 from neutrophils, although the mechanism that regulates release MMP-9 remains poorly understood.[16] Interleukin-8, chemokine CXC, is a powerful activator of neutrophils. Interleukin-8 binds CXC chemokine receptors 1 (CXCR1) and CXCR2, which are very much expressed on the surface of neutrophils. Stimulation with IL-8 triggers intracellular calcium flux and activates various signal kinases into human neutrophils, causing the release of primary, secondary, and tertiary granule contents. Of the many signal pathways that are activated by IL-8 stimulation in neutrophils, the signals of the Src ERK1 / 2 family and the PKC are strongly associated with MMP-9.[23.24]

Chakrabakti *et al*. (2005) showed that blocking CXCR1 had no effect on IL-8 which mediated the release of MMP-9, whereas inhibition of CXCR2 significantly reduced the release of MMP-9. This process is not affected by changes in intracellular calcium concentration. The mutually exclusive Src-kinase pathway and protein kinase C regulate the release of MMP-9 mediated by IL-8. More convincingly, they found that inhibition of ERK1 / 2 and PKC blocked the release of IL-8MMP-9-mediated. No effect of inhibition of mitogen-activated p38 kinase protein for the release of MMP-9.[18] Inoue *et al*. (2000) found that MMP-9 activity correlated with the expression of IL-8 in cancer cells. They conducted a study by incubating PC-3P cells with different doses of rIL-8 and MMP-9 activity determined by zimography after normalizing the volume of the supernatant based on cell numbers. The results showed that IL-8 caused an increase in MMP-9

activity. In addition, increased MMP-9 activity by rIL-8 was inhibited by neutralization with anti-IL-8 antibodies. Furthermore, when changing the expression of IL-8 by transfection of sense and antisense, a corresponding change in the expression of MMP-9 and activity both in vitro and in vivo was found. MMP-9 is transfected by biologically active activity, due to increased collagenase activity and increased cellular invasion through Matrigel. MMP-9 activity decreases after antisense transfection, both collagenase activity and Matrigel invasion decrease. [18] Regarding the relationship of IL-8 and MMP-2, Luca *et al*. (1997) found this relationship in melanoma cells in vivo. They found that when MMP-2 promoters were associated with increased release of chloramphenicol acyltrasferase, which increased its regulation by IL-8 (CAT), reporter genes showed that IL-8 was involved in MMP-2gene transcription.[23,13] In trophoblasts, IL8 was thought to increase migration and invasion of cells by increasing MMP and integrins. Jovanovic *et al*. (2010) found that exogenous IL8 triggered migration and invasion of HTR-8 / SVneo cells with stimulation of MMP2 and MMP9 of 182% ($P < 0.01$) and 134%, respectively ($P < 0.01$).

They conducted a study on the HTR-8 / SVneo cell line with a monolayer test and Matrigel invasion test. The effects of IL8 on MMPs were found to be significant in zymography and western blot examinations. Li *et al*. (2005) showed that antibodies to IL-8, CXCR1 or CXCR2 inhibited endothelial cell proliferation and MMP-2 production compared to culture cells with their own media or antibody control. In addition, it was found that the number of apoptotic cells was significantly higher in cells given anti-IL-8 exposure, anti-CXCR1 and anti-CXCR2.[21,25] Thus, the availability of IL8 was found to affect the production of MMP-2. In the cancer model, IL8 causes decreased regulation of MMP-2 and MMP-9 activities through modulation of NF-kB expression and transcription activity. In the pancreatic cancer cell line, IL8 was also found to trigger MMP-2 and MMP-9 activities. Other cytokines modulate MMP2 and MMP9 activities, such as IL-1, IL-6, and TGF-beta.[19] In TIMP, Moreau *et al*. (1999) found IL-8 (2.5 ng / mL) triggered maximum TIMP-1 inhibition. They examined the effects of these particles on MMP-1, MMP-3, MMP-9, TIMP-1, and TIMP-2 levels in human monocyte macrophage culture media. The TIMP-1 gene was found to be highly induced at the transcription level, in response to several proinflammatory cytokines.[24,26] Kostamo *et al*. (2005) also found an association of IL-8 and collagenase. Kostamo *et al*. assess the relationship of IL-8 and collagenase. The study was conducted on bronchial cells of chronic rhinosinusitis patients with nasal polyps. MMP-8 concentration and activation levels were analyzed by immunofluorometrics and Western blotting in mucus samples from CRSwNP patients on nasal lavage from healthy controls in relation to interleukin-8 (IL-8) inductive cytokines and tumor necrosis factor-alpha (TNF-alpha). MMP-8 and IL-8 significantly increase, especially cholegenase in mesenchymal type cells. IL-8 and MMP-8 which appear to be the pathogenesis of the inductive cytokine-proteinase cascade in CRSwNP.[27]

Conclusion

Most of the subjects in the pelvic organ prolapse group were> 60 years old, mutipara, and all had menopause and most had third degree pelvic organ prolapse. From the results of data analysis, a significant relationship between menopausal status with IL-8 expression based on Allred scores was found along with a significant relationship between parity and IL-8 expression based on Allred scores and statistically significant differences between IL-8 expression based on Allred scores in pelvic organ prolapse group and non pelvic organ prolapse.

Suggestion: IL-8 is recognized to have been associated with the mechanism of the occurrence of pelvic organ prolapse so that its potential role can be considered in the management of these cases.

Conflict of Interest: The authors declare that they have no competing interests.

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