

MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2, MUC1 and CD29 Expression in Endometrial Polyps at Implantation Window Compared to Neighbouring Normal Endometrium

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ABSTRACT

Purpose: The aim of this study was to evaluate the expression of potential mediators of embryo implantation and invasion on polyps such as Matrix metalloproteinases (MMPs), Tissue inhibitors of Metalloproteinases (TIMPs), Mucin-1 and CD29 in women with uterine polyps compared to healthy adjacent endometrium to elucidate causal relationship between infertility and endometrial polyps (EPs).

Methods: The study was conducted on 15 women presented with unexplained infertility and EPs. Office hysteroscopy was performed, and the polyps were removed via scissors and these polyps formed the Study group (n=15). The endometrium adjacent to the polyps was punch-biopsied and formed the Control group (n=15). Sections were cut, antigens unmasked, and immunohistochemical staining was performed. Immunohistochemical staining in luminal and glandular epithelium cytoplasm was graded.

Results: MMP-1 immunostaining in luminal epithelium cytoplasm did not differ between the groups, whereas luminal epithelium cytoplasm had statistically significant ($p<0.01$) staining for MMP-2 and 9 among the study and control groups. TIMP-1 and TIMP-2 expression in polyps were statistically significantly prominent compared to the control group. While MUC1 expression did not differ between the groups, CD29 integrin expression was higher in the control group ($p<0.01$).

Conclusion: The presence of EPs in infertile women may adversely affect the delicate balance of mediators unfavorably. As it is well studied, the hyperreactivity of MMPs in chronic inflammatory states may decrease the chance of successful implantation. Consequently, we believe that removing even asymptomatic EPs may increase implantation success in infertile women.

Keywords: Endometrial polyps, MMP, TIMP, MUC1, CD29

INTRODUCTION

Endometrial polyps (EPs) were reported to be present in 3 to 10% of women of reproductive age.¹ The prevalence is reported to be even higher among infertile patients which is 8 to 34%.^{2,3} Although routine use of transvaginal ultrasonography during gynecologic examination may lead to the diagnosis of EPs in asymptomatic patients, sonohysterography in premenopausal women with and without abnormal bleeding is believed to be the best non-invasive method to diagnose EPs accurately. However, the gold standard for more accurate diagnosis is accepted to be hysteroscopy-guided biopsy.^{4,5}

A healthy uterine environment plays a pivotal role in facilitating the successful implantation of a viable embryo, underscoring

the importance of exploring factors that may influence uterine receptivity. Uterine polyps are benign growths within the uterine cavity often associated with abnormal uterine bleeding, recurrent miscarriage, and impaired fertility. Despite their prevalence among infertile patients, the mechanisms linking uterine polyps to infertility are not thoroughly elucidated.^{6,7}

Furthermore, uterine polyps have been speculated to exert detrimental effects on fertility through various mechanisms, including hindrance of sperm transport, obstruction of embryo implantation due to their space-occupying nature, and induction of local inflammatory changes.⁸⁻¹⁰ These inflammatory responses, characterized by an increased presence of mast cells and upregulation of Matrix metalloproteinase 2 (MMP2) and MMP9 activity, suggest a potential association between



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uterine polyps and altered molecular pathways implicated in infertility.^{6,7}

MMPs are a family of calcium-dependent homologous enzymes containing zinc in the active site and capable of degrading extracellular matrix (ECM) and basement membrane components.¹¹ They are essential for physiological processes such as implantation, placentation, and the dynamic remodeling of uterine structures during pregnancy. MMP-1 belongs to the collagenase group and is up-regulated by trophoblast invasion while MMP-2 and MMP-9 belong to the gelatinase group. MMP-1 as a collagenase, breaks down collagen fibers in the ECM, while MMP-2 and MMP-9 are gelatinases that primarily degrade gelatin substrates in the ECM.¹²

Recent studies suggest a potential association between altered MMP activity and infertility, with elevated intrauterine levels observed in infertile women compared to fertile ones.¹³ MMP enzymes are regulated transcriptionally, activated by proenzyme conversion, and regulated by tissue inhibitors [Tissue inhibitors of Metalloproteinases (TIMPs)]. There are genetically four different TIMPs in humans. TIMPs are expressed coordinately with MMPs. The hyperactivity of MMP-2 and MMP-9 is associated with an unfavorable uterine environment, as seen in endometrial inflammation. The simultaneous expression and inhibition of MMP-9 during the luteal phase are regulated by TIMP.^{14,15}

Mucin-1 (MUC1) is a member-associated protein that is highly expressed in the luminal and glandular epithelium during the implantation window.^{16,17} MUC1 is crucial in aiding embryo implantation and the establishment of pregnancy. It accomplishes this by safeguarding the uterine lining, regulating immune reactions, and fostering communication and attachment between the embryo and the mother. Fertile women showed a higher level of endometrium MUC1 expression than infertile patients.¹⁸ The reduction of anti-adhesive factors such as MUC1 in infertility cases may contribute to the increased adhesiveness of pinopods.¹⁹ CD29 assists in the initial bonding of the embryo to the uterine lining and promotes the invasive actions of trophoblast cells during implantation, thus playing a vital part in establishing pregnancy.²⁰

This study aimed to evaluate the expression of potential mediators of embryo implantation and invasion on polyps such as MMPs, TIMPs, MUC1, and CD29 in women with uterine polyps compared to healthy adjacent endometrium to elucidate the causal relationship between infertility and EPs.

METHODS

The study was conducted on 15 women presented with unexplained infertility complaints and ultrasound-diagnosed EPs which were consecutively removed via hysteroscopy for pathological examination. Office hysteroscopy was performed, and the polyps were removed via scissors and these polyps formed the Study group (n=15). The endometrium adjacent to the polyps was punch-biopsied and formed the Control group (n=15). The biopsy specimens were taken without dilatation of the cervix and without anesthesia.

EPs and endometrial biopsies of normal endometrial neighbouring the polyps were taken from the mid-uterine cavity on day 20-24 of the menstrual cycle, at the time of embryo implantation window in regularly menstruating women. Participation in the study was voluntary and all participants read and approved the informed consent form.

The biopsy specimens were fixed in 10% neutral buffered formalin for at least 6 hours. After overnight tissue processing, they were embedded in paraffin. The histologic sections were in 5 µm thickness and stained with Hematoxylin and Eosin. Sections for immunohistochemical examination were cut in 5 µm thickness and mounted onto adhesive-coated slides (Menzel-Glaser, Superfrost® Plus, Germany). For immunohistochemical staining, sections were kept at 56 °C overnight and then soaked in xylene for 30 minutes. After washing with a decreasing series of ethanol, sections were washed with distilled water and phosphate-buffered saline (PBS) for 15 minutes. After that, coated slides were dried in an incubator at 56 °C for 2 hours. Antigen unmasking was performed for the MMP-1 antibody, MMP-2 antibody, MMP-9 antibody, TIMP-1 antibody, and TIMP-2 antibody in a citrated buffer solution in the commercially available pressure cooker at 1 atmosphere pressure for 1.5 min.

After antigen unmasking, step slides were washed with PBS (pH: 7.4) and thereafter to block endogenous peroxidase activity, the slides were incubated in 3% hydrogen peroxide for 20 mins. Slides were washed with PBS for 5 mins. Sections were then blocked with Super Block (REF: AAA 125 LOT:12232) at room temperature for 15 mins and afterward washed with PBS. Slides were immunostained with commercially available rabbit polyclonal antibody MMP-1 (Collagenase-1) (Neo-Markers, Fremont, CA, USA, Cat No: RB-1536-P) at 1:50 dilution for 1 hour in the microwave, with commercially available rabbit monoclonal antibody MMP-2 (72kDa

Collagenase IV) Ab-4 (Neo-Markers Fremont, CA, USA, Cat No: MS-806-P) at 1:100 dilution for 1 hour in the pressure cooker, with commercially available rabbit polyclonal antibody MMP-9 (92 kDa Collagenase type IV) Ab-9 (Neo-Markers Fremont, CA, USA, Cat No: RB-1539-P) at 1:50 dilution for 2 hours, with commercially available mouse monoclonal antibody TIMP-1 (Novocastra™, NCL-TIMP1-485, Newcastle, UK) at 1:150 dilution for 1 hour, and with commercially available mouse monoclonal antibody TIMP-2 (Clone 3A4) (Neo-Markers Fremont, CA, USA, Cat No: MS-1485-P) at 1:25 dilution for 2 h at room temperature (20-25 °C).

Afterward, slides were washed with PBS and slides were incubated with UltraTek Anti-Polyvalent Biotinylated Antibody (REF: ABN 125, LOT:11461, ScyTek Laboratories, Utah, USA) at room temperature for 25 mins. Slides were washed with PBS again and incubated with UltraTek HRP (REF: ABL125, LOT:11460, ScyTek Laboratories, Utah, USA) at room temperature for 25 mins. Slides were washed with PBS and incubated with Ultravision Detection System Large Volume AEC Substrate System (RTU) (REF: TA-125-HA, LOT: AHA60718, LabVision, Fremont, CA, USA) at room temperature for 15 mins. The sections were finally counterstained using Mayer's hematoxylin and mounted in an aqueous medium.

Slides were analyzed with a BX50 conventional light microscope (Olympus, Tokyo, Japan) by BM at 100 and 200 magnifications two times. Staining intensity was graded as; “0 = no staining”, “+1 or 1-10% staining = weak staining”, “+2 or 10-49% staining = mild staining” and “+3 or 50-100% staining = strong staining”. Immunohistochemical staining in luminal and glandular epithelium cytoplasm were graded in 60 sections counting 100 cells separately at 400X magnification.

Statistical Analysis

The statistical analysis of the study data was performed using SPSS 23.0 for the Windows packet program. The age of the patients were normally distributed according to Shapiro-Wilk test and presented as mean and standard deviation (SD) of the mean. Classified data was presented as number and percentages. Analysis of classified data was performed using the chi-square test, Fisher's Exact test. Probability (p) value smaller than 0.05 was considered significant. All values reported are mean (\pm SD) or percentage.

RESULTS

The mean age of the patients was 32.4 ± 4.8 years. The mean menstrual cycle timing of the hysteroscopic polypectomy and concomitant endometrial biopsy was 22.2 ± 1 days. According to common wisdom, any hysteroscopic intervention is advised to be done in the early proliferative phase of the cycle. However, the so-called- implantation window, is believed to be around the 21st-23rd day of the cycle. So, we decided to do

hysteroscopic interventions around these days to elucidate the problem in implantation and avoid other confounders. MMP-1 immunostaining in luminal epithelium cytoplasm did not differ between the groups, whereas luminal epithelium cytoplasm had statistically significant ($p < 0.01$) staining for MMP-2 and 9 in the study group (Table 1). Not surprisingly the TIMP-1 and TIMP-2 expression in polyps were statistically significantly prominent compared to the control group (Table 2). While MUC1 expression did not differ between the groups, CD29 integrin expression was higher in the control group ($p < 0.01$) (Table 3).

DISCUSSION

EPs are frequently encountered in asymptomatic women during routine gynecologic examinations. However, the prevalence of EPs in infertile women seems to be somewhat higher than in the normal population.^{2,3} The causal relation between EPs and implantation failure has not been extensively studied in the literature. Despite huge advances in this field implantation is still believed to be the least understood phase of human reproduction. There are multiple delicate regulatory mechanisms with interactions ultimately leading to the hosting of a genetically diverse group of cells within the cells of the mother. Among these, cytokines, cell adhesion molecules, prostaglandins, and growth factors, MMPs are believed to play an integral role in human embryo implantation and are the main rate-limiting enzymes in ECM remodeling during implantation. Successful implantation is believed to depend on

Table 1. MMP-1, MMP-2, and MMP-9 expression in polyps and adjacent endometrium

Variable	Study group (n=15)	Control group (n=15)	p-value
MMP-1 immunostaining in luminal epithelium cytoplasm			
No stain	7 (46.7)	9 (60)	0.5
+1	3 (20)	4 (26.7)	
+2	4 (26.7)	2 (13.3)	
+3	1 (6.7)	0	
MMP-2 immunostaining in luminal epithelium cytoplasm			
No stain	13 (86.7)	0	<0.0*
+1	2 (13.3)	5 (33.3)	
+2	0	8 (53.3)	
+3	0	2 (13.3)	
MMP-9 immunostaining in luminal epithelium cytoplasm			
No stain	13 (86.7)	5 (33.3)	<0.01*
+1	1 (6.7)	4 (26.7)	
+2	1 (6.7)	4 (26.7)	
+3	0	2 (13.3)	
MMP-9 immunostaining in luminal epithelium stroma			
No stain	11 (73.3)	6 (0.4)	0.8
+1	2 (13.3)	6 (0.4)	
+2	1 (6.7)	2 (13.3)	
+3	0	1 (6.7)	

*Likelihood ratio, statistically significant, $p < 0.05$. MMP: Matrix metalloproteinase

Table 2. TIMP-1 and TIMP-2 expression in polyps and adjacent endometrium

Variable	Study group (n=15)	Control group (n=15)	p-value
TIMP-1 immunostaining in luminal epithelium cytoplasm			
No stain	11 (73.3)	2 (13.3)	<0.01*
+1	3 (20)	6 (40)	
+2	1 (6.7)	3 (20)	
+3	0	4 (26.7)	
TIMP-1 immunostaining in endometrial stroma			
No stain	10 (66.7)	0	<0.01*
+1	1 (6.7)	2 (13.3)	
+2	4 (26.7)	12 (80)	
+3	0	1 (6.7)	
TIMP-2 immunostaining in luminal epithelium cytoplasm			
No stain	15 (100)	11 (73.3)	0.04*
+1	0	3 (20)	
+2	0	1 (6.7)	
TIMP-2 immunostaining in endometrial stroma			
No stain	15 (100)	13 (86.6)	0.1
+1	0	2 (13.3)	
*Likelihood ratio, statistically significant, p<0.05. TIMP: Tissue inhibitors of Metalloproteinase			

*Likelihood ratio, statistically significant, $p < 0.05$. TIMP: Tissue inhibitors of Metalloproteinase

Table 3. MUC1 and CD29 integrin expression on polyps and adjacent endometrium

Variable	Study group (n=15)	Control group (n=15)	p-value
MUC-1			
+2	2 (13.3)	4 (26.7)	0.36
+3	13 (86.7)	11 (73.3)	
CD29 integrin			
No stain	10 (66.7)	1 (6.7)	<0.01*
+1	5 (33.3)	6 (40)	
+2	0	8 (53.3)	
*Likelihood ratio, statistically significant, p<0.05. MUC1: Mucin-1			

*Likelihood ratio, statistically significant, $p < 0.05$. MUC1: Mucin-1

a strict balance between activation and inhibition of MMPs.^{14,15} Hyperactivity of MMP2 and 9 are reported to be found in the endometrial tissues in chronic or mild inflammatory states.

In our study, we aimed to highlight the hyperexpression of MMP 2 and 9 in the tissues extracted from the EPs and compared them with normal neighboring endometrial tissue. The reason for not choosing a control group from the endometrium of fertile women is that there might be innate differences between the fertile and infertile women. To overcome other confounding factors, we preferred to sample the same uterus for comparison. It was not surprising to find the overexpression of TIMPs, since they usually reflect the increased levels of MMPs in response.

CD29 integrin assists in the initial bonding of the embryo to the uterine lining and promotes the invasive actions of trophoblast cells during implantation, thus playing a vital part in establishing pregnancy. The immunohistochemical staining for CD29 antigen in the study group was significantly less than

the control group. This finding may also constitute another explanation for implantation failure in the infertile patients diagnosed with EPs.

In our study, we did not include EPs dimensions. Hence, we could not compare and quantify whether the magnitude of the volume of the polyps has an effect on the staining levels of the markers that we studied. This might be regarded as a weakness of our study.

CONCLUSION

The presence of EPs in infertile women may adversely affect the delicate balance of mediators unfavorably. As it is well studied, the hyperreactivity of MMPs as seen in chronic inflammatory states may lead to a decrease in the chance of a successful implantation. Consequently, we believe that removing even asymptomatic EPs may increase implantation success in infertile women.

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Ethics

Ethics Committee Approval: The study received the approval of Kocaeli University Clinical Research Ethics Committee (approval number: 17/18, dated: 2008/124).

Informed Consent: Participation in the study was voluntary and all participants read and approved the informed consent form.

Authorship Contributions

Surgical and Medical Practices: E.K., B.M., Concept: B.M., Design: E.K., B.M., Data Collection or Processing: Ö.D.S., N.E.A., Analysis or Interpretation: Ö.D.S., N.E.A., Literature Search: E.K., Ö.D.S., Writing: E.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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