Original Article

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The Role of Matrix Metalloproteinases in Copper IUD-Induced Dysmenorrhea and Prolonged Menstruation

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ABSTRACT

Purpose: This study was conducted to determine the role of matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinase (TIMP) expression in copper intrauterine device (IUD)-induced dysmenorrhea and prolonged menstruation.

Methods: This prospective clinical study included 30 women who were willing to use copper IUD contraception. Endometrial biopsies were performed before and 3 months after the insertion of the IUD. Correlations between menstrual bleeding abnormalities, visual analog scale (VAS) dysmenorrhea scores related to copper-IUD usage and MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2 endometrial expression were investigated.

Results: The mean ± standard deviation VAS score at 3 months after IUD insertion increased compared with before IUD use (3.5±1.8 vs. 2.8±0.9; p=0.003). The mean duration of menstrual bleeding was significantly prolonged three months after IUD use compared to before use (4.5±1.1 days vs. 4.06±0.8; p<0.001). MMP-1 expression in the luminal epithelium was present in 1.4±2.98 (0-8) cells per 100 cells before IUD use and significantly increased to 14.7±20.7 (0-76) cells three months after IUD insertion (p<0.001). MMP-1 expression was positively correlated with the presence of dysmenorrhea VAS score (p<0.001) and duration of menstruation (p=0.04) three months after IUD insertion. MMP-9 expression was increased three months after IUD use (47.9±40.2, 4-90 cells) compared to before IUD use (26.8±23.5, 4-80 cells, p=0.01) in luminal epithelium. MMP-9 expression grade in luminal epithelium (p=0.003) and stroma glandular epithelium (p=0.001) increased significantly as the menstrual pattern progressed from light to heavy. As MMP-9 expression grade in luminal epithelium and stroma glandular epithelium increased the mean duration of menstrual bleeding became longer (p=0.01, p=0.01, respectively).

Conclusion: With the use of copper-IUDs, VAS score increased and duration of menstruation was prolonged. MMP-1 expression increased in cases with menstrual pain and duration of menstruation while MMP-9 expression increased as the duration and/or the severity of menstrual bleeding increased.

Keywords: Intrauterine device, menstrual bleeding, dysmenorrhea, matrix metalloproteinases, tissue inhibitors of matrix metalloproteinase

INTRODUCTION

Matrix metalloproteinases (MMPs), are a group of enzymes involved in matrix degradation.[1,2] The family comprises interstitial collagenases, gelatinases, stromelysins, and membrane-type MMPs. MMPs are inhibited either by specific tissue inhibitors of metalloproteinases (TIMPs), or less specifically, by alfa 2-macroglobulin.[3,4]

MMPs are involved in several key reproductive events, such as ovulation, embryo implantation, menstruation and postpartum uterine involution.[5-8] Progesterone withdrawal increases production of pro-MMP-2 by decidualized stromal cells and activates endometrial proteases MMP-1, -2, -3, and -9.[9] MMP-9 is found in the epithelium only during the early secretory phase, while during menstruation it is predominantly present in a variety of leukocytes.[10]

TIMPs are the major endogenous regulators of MMPs and consist of four homologous members (TIMP 1-4). TIMP-1 binds to and inhibits the active form of MMPs. TIMP-2 is differentially regulated from TIMP-1 and has been proposed to act selectively on different MMPs. TIMP-3 has a high affinity

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for MMP-9 and has the ability to inhibit membrane type-1 MMP (MT1-MMP). However, unlike TIMP-1 or TIMP-2, TIMP-3 is secreted and then bound to the extracellular matrix (ECM). TIMP-4 is a good inhibitor for all classes of MMPs without remarkable preference for specific MMPs.[11]

Copper-intrauterine devices (IUDs) cause a sterile inflammatory reaction in the endometrium.[12] The most common side effects of an IUD are increased menstrual bleeding and pain and the removal rate for these complaints within the first year of IUD use is 5-15%.[13] Labied et al.[14] investigated MMP expressions in the endometrium in levonorgestrel-releasing intrauterine system users. However, the effect of copper-IUD on these inflammatory systems remains unclear and how the copper-IUD causes side effect, such as abnormal menstrual bleeding, is unknown.

In this study, we aimed to determine the role of copper-IUD on the expression of MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 in luteal phase endometrium. We assessed the relationship between tissue expression of these MMPs and TIMPs in menstrual bleeding abnormalities and dysmenorrhea associated with copper-IUD usage.

METHODS

The study population consisted of women whose midluteal phase progesterone level was 10 ng/mL (31.8 nmol/L) and who were willing to use a copper-IUD for contraception. The women were menstruating regularly (cycle varying between 28-35 days). The exclusion criteria were pregnancy, acute or chronic pelvic inflammatory disease, metrorrhagia for unknown reason, cervicitis, dysplasia in the cervix, genital tumor, copper allergy, usage of contraceptive pills within the previous three months, lactating women, hypothyroidism, hyperthyroidism, diabetes mellitus, hyperprolactinemia, abnormalities in blood clotting and severe dysmenorrhea. Menstrual day-3 serum thyroid stimulating hormone levels were 1.7±1.5 μIU/mL, follicule stimulating hormone levels were 7.2±2.4 mU/mL, luteinizing hormone levels were 5.1±2.1 mU/mL, estradiol levels were 42.7±24.6 pg/mL, international normalized ratios were 1.02±0.07, fasting blood glucose levels were 92±4.3 mg/dL, and prolactin levels were 14.9±6.7 ng/mL. Informed consent was taken from the women and the study was approved by the the Ethics Committee of the Kocaeli University Faculty of Medicine (approval number: KOÜ 2008/124, date: 2008).

All patients underwent gynecological examination and had a Papanicolaou smear taken during the previous 12 months. Menstruation period, bleeding quantity and dysmenorrhea was recorded before and three months after the IUD insertion. A menstrual calendar was used to evaluate the amount and the duration of menstrual bleeding. The amount of menstrual bleeding was defined as mild (<1/4 of tampon or pad surface stained with blood), moderate (1/4-3/4 of tampon or pad surface stained with blood) or heavy (>3/4 of tampon or pad surface stained with blood). Dysmenorrhea were estimated using the visual analogue scale (VAS) at the initial examination and after IUD insertion. The VAS score (0-10) of each patient was calculated as an arithmetic mean of the first three days of the menstrual cycle. Prolonged menstruation was defined as a menstrual period lasting more than eight days according to the Federation of Gynecology and Obstetrics ovulatory disorders classification.[15]

The biopsy specimens were taken with a Pipelle curette without dilatation of cervix and without anesthesia. Endometrial biopsies were taken from the mid-uterine cavity before and three months after the insertion of the IUD, on day 20-24 of the first and third cycle. The biopsy specimens were fixed in 10% neutral buffered formalin for at least six hours. After overnight tissue processing they were embedded in parafin. Tissue sections for histological examination were 5 μm thick and stained with hematoxylene and eosin. Sections for immunohistochemical examination were also 5 μm thick and mounted onto adhesive-coated slides (Superfrost® Plus, Menzel-Gloser, Germany). For immunohistochemical staining, sections were kept at 56 °C overnight and then soaked in xylene for 30 mins. After washing with a decreasing series of ethanol, sections were washed with distilled water and phosphate-buffered saline (PBS) for 15 minutes. Then, coated slides were dried in an incubator at 56 °C for two hours. Antigen unmasking was performed for 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 90 seconds.

After antigen unmasking, step slides were washed with PBS (pH 7.4) and then in order to block endogenous peroxidase activity, the slides were incubated in 3% hydrogen peroxide for 20 minutes. Slides were washed again with PBS for five minutes. Sections were then blocked with Super Block (REF:AAA 125 LOT:12232, CP Lab Safety, California, USA) at room temperature for 15 minutes and afterwards washed with PBS. Slides were immunostained with rabbit polyclonal antibody against human MMP-1 (Collagenase-1) (Neo-Markers, Fremont, CA, USA, Cat No: RB-1536-P) at 1:50 dilution for one hour in the microwave, with rabbit monoclonal antibody against MMP-2 (72kDa Collagenase IV) Ab-4 (Neo-Markers Fremont, CA, USA, Cat No: MS-806-P at 1:100 dilution for one hour in the pressure cooker, with rabbit polyclonal antibody against MMP-9 (92 kDa Collagenase type IV) Ab-9 (Neo-Markers Fremont, CA, USA, Cat No:RB-1539-P) at 1:50 dilution for two hours, with mouse monoclonal antibody against TIMP-1 (NCL-TIMP1-485, Novacastra, Newcastle, UK) at 1:150 dilution for one hour and with mouse monoclonal antibody against TIMP-2 (Clone 3A4) (Neo-Markers Fremont, CA, USA, Cat No:MS-1485-P) at 1:25 dilution for two hours at room temperature (20-250C).

Afterwards, 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 minutes. Slides were washed with PBS again and incubated with UltraTek horse radish peroxidase (REF:ABL125, LOT:11460, ScyTek Laboratories, Utah, USA) at room temperature for 25 minutes. Slides were washed with PBS and incubated with Ultravision Detection System Large Volume AEC Substrate System (REF:TA-125-HA, LOT:AHA60718, LabVision, Fremont, CA, USA) at room temperature for 15 minutes. 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 magnification twice. Staining intensity was graded as; "0= no staining", "+1 or 1-10% cells stained = weak staining", "+2 or 10-49% cells stained = mild staining" and "+3 or 50-100% cells stained = strong staining". Immunohistochemical staining in luminal and stromal 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, version 11.5, for Windows (IBM Inc., Armonk, NY, USA). All values reported are mean (± SD) or percentage. The McNemar Bowker test was used for immunostaining grade and intensity before and three months after IUD insertion. Analysis of classified data was performed using chi-square test and/or Fisher's exact test. Comparison of classified data was performed using McNemar-Bowker test before and after IUD use. Correlation of the data was determined using Pearson correlation test. Comparison of continuous variables between different grades of staining was performed using Kruskall-Wallis analysis. Probability (p) values <0.05 were considered statistically significant.

RESULTS

The study group numbered 30 women with a mean ± SD age of 32.8±5.3 years, ranging from 25 to 40 years. The first biopsy was made at cycle day 22.7±1.3 before IUD insertion and the second biopsy was made at cycle day 22.1±1.5 three months after IUD insertion (p=0.06). Before IUD insertion, menstrual bleeding was mild in 3 (10%) patients and moderate in 27 (90%) patients while it was mild in 2 (6.7%) patients, moderate in 20 (66.7%) patients, and heavy in 8 (26.7%) patients three months after IUD (p=0.1). The mean ± SD VAS score at the cycle three months after IUD insertion increased significantly compared to before IUD use (3.5±1.8 vs. 2.8±0.9, p=0.003). The mean ± SD duration of menstrual bleeding was longer three months after IUD use compared to before use (4.5±1.1 days vs. 4.06±0.8, p<0.001).

MMP-1 expression of the luminal epithelium was present in 1.4±2.98 (0-8) cells per hundred cells before IUD use and significantly increased to 14.7±20.7 (0-76) cells three months after IUD insertion (p<0.001). MMP-1 expression was positively correlated with the VAS score (p<0.001) three months after IUD insertion. Three months after IUD insertion, women with dysmenorrhea (n=9) had significantly higher mean luminal epithelium cytoplasm MMP-1 expression of 21.6±24.2% compared to women without dysmenorrhea (n=21) in whom the expression was 1.9±2.4% (p=0.04). Grade of expression of MMP-1 in the luminal epithelium with respect to menstrual bleeding amount and duration is presented in Table 1. As MMP-1 expression increased, the mean duration of menstruation became longer (p=0.04), but the severity of menstrual bleeding was similar.

MMP-2 expression of the luminal epithelium was similar before compared to three months after IUD insertion (36±26.7, 5-80 cells versus 41.2±22.6, 6-80 cells, respectively; p=0.41). No correlation was found between MMP-2 expression in the luminal epithelium and the mean duration of menstruation, the severity of menstrual bleeding or VAS score (Table 2).

MMP-9 expression increased three months after IUD use compared to before IUD use (47.9±40.2, 4-90 cells versus 26.8±23.5, 4-80 cells, respectively; p=0.01) in luminal epithelium. MMP-9 expression in the stroma was similar before compared to three months after IUD use (11.3±20.7, 0-70 cells versus 13.7±17.4, 0-40 cells, respectively; p=0.6). No correlation was found between increase of the MMP-9 expression of the luminal epithelium or the endometrial

Table 1. Menstrual bleeding volume and duration with respect to the expression grade of the MMP-1 antibody in the luminal epithelium

Grade of MMP-1 immunostaining in luminal epithelia	0	+1	+2	+3	p-value
Menstrual bleeding (n) Light	1	1	0	0	
Moderate	8	10	2	0	0.7
Heavy	3	4	0	1	
Duration of menstrual Bleeding (days)	4.3±1.1	4.4±1.1	5.5±0.7	7	0.04*

MMP: Matrix metalloproteinases, *p<0.05, statistically significant

Table 2. Menstrual bleeding volume and duration with respect to MMP-2 expression grade in luminal epithelium

Grade of MMP-2 immunostaining in luminal epithelia	0	+1	+2	+3	p-value
Menstrual bleeding (n) Light	0	1	1	0	
Moderate	0	3	10	1	0.08
Heavy	0	0	11	53	
Duration of menstrual bleeding (days)	0	4.5±1.2	4.3±1.1	5.1±1.4	0.4

MMP: Matrix metalloproteinases

stroma glandular epithelium and VAS score. Menstrual bleeding amount and duration of menstrual bleeding with respect to expression grade of the MMP-9 antibody in the luminal epithelium and in the endometrial stroma glandular epithelium is presented in Table 3. MMP-9 expression grade in luminal epithelium (p=0.003) and stroma glandular epithelium (p=0.001) increased significantly as the menstrual pattern progressed from light to heavy. As MMP-9 expression grade in luminal epithelium and stroma glandular epithelium increased the mean duration of menstrual bleeding become longer (p=0.01, p=0.01, respectively).

Expression grade of TIMP-1 in the luminal epithelium and endometrial stroma glandular epithelium is presented in Table 4. TIMP-1 expression in luminal epithelium and stroma glandular epithelium was similar after three months compared to before IUD insertion (p=0.1, p=0.06 respectively). No correlation was found between TIMP-1 expression in the luminal epithelium and endometrial stroma glandular epithelium and the VAS score (p=0.7 and p=0.36, respectively).

Expression grade of TIMP-2 in the luminal epithelium and endometrial stroma is presented in Table 4. TIMP-2 expression in luminal epithelium and stroma glandular epithelium was similar after three months compared to before IUD insertion (p=0.1 and p=0.06, respectively). No correlation was found between TIMP-2 expression in the luminal epithelium and endometrial stroma glandular epithelium and the VAS score (p=0.6 and p=0.43, respectively).

DISCUSSION

The World Health Organisation advises that copper-IUD is one of the most effective contraceptive methods used to date.[16] Menstrual abnormalities, including spotting and/or mild bleeding or heavy and/or prolonged bleeding were reported to be common in the first 3-6 months of IUD use, and persisted thereafter in a minority of women.[17] Studies have shown that menstrual bleeding alone and bleeding with pain were the most common reasons for requesting IUD removal.[18,19]

Table 3. Menstrual bleeding volume and duration with respect to MMP-9 expression grade in luminal epithelium and in endometrial stroma glandular epithelium

Immunostaining of MMP-9 in luminal epithelium	**0**	**+1**	**+2**	**+3**	**p-value**
Menstrual bleeding (n) Light	0	2	0	0	
Moderate	0	9	11	0	0.003*
Heavy	0	1	4	3	
Duration of menstrual bleeding (days)	0	3.9±0.8	4.6±1.1	6.3±0.5	0.01*
Immunostaining for MMP-9 in the endometrial stromal glandular epithelium	0	+1	+2	+3	p-value
Menstrual bleeding (n) Light	0	2	0	0	
Moderate	0	3	17	0	0.001*
Heavy	0	1	4	3	
Duration of menstrual bleeding (days)	0	3.6±0.5	4.5±1.1	6.6±0.5	0.01*

MMP: Matrix metalloproteinases, *p<0.05, statistically significant

Table 4. Expression grades of TIMP-1 and TIMP-2 in luminal and endometrial stromal glandular epithelium

Antibody	**Expression**	**Before IUD (n=30) n (%)**	**After IUD (n=30) n (%)**	**p-value**
Grade of TIMP-1 immunostaining in luminal epithelia	0 +1 +2 +3	18 (60) 9 (30) 2 (6.6) 1 (3.3)	26 (86.7) 4 (13.3)	0.1
Grade of TIMP-1 immunostaining in the endometrial stromal glandular epithelium	0 +1 +2 +3	24 (80) 4 (10) 2 (10)	27 (90) 3 (10)	0.06
Grade of TIMP-2 immunostaining in luminal epithelia	0 +1 +2 +3	21 (70) 5 (16.6) 3 (10) 1 (3.3)	25 (83) 5 (16.6)	0.1
Grade of TIMP-2 immunostaining in the endometrial stromal glandular epithelium	0 +1 +2 +3	25 (83) 3 (10) 2 (6.6)	28 (93) 2 (6.6)	0.07

p<0.05, statistically significant, TIMP: Tissue inhibitors of metalloproteinase, IUD: Intrauterine device

It has been reported that endometrial explants from metrorrhagic women released considerably more MMP-1, -2, -3, and -9, and lower amounts of TIMP-1.[20,21] In the literature review, we found only one report investigating the relationship between Copper-IUD and MMP-1. It suggested that the Copper-IUD may enhance the activity of MMPs in human endometrium and that MMPs may participate in the development of IUD-induced menorrhagia.[22] In a limited number of studies, it has been reported that the increase in MMP expressions due to IUD use is correlated with bleeding disorders.[14,23] In the present study, after three months of IUD usage, MMP-1 expression in endometrial luminal epithelium cytoplasm increased significantly. While the increase was not correlated with heavier menstruation, it was found to be positively correlated with the mean duration of menstruation as it became longer and the *de novo* dysmenorrhea VAS score related to IUD use.

MMP-2 and MMP-9 are synthesized by various stromal cells, including macrophages, fibroblasts, and endothelial cells.[24] One of the aims of this study was to investigate the effect of copper-IUD insertion on MMP-2 expression in luteal phase endometrium. Menstruation is characterized by the lysis of collagen-rich argyrophilic fibers in the endometrial stroma, followed by tissue collapse and fragmentation, collectively described as "stromal breakdown".[25,26] Thus, MMPs are prime candidates for triggering endometrial bleeding because they are able to collectively degrade most proteins of the ECM at neutral pH.[27] In the present study, while MMP-2 expression in endometrial luminal epithelial cytoplasm did not change significantly after three months of IUD usage, MMP-9 expression increased in luminal epithelium significantly after three months of IUD usage. No correlations were found between MMP-2 expressions in the luminal epithelium and prolonged and/or heavier menstruation. Furthermore, MMP-2 and MMP-9 expressions after IUD insertion were not correlated with the VAS score.

In normal endometrium, epithelial expression of MMP-2, MMP-9 and TIMP-2 were reported to increase during the proliferative phase of the menstrual cycle, and MMP-2 expression was negatively correlated with TIMP-2 expression. MMP-9 and TIMP-2 expression had been found not to vary with the phase of the menstrual cycle.[28] Studies reported that more MMP-1, -2, -3, and -9 and lower amounts of TIMP-1 were released when the endometrium was sampled during bleeding episodes.[20,21] At least two studies have indicated that TIMP-1 and TIMP-2 were expressed in small arteriolar and capillary vascular tissues in the secretory endometrium, suggesting that TIMPs might be involved in stabilization of uterine vasculature during the reproductive cycle and pregnancy.[29,30] Although the role of TIMP expression during endometrial vascularization remains to be fully elucidated, TIMP-1 and TIMP-2 have each been recently reported to have anti-angiogenic activity that may be related to an inhibition of vascular endothelial growth factor expression.[31] We found that TIMP-1 and TIMP-2 expression in endometrial luminal epithelial cytoplasm and endometrial stroma did not change significantly after three months of IUD use. Furthermore, luminal epithelial and stromal TIMP-1 and TIMP-2 expressions were not found to be correlated with prolonged and/or heavier menstruation and VAS score.

CONCLUSION

In conclusion, the pathogenesis of menstrual bleeding disturbances and dysmenorrhea associated with copper-IUD use is multifactorial and not dependent on a single variable. Our results show a positive correlation of MMP-1 expression with increased VAS score and prolonged mean menstrual duration. IUD-related prolonged menstruation and severity of menstrual bleeding appear to be associated with changes only in MMP-9 expression in luminal epitelium, while no associations were found with MMP-2, TIMP-1, TIMP-2 expressions in the endometrium. There is a need to conduct further studies with MMPs and develop new treatment modalities for IUD-related menstrual bleeding disorders and dysmenorrhea, which are the main reasons for the removal of copper IUDs.

Footnote

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Ethics Committee Approval: This study was approved by the Ethics Committee of the Kocaeli University Faculty of Medicine (approval number: KOÜ 2008/124, date: 2008).

Informed Consent: Informed consent was obtained.

Authorship Contributions

Surgical and Medical Practices: H.U.Ş., B.M., Concept: H.U.Ş., Ö.D.S., B.M., A.Ç., Design: H.U.Ş., Ö.D.S., B.M., A.Ç., Data Collection or Processing: H.U.Ş., E.C., Analysis or Interpretation: H.U.Ş., B.M., A.Ç., Literature Search: H.U.Ş., Ö.D.S., E.C., Writing: H.U.Ş., Ö.D.S., E.C., A.Ç.

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