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Effect of proinflammatory cytokines on endometrial collagen and metallopeptidase expression during the course of equine endometrosis

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ABSTRACT

Equine endometrosis (endometrial fibrosis) is a degenerative chronic process that occurs in the uterus of the mare and disturbs proper endometrial function. Fibrosis is attributed to excessive deposition of extracellular matrix (ECM) components. The turnover of ECM is mediated by matrix metallopeptidases (MMP). Previously, it was shown that cytokines modulate MMP expression in other tissues and may regulate fibrosis indirectly by attracting inflammatory cells to the site of inflammation and directly on various tissues. However, the regulation of MMP expression in equine endometrosis is still relatively unknown. Thus, our aim was to determine if interleukin (IL)-1β and IL-6 regulate ECM, MMPs, or their inhibitors (TIMPs) and whether this regulation differs during endometrosis in the mare. Endometrial fibrosis was divided into four categories according to severity: I (no degenerative changes), IIA (mild degenerative changes), IIB (moderate degenerative changes) and III (severe degenerative changes) according to Kenney and Doig classification. Endometrial explants (n = 5 for category I, IIA, IIB and III according to Kenney and Doig) were incubated with IL-1β (10 ng/ml) or IL-6 (10 ng/ml) for 24 h. Secretion and mRNA transcription of collagen type 1 (*Col1a1)* and type 3 (*Col3a1)*, *fibronectin* (*Fn1*), *Mmp-1, -2, -3, -9, -13*, *Timp-1, -2* were analyzed by real-time PCR and ELISA, respectively. IL-1β treatment up-regulated secretion of COL1, MMP-2, TIMP1, and TIMP2 in category I endometrial fibrosis tissues ($P < 0.05$). IL-6 treatment up-regulated secretion of ECM, MMP-2, and MMP-3 and down-regulated secretion of MMP-9 in category I tissues ($P < 0.05$). In category IIA tissues, IL-1β and IL-6 treatment up-regulated secretion of COL3 ($P < 0.05$; $P < 0.05$), and IL-6 treatment also down-regulated secretion of MMP-9 ($P < 0.05$). In category IIB tissues, IL-1β treatment down-regulated secretion of COL3 ($P < 0.05$) and up-regulated secretion of MMP-3 ($P < 0.01$), while IL-6 treatment up-regulated secretion of MMP-3, MMP-9, and MMP-13 ($P < 0.05$). In category III tissues, IL-1β treatment up-regulated secretion of COL1, MMP-1, MMP-9 and TIMP-2 ($P < 0.05$), and IL-6 up-regulated secretion of all investigated ECM components, MMPs and TIMPs. These results reveal that the effect of IL-1β and IL-6 on equine endometrium differs depending on the severity of endometrial fibrosis. Our findings indicate an association between inflammation and development of endometrosis through the effect of IL-1β and IL-6 on expression of ECM components, MMPs, and TIMPs in the mare.

1. Introduction

Equine endometrosis (endometrial fibrosis) is a degenerative chronic process of the uterus. This condition may be described as an active or inactive process that develops around the endometrial glands and/or in the stroma [1,2]. Endometrosis is associated with disorders of uterine morphology and dysfunction that can lead ultimately to embryo loss [3–7]. The effect of equine endometrosis on reproductive outcome depends upon its severity, which can be classified based on histological characteristics of endometrium [1,2], category I (no degenerative alteration), category IIA (mild degenerative alteration), category IIB (moderate degenerative alteration), and category III (severe

Abbreviations: ANOVA, Analysis of variance; COL1, collagen type 1; *col1a1*, collagen type 1 chain alpha 1; COL3, collagen type 3; *col3a1*, collagen type 3 chain alpha 1; ECM, extracellular matrix; FN, fibronectin; IL-1β, interleukin 1β; IL-6, interleukin 6; MMP-1, matrix metallopeptidase 1; MMP-2, matrix metallopeptidase 2; MMP-3, matrix metallopeptidase 3; MMP-9, matrix metallopeptidase 9; PBS, phosphate-buffered saline; PPBE, persistent post-breeding endometritis; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; P_4, progesterone; SDHA, succinate dehydrogenase complex subunit A; TIMP-1, metallopeptidase inhibitor 1; TIMP-2, metallopeptidase inhibitor 2

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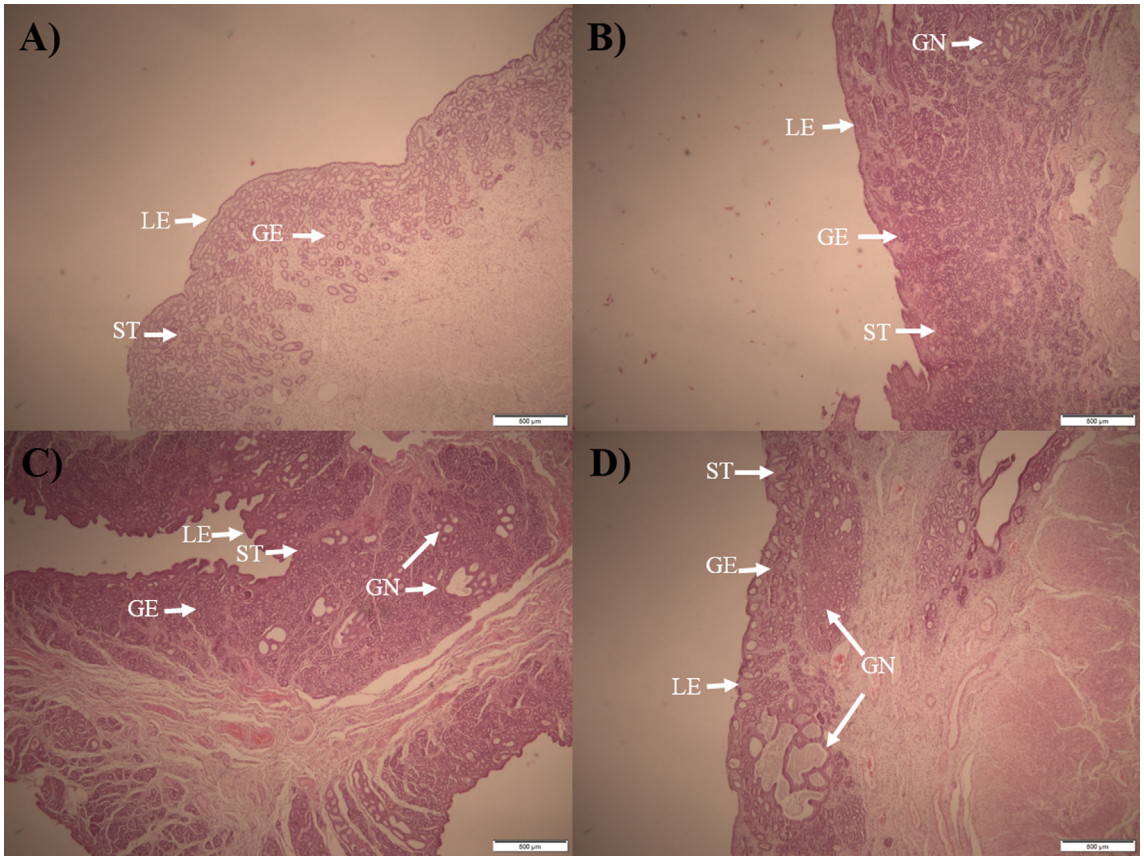

Fig. 1. Representative equine endometrial biopsies classified as (A) category I; (B) category IIA; (C) category IIB, and (D) category III, according to Kenney and Doig (1986). LE, luminal epithelium; GE, glandular epithelium; ST, stromal cells; GN, glandular nests. Hematoxylin and eosin staining. Scale bar = 500 μm (magnification: 4×).

Table 1
Primer sequences used in Real-time PCR.

Gene name	Primer sequence (5′–3′), forward/reverse	Amplicon length (bp)	Accession Number (GeneBank)
Mmp1	TCTCACAGCTTCCCAACAGT CATCTGGGCTGCTTCATCAC	230	NM_001081847.2
Mmp2	ACCCCACTACGGTTTTCTCG TACTTCACACGGACCACTTGC	212	XM_023637007.1
Mmp3	TCACTCGACCAACACCGAAG CAGGGGGAGGTCCATAGAGG	122	NM_001082495.2
Mmp9	GGCCAGTTCCAGACCTTTGA CCATCTCCGTGCTCCCTAAC	221	NM001111302.1
Mmp13	GACAAGCAGTTCCAAAGGCTAC GGGATGTTTAGGGTTCGGGT	214	NM_001081804.1
Timp1	AACCAGACCACCTTACAGCG GTCCAATAGTTGTCCGGCGA	186	NM_001082515.1
Timp2	CCCCATCAAGCGGATTCAGT GCCTTTCCTGCGATGAGGTA	156	XM_023651899.1

degenerative alteration) according to Kenney and Doig [2] The foaling rate in each category is around 80–90% (I), 50–80% (IIA), 10–50% (IIB), and 10% (III). Thus, endometrosis is a serious problem in horse reproduction.

The etiology and pathogenesis of equine endometrosis is not clearly understood. Generally, fibrosis is attributed to excessive deposition of collagen (COL) and other extracellular matrix (ECM) components in the endometrium [4]. The turnover of COL and remodeling of ECM is regulated by different matrix metallopeptidases (MMPs) and their inhibitors (TIMPs). It was shown that cytokines modulate MMP in the human endometrium [8–10], but the regulation of MMPs and their inhibitors in the equine endometrium is not well described. Recently, we showed that transforming growth factor (TGF)-β1 and neutrophil extracellular traps (NETs) components (myeloperoxidase, elastase, and cathepsin G) up-regulated COL1 and COL3 expression in the equine endometrium [11–13]. Studies about the process of fibrosis in many organs of other species indicate that pro-inflammatory cytokines may regulate fibrosis indirectly through attraction of inflammatory cells to the site of inflammation, but also by acting directly on various tissues reviewed by [14]. Previously, we showed that endometrial expression of interleukin (IL)-1β and IL-6 protein is up-regulated during the endometrosis progression in the mare [6]; however, to the best of our knowledge the role of these proinflammatory cytokines on expression of ECM components and MMPs in the equine endometrium has not been described to date. Interleukin 6 seems to contribute to fibrotic diseases [15–17]. Depending on the tissue type, IL-6 may affect proliferation of fibroblasts [18–19] and increase synthesis of ECM components, such as COL and glycosaminoglycan, from fibroblasts [20]. Additionally, IL-6 affected expression of MMPs, such as MMP-1 and MMP-9, and MMP inhibitors, such as TIMP-1, in fibroblasts [21–22]. Furthermore, IL-6 supports tissue repair by shifting acute inflammation into a more chronic, profibrotic state through the induction of Th1 cell responses that result from recurrent inflammation [15].

Moreover, IL-1β has been shown as one of the important players in the development of fibrosis in the lung, liver, and heart [23–25]. In in vivo studies, neutralization or other means of knocking down IL-1β expression caused attenuation of fibrosis. Likewise, increased IL-1β expression augmented the severity of fibrosis [23–28]. In *in vitro* studies, IL-1β showed putative profibrotic effects by augmenting fibroblast proliferation, and ECM and MMP secretion, from renal, cardiac, and endometrial fibroblasts [10,29–30].

Table 2
The list of ELISA kits used in the study.

	ELISA kit Name	Organism Species	Manufacture, product no	Curve range
COL1	ELISA Kit For Collagen Type I (COL1)	Equus caballus	Cloud-clone; SEA571Eq	6.25–400 ng/ml
COL3	ELISA Kit For Collagen Type III (COL3)	Equus caballus	Cloud-clone; SEA176Eq	6.25–300 ng/ml
FN	ELISA Kit for Fibronectin (FN)	Equus caballus	Cloud-clone; SEA037Eq	12.50–800 ng/ml
MMP-1	ELISA Kit For Matrix Metalloproteinase 1 (MMP-1)	Equus caballus	Cloud-clone; SEA097Eq	0.312–20 ng/ml
MMP-2	ELISA Kit For Matrix Metalloproteinase 2 (MMP-2)	Equus caballus	Cloud-clone; SEA100Eq	1.56–100 ng/ml
MMP-3	ELISA Kit for Matrix Metalloproteinase 3 (MMP-3)	Equus caballus	Cloud-clone; SEA101Eq	31.25–2000 pg/ml
MMP-9	ELISA Kit For Matrix Metalloproteinase 9 (MMP-9)	Equus caballus	Cloud-clone; SEA553Eq	0.625–40 ng/ml
MMP-13	ELISA Kit For Matrix Metalloproteinase 13 (MMP-13)	Equus caballus	Cloud-clone; SEA099Eq	0.781–50 ng/ml
TIMP-1	ELISA Kit for Tissue inhibitors of metalloproteinase 1 (TIMP-1)	Equus caballus	Cloud-clone; SEA552Eq	0.625–40 ng/ml
TIMP-2	ELISA Kit For Tissue inhibitors of metalloproteinase 2 (TIMP-2)	Equus caballus	Cloud-clone; SEA128Eq	7.8–500 ng/ml

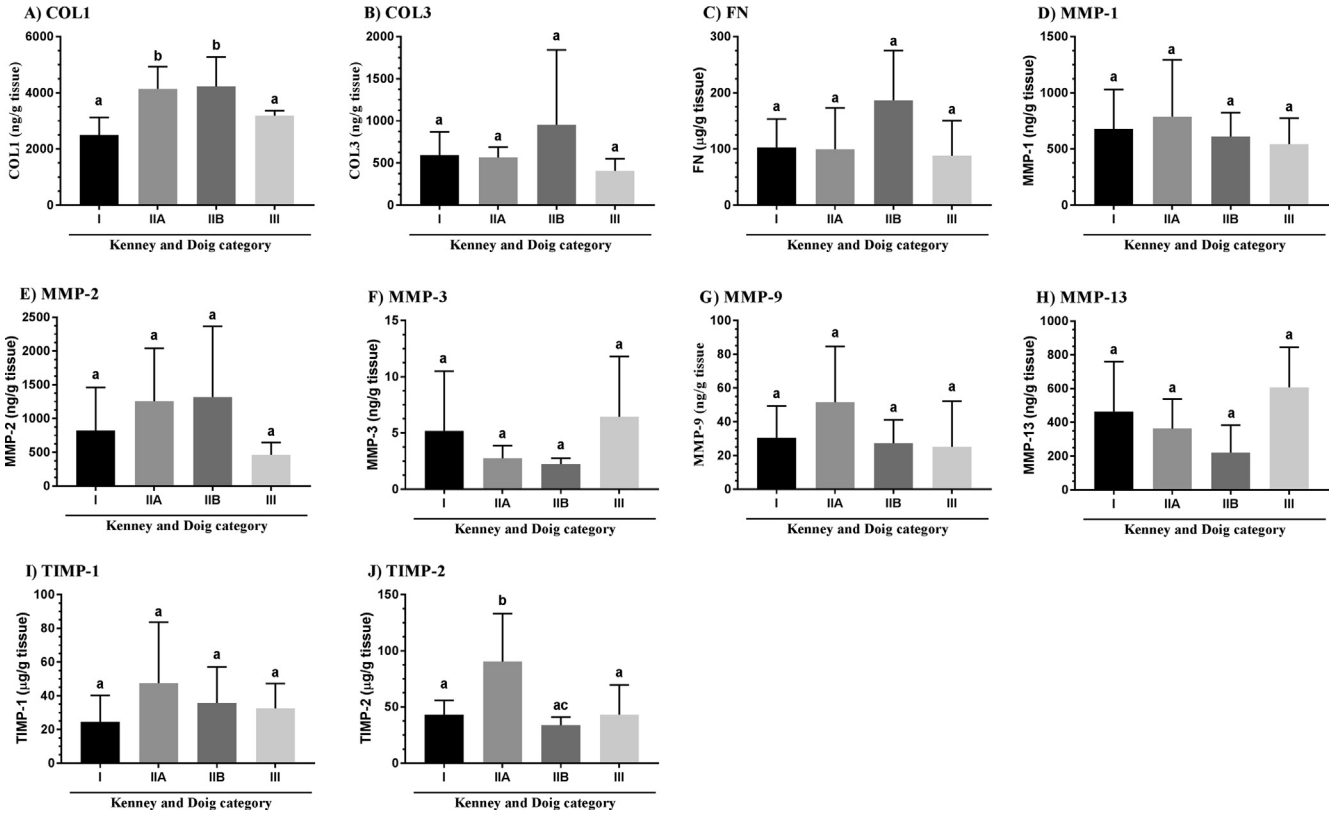

Fig. 2. The basal secretion of (A) COL1, (B) COL3, (C) FN, (D) MMP-1, (E) MMP-2, (F) MMP-3, (G) MMP-9, (H) MMP-13, (I) TIMP-1, and (J) TIMP-2 by endometrial explants *in vitro* classified as category I (n = 5), IIA (n = 5), IIB (n = 5), or III (n = 5) according to Kenney and Doig [2] in a mid-luteal phase of the estrous cycle. The comparison between basal secretion of all factors in each category was made using Tukey's multiple comparisons test. Letters indicate statistical differences ($P < 0.05$).

The objective of this study was to determine if proinflammatory interleukins (IL-1β and IL-6) regulate expression of ECM, MMPs, or TIMPs in equine endometrial tissue and whether these effects vary with the severity of equine endometrosis. Knowledge about the regulation of MMPs and TIMPs is essential for understanding the pathogenesis of endometrosis in the mare.

2. Material and methods

2.1. Endometrial tissue collection

Endometrial tissue was collected as described recently by Szóstek-Mioduchowska [13]. Briefly, uteri were obtained *post-mortem* from cyclic mares at a local slaughterhouse (Słomniki, Poland). The procedure for collection of the tissue was approved by the local Animal Care and Use Committee in Olsztyn, Poland (Agreement No. 51/2011). The mares used in this study were declared clinically healthy by the official government veterinary inspection and by individual historical records of animal health. The mares were slaughtered to obtain meat as part of routine slaughterhouse protocols, and endometrial samples were collected within 5 min. of the animal death. For progesterone (P_4) analysis, peripheral blood samples were collected into heparinized tubes immediately before the animal slaughter. The phases of the estrous cycle were defined based on P_4 analysis of blood plasma and macroscopic observation of the ovaries [31]. At the mid-luteal phase of the estrous cycle, a well-developed corpus luteum (CL) was associated with follicles 15–20 mm in diameter and $P_4 > 6$ ng/ml. For histological analysis, a cross-section (1 × 1 cm) of endometrial tissue was fixed overnight in 4% paraformaldehyde and dehydrated in an ethanol gradient [31]. The fixed tissue was processed for hematoxylin-eosin staining. All endometria were classified as category I, IIA, IIB, or III according to the Kenney and Doig classification [2]. Endometria without degenerative alterations were graded as category I, while categories IIA, IIB, and III corresponded to mild, moderate, and severe fibrosis, respectively [2];

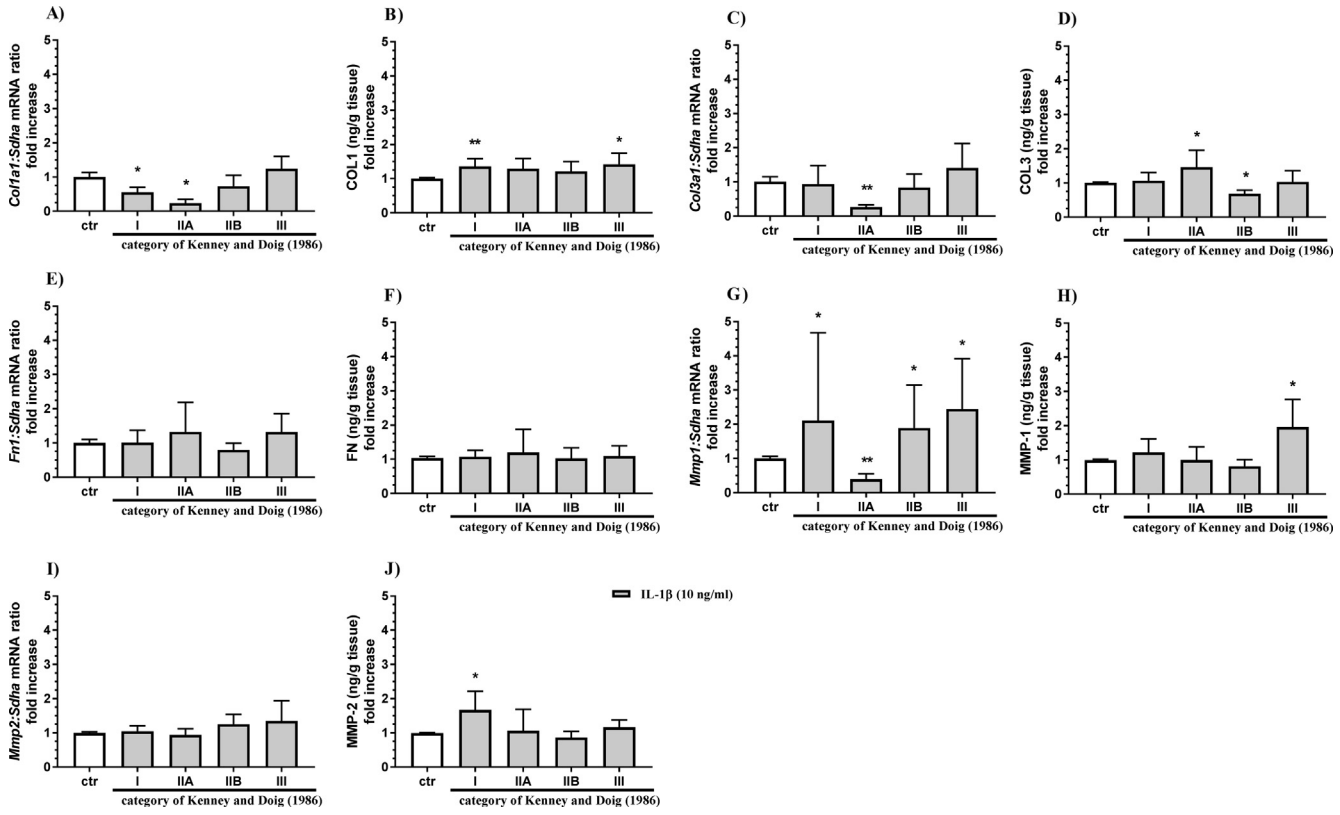

Fig. 3. The effect of IL-1β (10 ng/ml) on mRNA transcription of (A) *Col1a1*, (C) *Col3a1*, (E) *Fn1*, (G) *Mmp1*, (I) *Mmp2* and (B) COL1, (D) COL3, (F) FN, (H) MMP-1, (J) MMP-2 secretion in endometrial explants with or without endometrosis (classified as category I, IIA, IIB, or III according to Kenney and Doig [2]) in the mid-luteal phase of the estrous cycle. All values are expressed as an *n*-fold increase. The effect of IL-1β is reported relative to controls within each category and analyzed by nonparametric Mann-Whitney *U* test ($^{*}P < 0.05$; $^{**}P < 0.01$).

Fig. 1.

2.2. Endometrial tissue preparation

A total of 20 uteri from the mid-luteal phase of the estrous cycle (n = 5 for each category I, IIA, IIB, and III) were used. The tissue explants (~50 mg) for tissue culture were prepared as described previously by Szóstek et al. [6].

2.3. Endometrial tissue treatment

Endometrial tissue explants were washed in phosphate-buffered saline (PBS) followed by pre-incubation in 1 ml Dulbecco's Modified Eagle's medium (DMEM) without phenol red (Sigma–Aldrich; D2960) containing 0.1% bovine serum albumin (BSA) and 1% antibiotic/antimycotic (Sigma-Aldrich; A9909) on a shaker inside a tissue culture incubator at 38.0 °C with 5% CO_2 atmosphere. After 6 h of pre-incubation, the medium was replaced with fresh DMEM supplemented with 0.1% BSA and antibiotic/antimycotic. Subsequently, endometrial tissues were further incubated for 24 h with IL-1β (10 ng/ml) or IL-6 (10 ng/ml). The reactivity of tissues was tested by their incubation with oxytocin (OXT; 10^{-7} M) as a positive control for 24 h and measurement of prostaglandin (PGF)2α using the PGF2α ELISA kit (Enzo Life Sciences; ADI-900-069) (data not shown). All treatments were performed in triplicate for each sample. After incubation, culture media were collected in tubes for ELISA assay, and endometrial tissues were preserved in RNA-later and stored at −80 °C for mRNA extraction. The viability of endometrial explants was determined using Alamar-Blue in accordance with the manufacturer's instructions (Invitrogen; Burlington; Ontario, Canada; #DAL1025). For data normalization, the concentration of all investigated factors was assessed per 1 g of tissue.

2.4. Analytic methods

2.4.1. RNA extraction and cDNA synthesis

Total RNA was extracted using TRI Reagent® (Sigma-Aldrich; T9424) in accordance with the manufacturer's instructions. The concentration and quality of RNA was determined spectrophotometrically and by agarose gel electrophoresis. The ratio of absorbance at 260 and 280 nm (A260/280) was approximately 2. The RNA (1 µg) was reverse transcribed into cDNA using a QuantiTect Rev. Transcription Kit (Qiagen; 205313) in accordance with the manufacturer's instructions. The cDNA was stored at −20 °C until real-time PCR was carried out.

2.4.2. Real-time PCR

Real-time PCR was performed as described by Szóstek-Mioduchowska et al. [13]. The sequences for *succinate dehydrogenase complex, subunit A* (*Sdha*; reference gene) *Col1a1, Col3a1*, and *Fn1* were shown by Szóstek-Mioduchowska et al. [13]. Primer sequences are listed in Table 1. The data were analyzed as described by Zhao and Fernald [32]. The relative concentration of mRNA (R0) for each target and housekeeping gene (*Sdha*) was calculated using the equation $R0 = 1/(1 + E)^{Ct}$, where E is the average gene efficiency and Ct is the cycle number at threshold. The relative gene expression was calculated as $R0_{target\ gene}/R0_{reference\ gene}$ and was expressed in arbitrary units.

2.4.3. ELISA assay

All ELISA kits used in the study are listed in Table 2. In accordance with manufacturer information, the intra- and inter-assay coefficients of variation (CVs) for each ELISA kit were on average 10% and 12%, respectively.

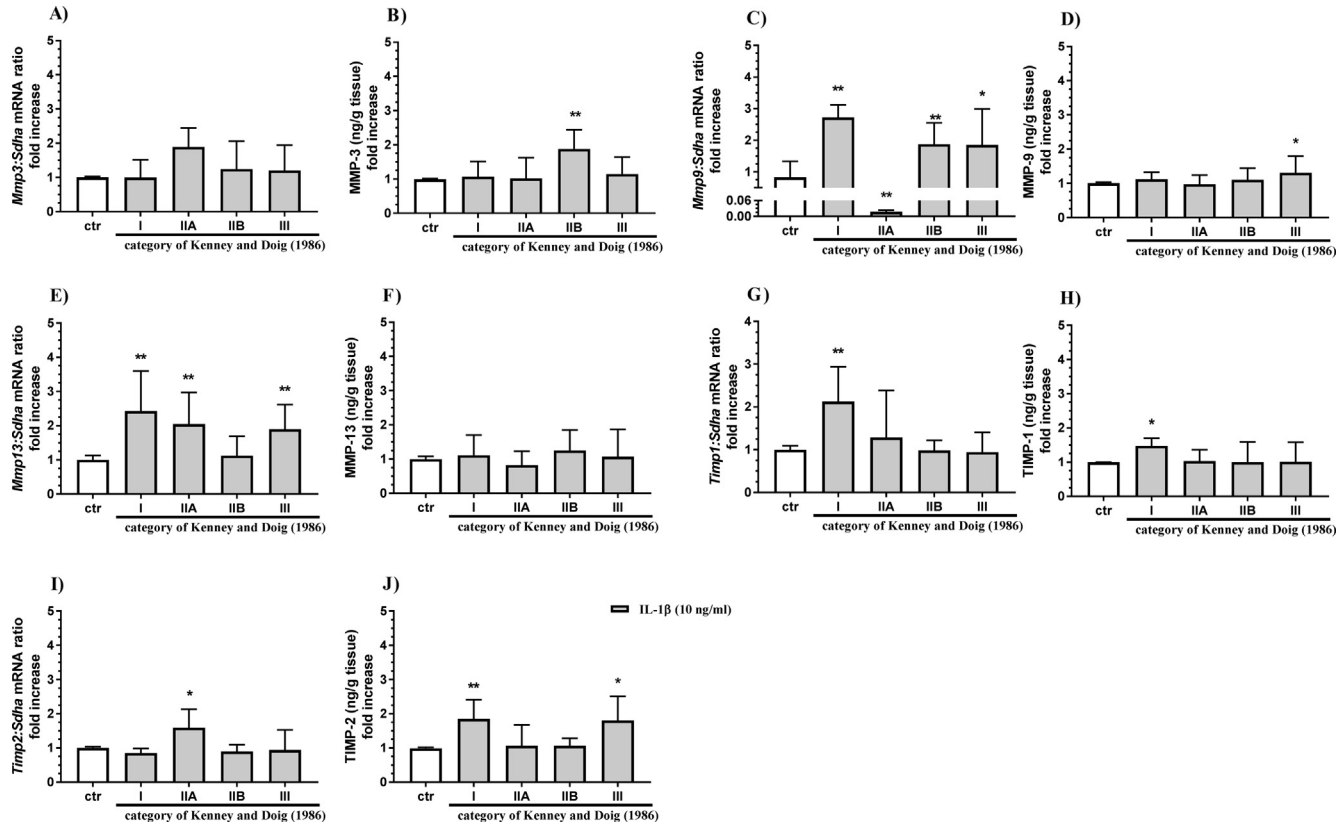

Fig. 4. The effect of IL-1β (10 ng/ml) on mRNA transcription of (A) *Mmp3*, (C) *Mmp9*, (E) *Mmp13*, (G) *Timp1*, (I) *Timp2* and (B) MMP-3, (D) MMP-9, (F) MMP-13, (H) TIMP-1, (J) TIMP-2 secretion by endometrial explants with or without endometrosis (classified as category I, IIA, IIB, or III according to Kenney and Doig [2]) in the mid-luteal phase of the estrous cycle. All values are expressed as an *n*-fold increase. The effect of IL-1β is reported relative to controls within each category and analyzed by nonparametric Mann-Whitney *U* test ($^{*}P < 0.05$; $^{**}P < 0.01$).

2.4.4. Radioimmunoassay

The concentrations of P_4 in mare plasma were measured by radioimmunoassay (RIA) (Diasource; KIP1458). The P_4 standard curve ranged from 0.12 to 36 ng/ml. The intra- and inter-assay CVs were on average 7% and 10%, respectively.

2.5. Statistical analysis

The experiment was performed five times each in three technical replicates. For each analysis, the Gaussian distribution of results was tested using the D'Agostino & Pearson normality test (GraphPad Software version 7; GraphPad, San Diego, CA). Whenever the assumptions of normal distribution were not met, nonparametric statistical analyses were done. The comparison between basal secretion (Mean ± SD) of all factors in each category was analyzed using Tukey's multiple comparisons test. The effect of each IL on genes and proteins of interest in comparison to control group within each category was analyzed by nonparametric Mann-Whitney *U* test. The results were considered significantly different at $P < 0.05$.

3. Results

3.1. The basal secretion of ECM components, MMPs, and TIMPs during of endometrosis

The basal secretion of ECM components, MMPs, and TIMPs by endometrial explants classified as categories I, IIA, IIB, and III endometria is presented in Fig. 2. The basal secretion of COL1 was up-regulated in categories IIA and IIB endometrial explants compared with those from categories I and III ($P < 0.05$; $P < 0.01$; Fig. 2A). The basal secretion of TIMP-2 was up-regulated in category IIA endometrial explants compared with those from categories I, IIB, and III ($P < 0.05$; Fig. 2J).

3.2. Effect of IL-1β on ECM, MMPs, and TIMPs

In category I endometrial explants, IL-1β treatment down-regulated mRNA transcription of *Col1a1* (Fig. 3A; $P < 0.05$) and up-regulated mRNA transcription of *Mmp1* (Fig. 3G; $P < 0.05$), *Mmp9* (Fig. 4C; $P < 0.01$), *Mmp13* (Fig. 4E; $P < 0.01$) and *Timp1* (Fig. 4G; $P < 0.01$) relative to the control group. Additionally, in category I endometrium explants, IL-1β treatment increased secretion of COL1 (Fig. 3B; $P < 0.01$), MMP-2 (Fig. 3J; $P < 0.05$), TIMP-1 (Fig. 4H; $P < 0.05$) and TIMP-2 (Fig. 4J; $P < 0.05$) compared with the control group.

In category IIA endometrium explants, IL-1β treatment down-regulated mRNA transcription of *Col1a1* (Fig. 3A; $P < 0.05$), *Col3a1* (Fig. 3C; $P < 0.001$), *Mmp1* (Fig. 3G; $P < 0.01$) and *Mmp9* (Fig. 4C; $P < 0.01$) and up-regulated mRNA transcription of *Mmp13* (Fig. 4E; $P < 0.01$) and *Timp2* (Fig. 4I; $P < 0.05$). In the same category, IL-1β treatment increased secretion of COL3 (Fig. 3D; $P < 0.05$).

In category IIB endometrium explants, IL-1β treatment up-regulated mRNA transcription of *Mmp1* (Fig. 3G; $P < 0.05$) and *Mmp9* (Fig. 4C; $P < 0.01$). In this group, IL-1β treatment also decreased secretion of COL3 (Fig. 3D; $P < 0.05$) and increased secretion of MMP-3 (Fig. 4B; $P < 0.01$).

In category III endometrium, IL-1β treatment up-regulated mRNA transcription of *Mmp1* (Fig. 3G; $P < 0.05$), *Mmp9* (Fig. 4C; $P < 0.05$), and *Mmp13* (Fig. 4E; $P < 0.01$). Additionally, IL-1β treatment of category III endometrium explants increased secretion of COL1 (Fig. 3B; $P < 0.05$), MMP-1 (Fig. 3H; $P < 0.05$), MMP-9 (Fig. 4D; $P < 0.05$), and TIMP-2 (Fig. 4J; $P < 0.05$).

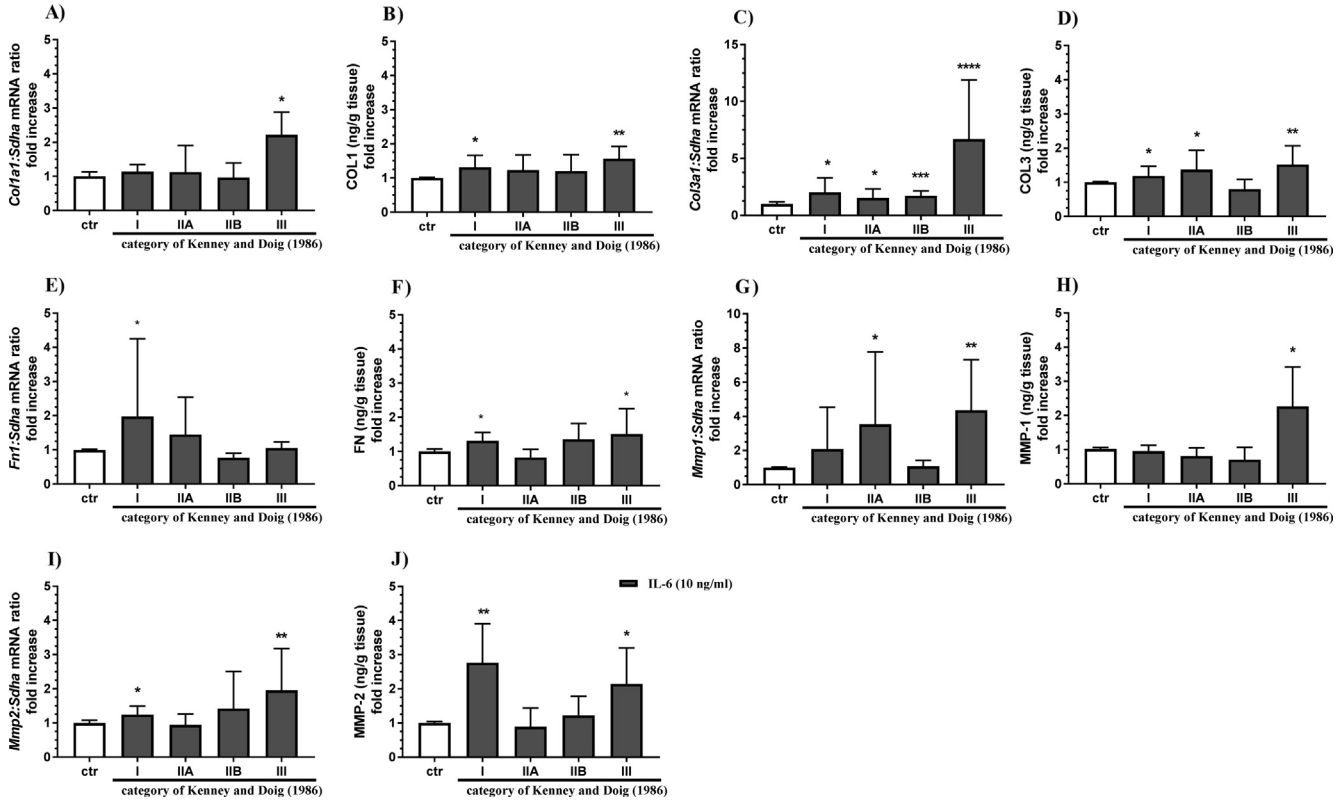

Fig. 5. The effect of IL-6 (10 ng/ml) on mRNA transcription of (A) *Col1a1*, (C) *Col3a1*, (E) *Fn1*, (G) *Mmp1*, (I) *Mmp2* and (B) COL1, (D) COL3, (F) FN, (H) MMP-1, (J) MMP-2 secretion by endometrial explants with or without endometrosis (classified as category I, IIA, IIB, or III according to Kenney and Doig [2]) in the mid-luteal phase of the estrous cycle. All values are expressed as a *n*-fold increase. The effect of IL-6 is reported relative to controls within each category and analyzed by nonparametric Mann-Whitney *U* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$).

3.3. Effect of IL-6 on ECM, MMPs, and TIMPs

In category I endometrium explants, IL-6 treatment down-regulated mRNA transcription of *Mmp3* (Fig. 6A; $P < 0.05$) and *Timp2* (Fig. 6I; $P < 0.01$) and up-regulated mRNA transcription of *Col3a1* (Fig. 5C; $P < 0.05$), *Mmp2* (Fig. 5I; $P < 0.05$), *Mmp9* (Fig. 6C; $P < 0.01$), and *Timp1* (Fig. 6G; $P < 0.01$). In these same explants, IL-6 treatment decreased secretion of MMP-9 (Fig. 6D; $P < 0.05$) and increased secretion of COL1 (Fig. 5B; $P < 0.05$), COL3 (Fig. 5D; $P < 0.05$), FN (Fig. 5F; $P < 0.05$), MMP-2 (Fig. 5J; $P < 0.01$) and MMP-3 (Fig. 6B; $P < 0.01$).

In category IIA endometrium explants, IL-6 treatment down-regulated mRNA transcription of *Mmp9* (Fig. 6C; $P < 0.01$) and *Timp1* (Fig. 6G; $P < 0.01$) and up-regulated mRNA transcription of *Col3a1* (Fig. 5C; $P < 0.05$), *Mmp1* (Fig. 5G; $P < 0.05$), *Mmp3* (Fig. 6A; $P < 0.05$), *Mmp13* (Fig. 6E; $P < 0.01$), and *Timp2* (Fig. 6I; $P < 0.05$). Additionally, IL-6 treatment of category IIA explants increased secretion of COL3 (Fig. 5D; $P < 0.05$) and decreased secretion of MMP-9 (Fig. 6D; $P < 0.05$).

In category IIB endometrium explants, IL-6 treatment down-regulated mRNA transcription of *Mmp3* (Fig. 6A; $P < 0.05$), *Mmp13* (Fig. 6E; $P < 0.001$), *Timp1* (Fig. 6G; $P < 0.01$), and *Timp2* (Fig. 6I; $P < 0.001$) and up-regulated mRNA transcription of *Col3a1* (Fig. 5C; $P < 0.001$) and *Mmp9* (Fig. 6C; $P < 0.01$). Additionally, IL-6 treatment of category IIB explants increased secretion of MMP-3 (Fig. 6B; $P < 0.05$), MMP-9 (Fig. 6D; $P < 0.05$), and MMP-13 (Fig. 6F; $P < 0.05$).

In category III endometrium, IL-6 treatment down-regulated of mRNA transcription of *Mmp13* (Fig. 6E; $P < 0.01$) and up-regulated mRNA transcription of *Col1a1* (Fig. 5A; $P < 0.05$), *Col3a1* (Fig. 5C; $P < 0.0001$), *Mmp1* (Fig. 5G; $P < 0.01$), *Mmp2* (Fig. 5I; $P < 0.01$), and *Timp1* (Fig. 6G; $P < 0.05$). Additionally, IL-6 treatment of category III explants increased secretion of COL1 (Fig. 5B; $P < 0.01$), COL3 (Fig. 5D; $P < 0.01$), FN (Fig. 5F; $P < 0.05$), MMP-1 (Fig. 5H; $P < 0.05$), MMP-2 (Fig. 5J; $P < 0.05$), MMP-3 (Fig. 6B; $P < 0.05$), MMP-9 (Fig. 6D; $P < 0.05$), MMP-13 (Fig. 6F; $P < 0.05$), TIMP1 (Fig. 6H; $P < 0.05$) and TIMP-2 (Fig. 6J; $P < 0.001$).

Simplified results are presented in Supplementary data (Tab. 1).

4. Discussion

The etiology of equine endometrosis is not known, but one hypothesis suggests it could occur as a consequence of repeated chronic inflammation due to insults associated with breeding, foaling, and veterinary intervention [1]. This hypothesis has recently been the subject of extensive research, including this study [6,11–13,33]. Inflammation seems to be associated with fibrosis through paracrine signaling via secretion of profibrotic chemokines, cytokines, and other factors from injured cells and inflammatory cells reviewed by [14]. Inflammatory mediators act on resident cells, such as fibroblasts, and affect fibrogenesis and ECM remodeling reviewed by [14]. Here, we demonstrated that IL-1β and IL-6 directly modulate ECM, MMP, and TIMP expression in equine endometria. Thus, these cytokines could affect ECM remodeling, and be important regulators of the mechanisms involved in the pathogenesis of fibrosis. Our findings imply that there is a link between inflammation and development of endometrosis in the mare. Other studies showed that mRNA transcription of *IL-1β* and *IL-6* is higher endometrial biopsies from mares susceptible to persistent post-breeding endometritis (PPBE) than in those from mares resistant to PPBE [34] and is up-regulated during equine endometrosis [6]. Additionally, IL-6 concentration, as well as mRNA transcription of *IL-1β* and *IL-6*, increased in incubated endometrial biopsies of mares suffering from subclinical endometritis compared with healthy mares [35]. Furthermore, it was demonstrated that the equine endometrium could

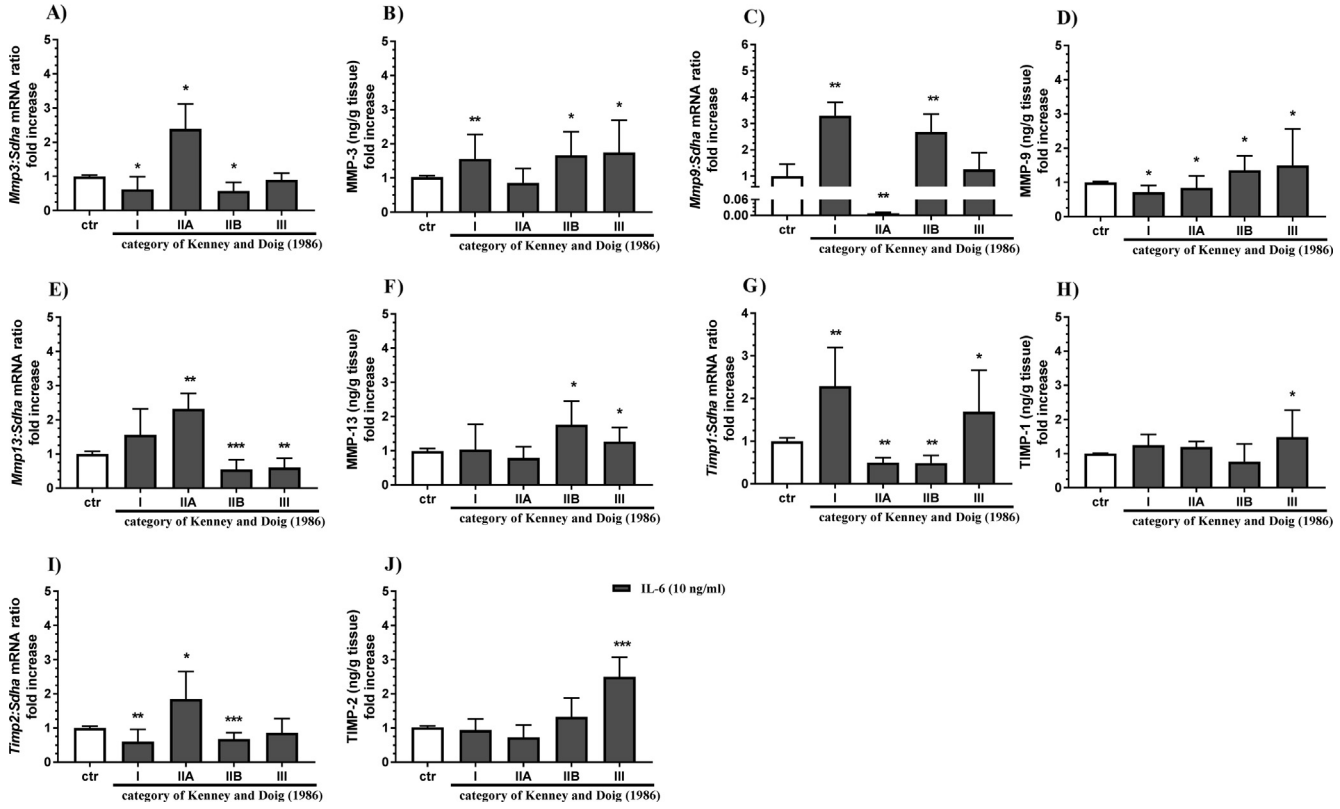

Fig. 6. The effect of IL-6 (10 ng/ml) on mRNA transcription of (A) *Mmp3*, (C) *Mmp9*, (E) *Mmp13*, (G) *Timp1*, (I) *Timp2* and (B) MMP-3, (D) MMP-9, (F) MMP-13, (H) TIMP-1, (J) TIMP-2 secretion by endometrial explants with or without endometrosis (classified as category I, IIA, IIB, or III according to Kenney and Doig [2]) in the mid-luteal phase of the estrous cycle. All values are expressed as an *n*-fold increase. The effect of IL-6 is reported relative to controls within each category analyzed by nonparametric Mann-Whitney *U* test ($^{*}P < 0.05$; $^{**}P < 0.01$).

remodel itself in response to inflammation, involving the expression, secretion and activation of MMPs [36]. We demonstrated that IL-6 treatment increased ECM secretion, and IL-1β treatment increased COL1 secretion in equine endometrial explants without degenerative changes and those with severe degenerative changes. The effect of IL-1β treatment on COL3 was also dependent on endometrosis stage. The effects of ILs on COLs and FN were confirmed previously in other tissue types [37–41]. It is interesting that the basal secretion of COL1 in equine endometrosis increased in category IIA and IIB endometria compared with healthy tissues. A similar phenomenon exists in other fibrotic tissues, where cytokines act as amplifiers of mechanisms leading to a profibrotic cascade. Fibroblasts derived from diseased pulp synthesized greater amounts of collagen than fibroblasts from healthy pulp [39].

In our study, IL-1β treatment increased secretion of MMP-2 and TIMPs, whereas IL-6 treatment increased secretion of MMP-2 and MMP-3 secretion and decreased secretion of MMP-9 by equine endometrial explants without degenerative changes (category I). However, the effects of IL-1β and IL-6 treatments on endometrial MMP and TIMP secretion were different depending on the severity of endometrosis, suggesting that endometrial degeneration disturbs proper endometrial function. Modulation of MMPs and their inhibitors by IL-1β and IL-6 treatment has been shown in endometrial human and bovine fibroblasts and seems to be tissue-specific [9–10,42–43] The changes in ECM, MMP, and TIMP expression in response to IL-1β and IL-6 treatment during endometrosis suggest that endometrial cells are altered by some intracellular mechanisms that are associated with the cells' ability to respond in the presence of effector cytokines. Additionally, the deregulations of ECM, MMP, and TIMP expression in response to IL-1β and IL-6 treatment during equine endometrosis reflect complex changes that occur to maintain homeostasis of ECM. Matrix metallopeptidases were considered to be primarily responsible for turnover and degradation of ECM substrates that should prevent excessive deposition of ECM, and thus, fibrosis. But taking into consideration our results, increasing expression of MMPs can be a cellular response to excessive ECM production and disruption in the normal regulation of endometrial MMP expression. Additionally, MMPs were shown to have other cellular activities, such as effects on proliferation and survival, gene expression, and multiple aspects of inflammation that affect outcomes related to fibrosis [44]. The level of MMP in response to IL-6 treatment during endometrosis, in category III endometria suggest that MMPs contribute to modifications in the endometrial microenvironment, thus enhancing fibrosis. The increased expression of MMPs can be related to transforming growth factor (TGF)-β1 and other MMP activators [45–49], myofibroblast differentiation [50], and cell proliferation [47,51]. However, further studies are needed to explain this phenomenon.

To summarize, the exposure of endometrial tissue to IL causes increased ECM components, as well as MMP and TIMP secretion. Endometrial degeneration disturbs proper endometrial function, and the effects of cytokines on equine endometrium differ depending on the severity of endometrosis. Our findings indicate an association between inflammation and the development of endometrosis, through which IL-1β and IL-6 affect expression of ECM components, MMPs, and TIMPs in the mare.

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Author Contributions

ASz-M – developed the study concept, carried out experiments; analyzed and interpreted the results, and wrote the manuscript; DJS, KO – contributed to the study concept, planned the experiments, interpreted the results, and revised the manuscript; AB carried out and analyzed part of the ELISA measurements and real-time PCR.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.154767.

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