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Abstract

Interleukin-8 (IL-8) and 10 (IL-10) mRNA transcriptions in the endometrium of normal mares and mares susceptible to persistent post-breeding endometritis☆

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1. Introduction

Breeding normal mares results in endometritis with a transient neutrophilic inflammation that typically is resolved within 48 h post-breeding (Watson, 2000). In some mares, referred to as susceptible mares, a persistent post-breeding endometritis (PPBE) develops resulting in a persistent accumulation of inflammatory fluid within the uterus. PPBE reduces fertility resulting in serious economic losses. In a previous study, we have documented a greater induction of pro-inflammatory cytokines interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF-α) in susceptible compared to normal mares during the post-breeding period. We have also demonstrated that the administration of an immunomodulator to susceptible mares prior to artificial insemination (AI) results in a significant decrease in IL-1β mRNA expression (Fumuso et al., 2003). Interleukin-8, a chemokine, plays a central role in chemotaxis acting as a pro-inflammatory mediator. In contrast, IL-10 has a pronounced anti-inflammatory effect by inhibiting pro-inflammatory cytokine production. The

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balance between these pro- and anti-inflammatory cytokines is likely to play a role in PPBE. The aim of this study was to measure and compare IL-8 and IL-10 expression in the endometrium of susceptible and normal mares. The observations were conducted in untreated mares, after artificial insemination (AI), and after AI combined with immunomodulation.

2. Materials and methods

Sixteen mares (eight normal and eight susceptible), were selected on the basis of their reproductive records and capacity to clear an intrauterine bacterial infusion. Three experiments were performed over three consecutives estrous cycles: (1) after no AI or treatment, (2) after artificial insemination, and (3) after artificial insemination and immunomodulation. Endometrial biopsies were taken: in experiment 1 during estrus, with follicles $\geq$35 mm, and in diestrus (7 ± 1 days after ovulation); in experiment 2 during estrus (24 h post-AI with dead spermatozoa) and in diestrus and in experiment 3 during estrus (24 h after AI and a simultaneous intravenous treatment of 1.5 mg of *Mycobacterium phlei* cell wall extract (MCWE; Settle^{TM}, Bioniche AH, USA Inc.) and during diestrus. Biopsy samples were kept frozen in liquid nitrogen until processed for the cytokine assays. Biopsy samples were thawed at room temperature in a dry incubator for 10 min, and then 50 mg of tissue was placed into 1 ml of stabilization reagent to be disrupted and homogenized. RNA extraction was performed using the RNeasy Mini Kit (Qiagen). RNA samples were treated with DNAse 1 (Clontech, CA) to remove any trace of genomic DNA contamination. Real-time PCR was performed using gene-specific primers and internal oligonucleotide probes, selected from equine cDNA sequences for IL-8, IL-10 and glyceraldehyde-3-phosphate dehydrogenase using the ABI Prism 7700 Sequence Detection System (PE Biosystems). Relative quantification between IL-8 and IL-10 samples was achieved by the comparative threshold cycle method. Results are reported as the *n*-fold difference relative to cytokine mRNA expression in the sample with the lowest expression, which was arbitrarily given the value of 1.0. Analysis of variance using PROC GLM with SASV8 for double split plot experimental design was used to compare normal versus susceptible mares. Because the data were not normally distributed, an exponential transformation was applied using medians and quartile ranges rather than means and standard deviation.

3. Results

Results are summarized in Table 1. In experiment 1 (untreated mares), susceptible mares in estrus had greater IL-8 and lower IL-10 concentrations than did normal mares. In diestrus, susceptible mares had greater concentrations of IL-8 and IL-10 compared to their normal counterparts. In experiment 2, susceptible mares had greater concentrations of IL-8 and lower levels of IL-10 compared to normal mares. During diestrus in the same estrous cycle, susceptible mares had greater concentrations of both cytokines. In experiment 3, during estrus 24 h after the MCWE treatment and AI, susceptible mares had greater concentrations of IL-10 compared to normal mares and no difference was detected for IL-8. In diestrus (third cycle), no significant differences were detected for either IL-8 or IL-10 between susceptible and normal mares.

4. Discussion

The present study demonstrates for the first time IL-8, a chemokine playing a key role in neutrophil chemotaxis, and IL-10, an important anti-inflammatory cytokine, in the mare's endometrium. Results from the present study also demonstrated different profiles for the transcrip-

Table 1
IL-8 and IL-10 transcription in endometrial tissue of normal and susceptible mares

Exp.	Stage of cycle	IL-8			IL-10		
		Normal mares	Susceptible mares	*P*-value	Normal mares	Susceptible mares	*P*-value
1	Estrus	69.3 (11.4–145.8)	20101 (18197–36707)	0.05	1667 (1233–3420)	5.2 (3.4–102.1)	0.0001
	Diestrus	10.1 (6.4–30.9)	82.2 (44.8–1464)	0.0001	2.7 (1.9–6.3)	1163 (967–1535)	0.0001
2	Estrus 24 h post-AI	168.8 (114–1905)	14859 (6797–16437)	0.0009	22.5 (11.8–456)	4.5 (3.2–6.6)	0.01
	Diestrus	4.1 (4.1–6.8)	832.2 (392–8075)	0.0001	2.1 (1.1–2.4)	20 (5.5–214.4)	0.02
3	Estrus 24 h post-AI and immunomodulation	173.8 (24.4–10512)	23460 (650–46718)	0.28	6.5 (3.7–12.1)	1941 (607–7859)	0.005
	Diestrus	16.9 (10.3–25.4)	13.8 (4.0–31.5)	0.23	5.5 (1.7–8.3)	4.7 (2.1–7.0)	0.92

The values represent median (Q1 − Q3) for relative mRNA within each group. Q, quartile; Exp., experiment.

tion of pro-inflammatory IL-8 and anti-inflammatory IL-10 cytokines for normal and susceptible mares in the untreated estrous cycle. Susceptible mares appear to have a greater amount of pro-inflammatory IL-8 cytokine during estrus and diestrus, which may explain their susceptibility. The intense endometritis seen in the mares subjected to AI with frozen semen was presumably mainly due to intact or damaged spermatozoa which can produce a similar inflammatory reaction (Kotilainen et al., 1994). The increase in IL-8 induced by AI with frozen-dead spermatozoa in the second cycle decreased later in diestrus in normal mares but not in susceptible mares. MCWE treatment at the time of AI in the third cycle substantially reduced this difference between normal and susceptible mares. This reduction in IL-8 in diestrus in the susceptible mares may have been induced by the increase of the anti-inflammatory IL-10 that they exhibited during estrus in the third (treated) cycle. IL-10 has a pronounced anti-inflammatory impact on neutrophils, inhibiting the secretion of IL-8 mRNA and protein (Kasama et al., 1994). This increased IL-10 transcription in susceptible mares may have been due to compound(s) in the *Mycobacterium* cell wall (TDM, trehalose dimycolate; MA, mycolic acid) that bind to surface receptors of macrophages and dendritic cells resulting in cell activation, and subsequent cytokine induction (Means et al., 2001). In vitro cultures of macrophages, upon secondary exposure to inflammatory triggers and treated with MA and TDM, showed an increased secretion of IL-10 (Korf et al., 2005).

Summarizing, the different amounts of endometrial IL-8 and IL-10 observed between normal and susceptible mares in the untreated cycle were maintained after AI and surprisingly disappeared after the treatment with MCWE in the diestrus of the third cycle. It appears that treating susceptible mares with MCWE can help them restore their homeostatic uterine immune mechanisms and thereby resist post-breeding endometritis. The significance of this should be further investigated in terms of pregnancy rates.

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