Available online at www.sciencedirect.com

SCIENCE DIRECT®

Veterinary Immunology and Immunopathology 96 (2003) 31–41

Veterinary immunology and immunopathology

www.elsevier.com/locate/vetimm

Endometrial IL-1β, IL-6 and TNF-α, mRNA expression in mares resistant or susceptible to post-breeding endometritis Effects of estrous cycle, artificial insemination and immunomodulation

Elida Fumuso[a], Steeve Giguère[d], José Wade[e], Dragan Rogan[e], Ignacio Videla-Dorna[b], Raúl A. Bowden[c,*]

[a]Departamento de Clínica, Facultad de Ciencias Veterinarias, UNICEN, Pinto 399, 7000 Tandil, Argentina

[b]Departamento de Fisiopatología, Facultad de Ciencias Veterinarias, UNICEN, Pinto 399, 7000 Tandil, Argentina

[c]Departamento de Sanidad,Animal y Medicina Preventiva, Laboratorio de Inmunoquímica y Biotecnología, Facultad de Ciencias Veterinarias, UNICEN, Pinto 399, 7000 Tandil, Argentina

[d]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610, USA

[e]Bioniche AH, Clonee Co., Meath, Ireland

Received 8 January 2003; received in revised form 2 May 2003; accepted 9 June 2003

Abstract

Endometrial mRNA expression of the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) was assessed in mares resistant (RM) or susceptible (SM) to persistent post-breeding endometritis (PPBE). Eight RM and eight SM, were selected based on reproductive records and functional tests out of a herd of 2000 light cross-type mares. Three experiments were done to study transcription patterns in (i) basal conditions; (ii) after artificial insemination (AI); and (iii) after administration of an immunomodulator at time of artificial insemination. Endometrial biopsies were taken during consecutive cycles: (i) at estrus, when follicles reached 35 mm and at diestrus (7 ± 1 days after ovulation); (ii) at 24 h post-AI, with dead semen (estrus) and in diestrus; (iii) at 24 h after treatment with a *Mycobacterium phlei* cell-wall extract (MCWE) preparation and AI (with dead semen), and at diestrus. mRNA expression was quantitated by real time PCR. Under basal conditions, SM had significantly higher mRNA expression of all cytokines in estrus and of IL-1β and TNF-α in diestrus, compared to RM. After AI, there were no differences between RM and SM in estrus; however, mRNA expression for all three pro-inflammatory cytokines was higher than under basal conditions. In diestrus, RM showed significantly lower IL-1β and TNF-α mRNA expression than SM. When MCWE was administered at time of AI, no differences between cytokine induction from RM and SM were found. Globally, mRNA expression for all three cytokines correlated well among themselves when expression was high. The present study showed that (i) in basal conditions RM had lower mRNA expression of pro-inflammatory cytokines than SM with no effect of estrous cycle; (ii) AI upregulated mRNA expression for all three cytokines in both RM and SM, with persistance in diestrus in the latter; (iii) treatment with MCWE at time of AI down-regulated mRNA expression of IL-1 with significant effects in SM which behaved like RM. Immunomodulation with MCWE

* Corresponding author. Tel.: +54-2293-42-66-67; fax: +54-2293-42-66-67.
E-mail address: rbowden@vet.unicen.edu.ar (R.A. Bowden).

0165-2427/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0165-2427(03)00137-5

could be of help in restoring homeostatic local inflammatory mechanisms, thus assisting in the prophylaxis of post-breeding endometritis in mares.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Endometritis; PCR; Immunomodulation; Mares; Reproduction; Breeding

1. Introduction

Endometritis is a major cause of infertility in mares and thus has a serious economic impact on the horse breeding industry (Troedsson, 1997; Watson, 2000). Equine endometritis has various possible outcomes: (a) acute endometritis appearing after breeding or associated with venereal infection; (b) chronic infectious endometritis; (c) persistent post-breeding endometritis (PPBE); and (d) endometriosis (chronic degenerative endometritis) (Troedsson, 1997; Watson, 2000). While acute uterine infections recover either spontaneously or with appropriate local antimicrobial treatments, PPBE results in accumulation of inflammatory fluid within the uterine lumen and is difficult to treat (Troedsson, 1995). The reasons for why mares behave either as ‘‘susceptible’’ (SM) or ‘‘resistant’’ (RM) to PPBE are unknown and thus a matter of active research.

After natural mating or artificial insemination (AI), there is a physiological transient endometritis (Watson, 2000) triggered both by spermatozoa (Kotilainen et al., 1994) and by contaminant microorganisms in semen (reviewed by Troedsson et al., 2001). This transient inflammatory response should be cleared within 5 days (i.e. the time spent by the ovocytes in the Fallopian tubes before reaching the uterus) to provide a safe milieu for embryo implantation. SM characteristically fail in doing so, thus developing PPBE. In addition, inflammation by-products have been shown to be harmful for spermatozoa and affect their motility (Alghamdi et al., 2001). Therefore, mare’s fertility critically depends on the time taken to resolve this inflammation/infection (Katila, 1995; Watson, 2000; Troedsson, 1997).

Given the relevance of innate defense mechanisms in the development and persistence of endometritis, the effects of immunomodulators on the prevention and treatment of this condition have been evaluated. In a preliminary study, a *Mycobacterium phlei* cell-wall extract (MCWE) was administered, together with AI, to both RM and SM, and compared with AI alone as a control in a previous cycle; at 24 h post-AI alone both untreated RM and SM exhibited a massive polymorphonuclear infiltration of endometrium, which significantly decreased by diestrus only in RM. However, when SM mares received MCWE, there was a significant decrease in neutrophil numbers to similar values of RM (Fumuso et al., 2000). As PMNs are hallmarks of acute inflammation, this could indicate a beneficial effect of that treatment. However, the mechanisms controlling the reduction in PMN infiltration have not been investigated.

Cytokines are intercellular signaling proteins released by both immune and non-immune cells. Interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α), known as pro-inflammatory cytokines (PIC), modulate the acute phase response that involves potent systemic and local effects. In humans, reproductive processes including ovulation, implantation, cervical ripening and dilation at term are inflammatory in nature, and have been connected with the activity of these cytokines. Pro-inflammatory cytokines are produced in the uterus by leukocytes and endometrial cells (Barañao, 1997; Wade, 1995). Macrophages from mouse and human endometrium express mRNA for IL-1 during the late secretory phase of the menstrual cycle (Takacs et al., 1988; Kauma et al., 1990). IL-1β and IL-1 type 1 receptor have been both detected in human glandular and stromal cells (Simon et al., 1994) and TNF-α mRNA and its protein have also been detected in endometrium (Hunt et al., 1992). IL-1 and TNF-α act on endometrial stromal cell to stimulate the synthesis of IL-6 in vitro (Semer et al., 1991). Although these cytokines have been studied in various conditions affecting human and animal reproduction (Cadario et al., 2002), there are no reports relating them to susceptibility or resistance to endometritis in mares.

The objectives of the present work were to compare IL-1β, IL-6 and TNF-α mRNA expression in

endometrial biopsy samples obtained from both SM and RM and to evaluate the effects of estrous cycle, artificial insemination and the use of an immunomodulator on the expression of these pro-inflammatory cytokines.

2. Materials and methods

2.1. Selection of mares entering this study

Animals were selected from a herd of 2000 light Criollo cross-type mares aged between 6 and 16 years. From these, 161 non-pregnant mares were pre-selected considering good body and vulvar scores (Losinno et al., 1997), and their reproductive records of the two previous breeding seasons. Mares with normal uterine cytology, negative results on culture for bacteria and fungi, and a grade 1 or 2A uterine biopsy according to a previously established scoring system (Kenney and Doig, 1986) were tentatively classified as RM. In brief, the score is I: no pathologic changes; IIA: slight to moderate inflammatory, fibrotic, lymphatic or atrophic changes; IIB: widespread inflammatory and fibrotic changes, more intense than in IIA; III: widespread diffuse severe inflammatory changes, widespread fibrosis of glands, extensive lymphatic lacunae (Fig. 1).

Mares were evaluated for ultrasonographic evidence of intra-uterine fluid, endometrial cytology and culture results 72 h after intra-uterine inoculation with 10^6 CFU of *Streptococcus zooepidemicus*. Finally, the ability of the mares to become pregnant after two cycles of breeding with fertile stallions was assessed. Two groups of eight resistant and susceptible mares each were constituted. A summary of the pertinent traits exhibited by the mares that entered the study, is given in Table 1. The mares were maintained on pasture throughout the experimental period, which spanned from October to December (spring season, southern hemisphere).

2.1.1. *Streptococcus zooepidemicus* inoculation

The outcome of the infection by infusion of bacteria (*S. zooepidemicus* or *Pseudomonas* spp.) has been used as a criterium to classify mares as either susceptible or resistant to persistent or chronic endometritis (Troedsson, 1997). The mares were bacteriologically examined and found negative before this test thus excluding chronic endometritis. Inoculation was performed as described by Cadario et al. (1999) with minor modifications. A strain of *S. zooepidemicus* originally isolated from a mare with endometritis was grown overnight in Brain Heart Infusion Broth (Merck, Argentina) at 37 °C, washed twice in PBS, brought to 1×10^9 ml^{-1} and stored at −70 °C. Prior to inoculation, the culture was thawed and diluted in PBS to obtain a final concentration 5×10^6 CFU ml^{-1}. Fifty milliliter of this suspension was administered into the uterus through a plastic pipette when mares were in estrus. Clearance of *S. zooepidemicus* was evaluated at 72 h post-inoculation by (i) ultrasonographic detection of fluid within uterus; (ii) endometrial culture for bacteria; and (iii) endometrial cytology. The results of these tests are given in Table 1.

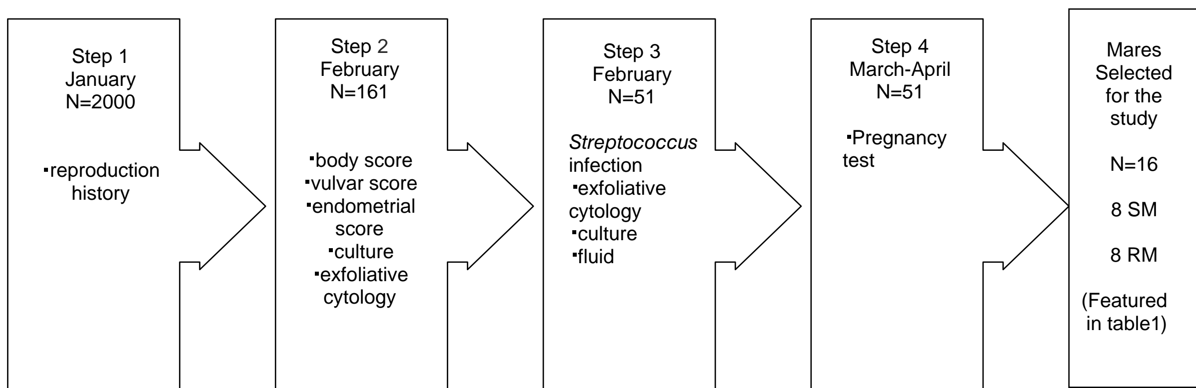

Fig. 1. Flow chart depicting the steps and tests used for selecting the mares entering this study.

Table 1
Summary of selected individual traits of the mares entering the trial

Mare's number	Age (years)	Reproductive history[a]	Body score[b]	Vulvar score[b]	Endometrial score[c]	Exfoliative cytology[d]	Culture result[e]	Uterine fluid[f]	Pregnancy test[g]
Resistant mares									
60	4	Good	4	4	2A	−	−	0	+
345	9	Bad	4	3	2A	−	−	1	+
421	11	Good	4	3	2A	−	−	1	+
423	9	Good	4	3	1	−	−	1	+
773	8	Good	4	3	2A	−	−	0	+
782	10	Good	4	4	2A	−	−	0	+
801	9	Good	4	3	2A	−	−	1	+
1417	4	Good	3	3	2A	−	−	0	+
Susceptible mares									
452	10	Bad	4	4	2B	+	+	1	−
562	12	Bad	3	3	3	−	+	1	−
928	11	Bad	4	3	2A	−	+	1	−
1006	5	Bad	3	3	2B	+	+	1	−
1015	11	Bad	4	3	2A	+	+	2	−
1043	5	Bad	3	3	2A	+	+	4	−
1114	8	Bad	4	3	2A	+	+	1	−
1440	11	Bad	3	3	2B	+	−	2	−

The results marked as (d, e, f) were obtained after experimental inoculation test with *Streptococcus zooepidemicus*.

[a] Reproductive history refers to the condition found in mares during the 2 years before entering the trial, i.e. "good" if pregnancy or "without endometritis" status had been observed, or "bad" if not.

[b] Body and vulvar scores according to Losinno et al. (1997).

[c] Endometrial score is indicated according to the scale of Kenney and Doig (1986). I: no pathologic changes; IIA: slight to moderate inflammatory, fibrotic, lymphatic or atrophic changes; IIB: widespread inflammatory and fibrotic changes more intense than in IIA; III: widespread diffuse severe inflammatory changes, widespread fibrosis of glands, extensive lymphatic lacunae.

[d] Exfoliative cytology: (+) and (−) indicates >12 or <12 polymorphonuclear neutrophil leukocytes per microscopic field at 400×, respectively.

[e] Culture results were given as (−) when no bacteria/yeast grew after culturing the endometrial swab content.

[f] Presence of fluid was ultrasonographycally registered and scored as described by McKinnon et al. (1988).

[g] Pregnancy column test result before entering the trial after two cycles with fertile stallions: (+) means pregnant and (−) non-pregnant.

2.1.2. Bacteriology

Uterine cervical samples were taken with guarded Culturvet swabs (Culturvet, AR) and placed in Amies's transport medium (Oxoid, London, UK). Bacterial pathogens were identified using conventional microbiology procedures.

2.1.3. Endometrial exfoliative cytology

Samples were taken with guarded Culturvet swabs, smeared on glass slides, dried at room temperature, and stained with Giemsa stain (15″ Biopur, Argentina). PMN were counted in 20 fields (400×) and the mean number of PMN per field was calculated. Mares with <12 or >12 mean PMN counts were considered as showing or not showing inflammation, respectively. This corresponded to 1–2 or 3–4°, respectively, according to Vásquez-López et al. (2000).

2.1.4. Pregnancy test

Mares were bred by natural service, spending two cycles with the stallions. Pregnancy was assessed by transrectal ultrasonography 15 days after breeding.

2.2. Experimental design

Three experiments spanning three consecutive cycles were done, in which the same groups of S and R mares were studied. Methods for biopsy collection, conservation of samples and data analysis (described below) were the same in all three experiments.

2.2.1. Experiment 1: baseline IL-1β, IL-6 and TNF-a mRNA expression in SM and RM, and effect of estrous cycle

Endometrial biopsy samples were taken from mares during estrus, when transrectal palpation and ultrasonography showed a relaxed cervix, a low uterine tonus, the presence of endometrial edema and a dominant ovarian follicle (≥35 mm). The day of ovulation was monitored and recorded, and the second sample (diestrus) was taken at 7 ± 1 days post-ovulation. Uterine biopsy samples were immediately frozen and stored in liquid nitrogen until used for total RNA extraction.

2.2.2. Experiment 2: effects of AI

Dead semen was used to study the inflammatory effect of semen deposition in the uterus while avoiding the otherwise superimposed effects of conception. Semen was collected from four healthy fertile stallions using the Missouri artificial vagina. Semen samples were pooled and diluted in Kenney's extender without antibiotics. Dose was adjusted to 5×10^8 spermatozoa with progressive motility. Spermatozoa were killed by two rounds of freezing and thawing, and then stored at −20 °C. Previous studies have shown that insemination with frozen semen provokes intense endometritis due mainly to spermatozoa (Kotilainen et al., 1994). Intact or damaged spermatozoa have been shown to have similar inflammatory-inducing capacity, whereas semen extender is innocuous (Kotilainen et al., 1994).

The mares were artificially inseminated once in estrus (see experiment 1). Uterine biopsy samples were taken 24 ± 8 h after insemination. The day of ovulation was monitored and recorded. The second sample (in diestrus) was taken on day 7 ± 1 post-ovulation.

2.2.3. Experiment 3: effects of immunomodulation

This experiment was similar to experiment 2 except that an immunomodulator was injected to mares at time of AI. An emulsion of a cell-wall skeleton preparation purified from *Mycobacterium phlei* (Equimune IV™, Bioniche, AH, US) (MCWE) and marketed for the treatment of equine respiratory infectious complex, was injected intravenously at a single dose of 1.5 mg. Mares were biopsy sampled 24 ± 8 h after insemination and MCWE treatment. The day of ovulation was registered and the second sample was taken at day 7 ± 1 post-ovulation (i.e. at diestrus).

2.2.4. Ultrasonography

Ultrasonography was used to identify pregnant and barren mares, to determine the number and size of dominant follicle(s), the presence of uterine fluid and edema, the state of the cycle and ovulation. All examinations were performed using a 5-MHZ linear array, real time, transducer (Portable Scanner 100 vet, Pie Medical Equip. BV, Maastricht, The Netherlands).

2.2.5. Endometrial biopsies

Endometrial biopsy samples were collected using a biopsy punch (Alligator Jaw Instrument, Pilling, PA). For histologic examination, samples were fixed in Bouin's solution, prepared for optical microscopy and scored according to a well-established grading system (Kenney and Doig, 1986). For cytokine assays, punches were kept frozen in liquid nitrogen until used for RNA extraction.

2.2.6. RNA isolation, DNAse treatment of RNA samples, and cDNA synthesis

Total RNA was isolated from 50 mg of endometrium using the RNeasy total RNA isolation kit (Qiagen Inc., Valencia, CA, USA). RNA concentration was measured by optical density at 260 nm. All RNA samples were treated with amplification grade DNAse I (Gibco BRL, Rockville, MD, USA) to remove any traces of genomic DNA. Briefly, 1 U of DNAse I and 1 μl of 10× DNAse I reaction buffer were mixed with 1 μg of total RNA in a 10 μl reaction. The mixture was incubated for 10 min at room temperature and then inactivated by adding 1 μl of 25 mM EDTA and heating at 65 °C for 10 min.

cDNA was synthesized with the Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA, USA) by using the protocol of the manufacturer. Briefly, 1 μg of total RNA was mixed with 1 μl of oligo $(dt)_{18}$ primer (20 μM) and heated at 70 °C for 2 min. After cooling to room temperature the following reagents were added: 4 μl of 5× reaction buffer (containing 250 mM Tris–HCl (pH 8.3), 375 mM KCl, and 15 mM $MgCl_2$), 1 μl of deoxynucleoside triphosphates (dNTP, 10 mM each), 0.5 μl of RNase inhibitor (40 U μl^{-1}) and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U μl^{-1}).

The mixture was incubated at 42 °C for 1 h, heated at 94 °C for 5 min, diluted to a final volume of 100 μl, and stored at −70 °C until used for PCR analysis.

2.2.7. Real time PCR

Gene specific primers and internal oligonucleotide probes for equine IL-1β, IL-6, TNF-α and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) have been previously described (Garton et al., 2002). The internal probes were labeled at the 5′ end with the reporter dye 6-carboxyfluoresceine, and at the 3′ end with the quencher dye 6-carboxytetramethyl-rhodamine. Amplification of 2 μl of cDNA was performed in a 25 μl PCR reaction containing 900 nM of each primer, 250 nM of Taqman probe and 12 μl of TaqMan Universal PCR Mastermix (Applied Biosystems). Amplification and detection were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) with initial incubation steps at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each samples was assayed in triplicate and the mean value was used for comparison. Samples without cDNA were included in the amplification reactions to determine background fluorescence and check for contamination. cDNA from 24 h Concanavallin A-stimulated equine blood mononuclear cells was used as positive control. To account for variation in the amount and quality of starting material, all the results were normalized to G3PDH expression. In preliminary experiments, the amplification efficiency of IL-1β, Il-6 or TNF-α primer and probe combinations were similar to that of G3PDH with slopes of the log input amount versus differences in threshold cycles <0.1. Relative quantitation between samples was achieved by the comparative threshold cycles method aspreviously described (Leutenegger et al., 1999). Results are reported as the n-fold difference relative to cytokine mRNA expression in the sample with the lowest expression.

2.2.8. Data presentation and statistical analyses

Data were presented as median and ranges in relative mRNA expression within each group ($n = 8$). Differences between paired groups (experiment 1 versus experiment 2 and experiment 2 versus experiment 3) were analyzed with the Wilcoxon ranks signed test. Differences between unpaired groups (SM versus RM) were analyzed with the Mann–Whitney U-test. The Spearman non-parametric correlation test was used for correlation analyses between cytokine mRNA expression. Results were considered statistically significant if the value of P was <0.05.

3. Results

3.1. Baseline IL-1β, IL-6 and TNF-a mRNA expression in SM and RM, and effect of estrous cycle

SM and RM were studied in estrus and in diestrus to establish their baseline cytokine mRNA expression. In estrus, IL-1β ($P = 0.002$), IL-6 ($P = 0.001$) and TNF-α ($P = 0.0006$) mRNA expression in endometrial biopsy samples was significantly higher in SM than in RM (Table 2). In diestrus, endometrial mRNA expression of IL-1β ($P = 0.005$) and TNF-α ($P = 0.007$) of SM was significantly higher than that of RM.

Table 2
Baseline relative IL-1β, IL-6 and TNF-α mRNA expression in endometrial tissue from RM and SM, and effect of estrous cycle

Period	Resistant mares			Susceptible mares		
	IL-1β	IL-6	TNF-α	IL-1β	IL-6	TNF-α
Estrus	4.49[a] (2.26–9.59)	6.86[a] (2.65–39.42)	14.93[a] (7.25–58.52)	16.21[a] (5.24–133)	156[a,b] (27.18–560)	127[a,b] (22.18–652)
Diestrus	4.15[a] (2.26–7.47)	2.38 (1–112426)	10.56[a] (2.4–30.79)	11.23[a] (4.5–60.31)	14.81[b] (3.15–236)	35.76[a,b] (7.08–288)

Relative quantitation between samples was achieved by the comparative threshold cycles method as previously described (Leutenegger et al., 1999). 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. The values represent median (range) relative mRNA expression within each group ($n = 8$).

[a] Indicates significant differences for each cytokine between resistant and susceptible mares ($P < 0.05$).

[b] Indicates significant differences between estrus and diestrus within each group ($P < 0.05$).

Table 3
Relative IL-1β, IL-6 and TNF-α mRNA expression in endometrial tissue from RM and SM, and effect of artificial insemination

Period	Resistant mares			Susceptible mares		
	IL-1β	IL-6	TNF-α	IL-1β	IL-6	TNF-α
24 h after AI	97.66 (8–511)	231 (12.25–10447)	317 (2.83–3063)	317 (31.22–4484)	1414 (39.79–4082088)	430 (18.26–24066)
Diestrus	14.2[a] (3–157)	43.36 (1.28–3806)	18.58[a] (0–478)	86.69[a] (18.43–2294)	87.17 (6.7–81671)	301[a] (30.22–18875)

Relative quantitation between samples was achieved by the comparative threshold cycles method as previously described (Leutenegger et al., 1999). 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. The values represent median (range) relative mRNA expression within each group ($n = 8$).

[a] Indicates significant differences for each cytokine between resistant and susceptible mares ($P < 0.05$).

IL-1β, IL-6 and TNF-α mRNA expression in RM was not significantly different between estrus and diestrus. In contrast, SM had significantly lower IL-6 ($P = 0.008$) and TNF-α ($P = 0.05$) mRNA expression in diestrus than in estrus.

3.2. Effects of AI

The effect of AI as an inflammatory stimulus was evaluated in two ways. First, RM and SM were compared in estrus versus diestrus following AI with dead semen (Table 3). Second, mRNA expression observed following AI in experiment 2 was compared to baseline values obtained from each respective group in experiment 1 (mares without treatment) (Table 5).

Differences in IL-1β, IL-6 and TNF-a mRNA expression between SM and RM in estrus 24 h following AI were not statistically significant (Table 3). In diestrus, mRNA expression of IL-1β ($P = 0.01$) and TNF-α ($P = 0.02$) was significantly higher in SM than in RM.

Twenty-four hours after AI, mRNA expression for the three cytokines was significantly increased in RM compared to baseline expression in the same mares during estrus of the previous cycle (without treatment) (Table 4). Higher IL-1β, IL-6 and TNF-α levels were found in SM compared to baseline expression in the same mares during estrus of the previous cycle (without treatment) (Table 4) although these differences were not significant. The same comparisons made during diestrus showed a significant increase in IL-1β mRNA expression in both RM ($P = 0.008$) and SM ($P = 0.04$).

3.3. Effects of immunomodulation

To evaluate the effect of an immunomodulator on semen-induced endometrial inflammation, MCWE was administered intravenously to mares at the time of AI. After immunomodulation, there were no significant differences between mRNA transcription for

Table 4
Relative IL-1β, IL-6 and TNF-α mRNA expression in endometrial tissue from RM and SM (comparisons between groups)

Comparison	Resistant						Susceptible					
	Estrus			Diestrus			Estrus			Diestrus		
	IL-1β	IL-6	TNF-α	IL-1β	IL-6	TNF-α	IL-1β	IL-6	TNF-α	IL-1β	IL-6	TNF-α
No treatment vs. AI	↑[a] **	↑ **	↑ **	↑ **	↑ ns	↑ ns	↑ ns	↑ ns	↑ ns	↑ **	↑ ns	↑ ns
AI vs. AI + MCWE	↓ ns	↓ ns	↓ ns	↑ ns	↓ ns	↑ ns	↓ **	↓ ns	↓ ns	↓ ns	↓ ns	↓ ns

Asterisks indicates significant differences ($P < 0.05$); ns, non-significant differences.

[a] Arrows indicate the effect of the treatment (upwards, augmentation; downwards, reduction).

Table 5
Relative IL-1β, IL-6 and TNF-α mRNA expression in endometrial tissue from RM and SM, and effect of artificial insemination plus immunomodulation

Period	Resistant mares			Susceptible mares		
	IL-1β	IL-6	TNF-α	IL-1β	IL-6	TNF-α
24 h after AI plus immunomodulator	37.99 (5–28210)	96.42 (4.36–27444)	90.43 (8.08–83379)	27.38 (4.3–114.9)	181 (2.13–1004)	78.14 (9.92–476)
Diestrus	31.51 (4.3–115)	17.94 (2.13–1004)	118 (9.92–466)	57.79 (2.54–161)	29.17 (8.48–139)	137 (26.49–373)

Relative quantitation between samples was achieved by the comparative threshold cycles method as previously described (Leutenegger et al., 1999). Results are reported as the *n*-fold difference relative to cytokine mRNA expression in the sample with the lowest expression, which was arbitrarly given the value of 1.0. The values represent median (range) relative mRNA expression within each group ($n = 8$).

any of the cytokines between RM and SM neither during estrus nor during diestrus (Table 5).

IL-1β, IL-6 and TNF-α mRNA expression in mares administered MCWE at time of AI was compared to expression in the same mares after AI without MCWE administration (Table 4). Administration of MCWE resulted in a significant decrease in IL-1β ($P = 0.02$) and lower IL-6 (ns, $P = 0.08$) mRNA expression in SM during estrus compared to the same mares not receiving MCWE at time of AI. Other comparisons did not identify statistically significant differences. However, it is worth to mention that maximum levels of mRNA transcription were much lower when SM received AI plus MCWE treatment (Tables 3 and 5).

3.4. Correlation of IL-1β with IL-6 and TNF-α mRNA levels in endometrial samples

To assess if the mRNA expression of the three cytokines varied mutually, a correlation analysis was made between IL-1β and IL-6 and between IL-1β and TNF-α.

No significant correlation were found between mRNA expression for the three cytokines in untreated RM. However, IL-1β and TNF-α mRNA expression were highly correlated ($r = 0.88$, $P = 0.007$) during both estrus and diestrus in SM. A lower correlation between IL-1β and IL-6 was found in SM during both estrus ($r = 0.66$, $P = 0.08$) and diestrus ($r = 0.64$, $P = 0.09$).

After AI, mRNA expression was highly correlated in both groups of mares during estrus and diestrus with the only exception being a low correlation between IL-1β and TNF-α in RM during diestrus. In response to administration of MCWE together with AI, there were different correlation degrees in both RM and SM in estrus (IL-6: $r = 0.74$, TNF-α: $r = 0.9$ and IL-6: $r = 0.95$, TNF-α: $r = 0.69$, respectively). In diestrus, the degrees of correlation were lower and not significant in both groups with the exception of TNF-α ($r = 0.74$) in SM.

4. Discussion

This work aimed to study mRNA expression of endometrial pro-inflammatory cytokines in mares resistant and susceptible to PPBE. Results of the present study demonstrated that these two groups of mares differed in their endometrial mRNA expression of IL-1β, IL-6 and TNF-α, particularly after the stimulus of AI. Furthermore, the altered profiles of mRNA transcription observed in SM were modified upon application of an immunomodulator.

Mares classified as RM on the basis of reproductive history, endometrial cytology and histopathology, and bacterial clearance experiments, exhibited lower endometrial mRNA expression of the three pro-inflammatory cytokines than SM. Previous studies have shown that SM have a sustained high number of inflammatory cells infiltrating their endometrium as a result of inflammatory stimuli (Cadario et al., 1999; Fumuso et al., 2000). Although not addressed in our study, macrophages may be responsible for the high pro-inflammatory cytokine mRNA expression observed in SM. Indeed, macrophages are a major source for these molecules (Dinarello, 1992; Murtaugh and Myers, 1995) and monocyte/macrophage cells are found in the endometrium of both RM and SM (Summerfield and Watson, 1998). However, mRNA

and protein for these pro-inflammatory cytokines have also been identified in human and rat luminal epithelial endometrial cells by in situ hybridization and immunohistochemistry, indicating the contribution of other cells to cytokine pool (Yelavarthi et al., 1991; Barañao, 1997). The current study does not address whether increased mRNA expression in SM was due to higher numbers (and variety) of transcribing cells or, instead, to the higher activity per cell. Therefore, these results should be taken as a measure of a global endometrial response. In the absence of stimuli (i.e. AI), the activation of the innate immune system—reflected here by transcription levels of PICs—is tightly controlled at endometrium in RM during estrus and early diestrus. Thus, RM produced an inflammatory response high enough to control an incipient infection (supported by data from previous bacterial clearance assays referred to in Section 2) and to eliminate spermatozoa (Kotilainen et al., 1994), yet transient enough as not to create a persistently hostile medium for the embryo nidation and development (Troedsson, 1999).

Resistant mares submitted to an intra-uterine inflammatory stimulus (experiment 2), here represented by AI with dead semen, significantly upregulated mRNA expression for pro-inflammatory cytokines. Results also indicated that during AI (and also most likely during natural breeding), cytokines play a regulatory role enhancing endometritis during estrus in both groups of mares and then down-regulating it during diestrus in RM but not in SM. This tendency was consistent with the behavior seen in basal conditions in experiment 1, and thus it most likely might represent a trait particular to the mare's phenotype instead of a characteristic of the evoking stimulus. In this respect, apparently opposite roles (i.e. pro- or counter-inflammatory) have been reported for IL-6 and recently an hypothesis has been postulated to connect those effects (Kaplanski et al., 2003). IL-6 would act initially promoting acute inflammation and PMN recruitment; later on, IL-6 would induce PMN apoptosis and phagocytosis by monocytes, which would become inflammatory macrophages, leading to the termination of the inflammation. In our study, both in basal conditions and after an inflammatory stimulus, IL-6 was not significantly upregulated in SM thus probably indicating a point of down-regulation failing in these mares.

Appropriate contractility of the myometrium is recognized to play a significant role in clearance of endometrial debris and inflammatory by-products (Nikolakopoulos and Watson, 1999). Interestingly, in humans and mice, high induction of pro-inflammatory cytokines have been associated with modulation of several mechanisms affecting myometrial contractility such as cell-to-cell communication failure (Semer et al., 1991), COX2-dependent increased synthesis of prostaglandins $F_2\alpha$ and E_2 (Erkinheimo et al., 2000); and, in cows, with the expression of oxytocin receptors (Leung et al., 2001). Thus, SM could probably present some of these defects, or a combination of them, which could be related with unpaired contractions.

On a global basis, there was a good correlation between expression of all three pro-inflammatory cytokine examined when expression was high (we considered as "low" or "baseline" levels those presented by untreated RM in experiment 1) as was the case 24 h after AI. Thus, it would appear that stimulus should exceed a threshold to induce a coordinate transcription of cytokines, and that in SM, such a threshold would be lower than in "normal" RM. Besides, lack of correlation found in a number of comparisons suggested that caution should be taken if a single cytokine is to be selected to monitor endometritis.

We sought to evaluate if immunomodulation would affect post-breeding endometritis. Among available candidates, MCWE was selected because it targets macrophages, a central cell to innate immunity, and it is safe and already approved for use in horses. Mares treated with MCWE at time of AI reverted the pattern of mRNA expression similar to baseline levels. Indeed, SM, did not show such a high up regulation as when just received AI, and, in fact, most of them behaved like the RM. This was clearly reflected by the lack of significant differences between both groups when treated with MCWE; in addition, giving the ample variation considering individual values, it is to note that in every case, MCWE treatment resulted in much lower maximum transcription values. Therefore, applying MCWE to mares subjected to AI, modulated inflammation by significantly down-regulating mRNA expression of IL-1β in SM. However, the inflammatory potential of each one of the cytokines studied has not been determined; thus probably the upregulation of one of them would suffice to provoke inflammation; for example, in vivo experiments in rats have shown

that IL-1β was more potent than IL-6 in inducing acute-phase hypothalamic effects (Wu et al., 1999). Although not yet studied in equine cells, the most likely mechanism of action of MCWE is by receptor signaling upon binding to TLR-4 and TLR-2 (Underhill et al., 1999). The same stimuli that generate pro-inflammatory cytokines might also evoke other mechanisms with opposite effects. For instance, TNF-α activity can be neutralized by a soluble receptor, IL-1 receptor antagonist competes with active IL-1 for receptor sites and high concentrations of IL-6 reduce IL-1 and TNF-α production (Murtaugh and Myers, 1995; MacKay, 2000). Further studies are needed to better appreciate the contribution of individual factors in regulation of endometritis.

Thus, according to our results, SM exhibited high and protracted mRNA expression for some pro-inflammatory cytokines as a significant feature of PPBE. Immune intervention with MCWE could be of help in restoring homeostatic local inflammatory mechanisms, thus assisting in the prophylaxis of this condition in mares.

Acknowledgements

Work supported by UNICEN (Argentina), Syntex S.A. (Argentina) and Bioniche Animal Health (Canada). E. Lee (Gainesville, USA) is acknowledged for her help with PCR procedures. We also thank Dr. Losinno and Dr. Aguilar for valuable help in experiment design, E.M. Rodriguez for advice in statistics, and M. Tallarico, V. Berestin, E. Inchausti and Dr. M. Aba for on farm collaboration, and to Dr. R. Guerrero, on behalf of the staff of Syntex's farm.

References

Alghamdi, A., Troedsson, M.H.T., Laschkewitsch, T., Xue, J.L., 2001. Uterine secretion from mares with post-breeding endometritis alters sperm motion characteristics in vitro. Theriogenology 55, 1019–1028.

Barañao, R., 1997. Citoquinas en el proceso reproductivo. Reproducción (Argentina) 1, 13–27.

Cadario, M., Thatcher, W., Klapstein, E., Merrit, A., Archbald, L., Thatcher, M., LeBlanc, M., 1999. Dynamics of prostaglandin secretion, intrauterine fluid and uterine clearance in reproductively normal mares and mares with delayed uterine clearance. Theriogenology 52, 1181–1192.

Cadario, M., Losinno, L., Giguère, S., Aguilar, J., Jack, T., Macpherson, M., Fitzpatrick, C., Uhl, E., 2002. Uterine expression of fibrogenic cytokines in the mare. Theriogenology 58, 449–452.

Dinarello, C., 1992. The biology of interleukin-1. In: Kishimoto, T. (Ed.), Interleukins: Molecular Biology and Immunobiology, Chemistry of Immunology, vol. 51. Karger Verlag, Basel, pp. 1–32.

Erkinheimo, T., Saukkonen, K., Narko, K., Jalkanen, J., Ylikorkala, O., Ristimaki, A. J. Clin. Endoc. Metab. 85, , 3468–3475.

Fumuso, E.A., González, C., Videla Dorna, I., Inchausti, E., Tallarico, M., Berestin, V., Wade, J., Aguilar, J., Losinno, L., Soto, P., Bowden, R.A., 2000. Effect of an immunomodulator in mares resistant and susceptible to endometritis. In: Proceedings of the Second International Conference on Experimental and Clinical Reproduction Immunobiology, 15–18 November. Elsevier, Amsterdam, The Netherlands, p. 104.

Garton, N.J., Gilleron, M., Brando, T., Dan, H.H., Giguère, S., Puzo, G., Prescott, J.F., Sutcliffe, I.C., 2002. A novel lipoarabinomannan from the equine pathogen *Rhodococcus equi*: structure and effect on macrophage cytokine production. J. Biol. Chem. 177, 31722–31733.

Hunt, J., Chen, H., Hu, X., Talibzadeh, S., 1992. Tumor necrosis factor α mRNA and protein in human endometrium. Biol. Reprod. 47, 141–147.

Kaplanski, G., Marin, V., Montero-Julian, F., Mantovani, A., Franarier, C., 2003. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends Immunol. 24, 25–29.

Katila, T., 1995. Onset and duration of uterine inflammatory response of mares after insemination with fresh semen. Biol. Repod. Mono. 1, 515–517.

Kauma, S., Matt, D., Strom, S., Eirman, D., Tuner, T., 1990. Interleukin 1β IL-1β, HLA-DR β in endometrium, placenta and placental membranes. Am. J. Obstet. Gynecol. 163, 1430–1436.

Kenney, R., Doig, P., 1986. Equine endometrial biopsy. In: Morrow, D. (Ed.), Current Therapy in Theriogenology, vol. 2. WB Saunders, Philadelphia, pp. 723–729.

Kotilainen, T., Huhtinen, M., Katila, T., 1994. Sperm-induced leukocytosis in the equine uterus. Theriogenology 16, 630–631.

Leung, S., Cheng, Z., Sheldrick, E., Derecks, K., Flint, A., Wathes, D., 2001. The effects of lipopolysaccharide and interleukins-1α, -2 and -6 on oxytocin receptor expression and prostaglandin production in bovine endometrium. J. Endocrinol. 168, 497–508.

Leutenegger, C.M., von Rechenberg, B., Huder, J.B., Zlinsky, K., Mislin, C., Akens, M.K., Auer, J., Lutz, H., 1999. Quantitative real-time PCR for equine cytokine mRNA in nondecalcified bone tissue embedded in methyl methacrylate. Calcif. Tissue Int. 65, 378–383.

Losinno, L., Woods, G., Miragaya, M., 1997. Uterine and oviductal inflammation in anovulatory mares with ultrasonographycally detected intrauterine luminal fluid. Theriogenology 48, 1361–1368.

MacKay, R., 2000. Inflammation. In: Lunn, P., Horohov, D. (Eds.). Vet. Clin. North Am. Equine Pract.: Immunol. 16, 15–27.

McKinnon, A., Squires, E., Harrison, B., Blach, E., Shideler, R., 1988. Ultarasonographic studies on the reproductive tract of

mares after parturition. Effect of involution and uterine fluid on pregnancy rates in mares with normal and delayed first postpartum ovulatory cycles. J. Am. Vet. Med. Assoc. 192, 350–353.

Murtaugh, M., Myers, M., 1995. Future directions in animal cytokine research and applications. In: Myers, M., Murtaugh, M. (Eds.), Cytokines in Animal Health and Diseases. Book News Inc., Portland, OR, pp. 449–457.

Nikolakopoulos, E., Watson, E.D., 1999. Uterine contractility is necessary for the clearance of intrauterine fluid but not bacteria after bacterial infusion in the mare. Theriogenology 52, 413–423.

Semer, D., Reisler, K., MacDonald, P., Casey, M., 1991. Responsiveness of human endometrial stromal cells to cytokines. Ann. N. Y. Acad. Sci. 622, 99–110.

Simon, C., Piquette, G., Frances, A., El-Danasouri, I., Irwin, J., 1994. The effect of interleukin-IP (IL-1β) on the regulation of IL1 receptor type messanger ribonucleic acid and protein levels in cultured human endometrial stromal and glandular cells. J. Clin. Endocrinol. Metab. 78, 675–681.

Summerfield, N., Watson, E., 1998. Endometrial macrophage populations in genitally normal mares at oestrus and dioestrus and in mares susceptible to endometritis. Equine Vet. J. 30, 79–81.

Takacs, L., Kavacs, E., Smith, M., Young, H., Durun, S., 1988. Detection of IL-1β gene expression by in situ hybridization tissue localization of IL-1 mRNA in the normal C57BL/6 mouse. J. Immunol. 141, 3081–3087.

Troedsson, M.H.T., 1995. Comparative treatment of mares susceptible to chronic uterine infection. Am. J. Vet. Res. 56, 468–472.

Troedsson, M.H.T., 1997. Therapeutic considerations for mating-induced endometritis. Pferdeheilkunde 13, 516–520.

Troedsson, M.H.T., 1999. Uterine clearence and resistance to persistent endometritis in the mare. Theriogenology 52, 461–471.

Troedsson, M.H.T., Loset, K., Alghamdi, A.M., Dahms, B., Crabo, B.G., 2001. Interaction between equine semen and the endometrium: the inflammatory response to semen. Anim. Reprod. Sci. 68, 273–278.

Underhill, D., Ozinnsky, K., Smith, K., Aderem, A., 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc. Natl. Acad. Sci. U.S.A. 96, 14459–14463.

Vásquez-López, M., Maffrand, C., Losinno, L., Maldonado, J. Rev. Col. Cienc. Pec. 13 (S), , 33.

Wade, J., 1995. Interacción entre la reproducción y el sistema inmunológico. Sus implicaciones en la investigación y el manejo reproductivo. Rev. Arg. Prod. Anim. 15, 151–158.

Watson, E., 2000. Post-breeding endometritis in the mare. In: Proceedings of the 14th International Congress on Animal Reproduction, 2–6 July 2000. Elsevier, Stockholm, pp. 221–232.

Wu, Y., Khan Shaghaghi, E., Jacquot, C., Pallardy, M., Gardier, A.M., 1999. Synergism between interleukin-6 and interleukin-1β in hypothalamic serotonin release: a reverse in vivo microdialysis study in F344 rats. Eur. Cytokine Network 10, 57–64.

Yelavarthi, K.K., Fishback, J.L., Hunt, J.S., 1991. Analysis of HLA-G mRNA in human placental and extraplacental membrane cells by in situ hybridization. J. Immunol. 146, 2847–2854.