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# Immune parameters in mares resistant and susceptible to persistent post-breeding endometritis: Effects of immunomodulation

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**Abstract**

Our objective was to characterize immune parameters in susceptible (SM) and resistant (RM) mares, with and without artificial insemination (AI) and immunomodulation. Eight RM and eight SM were selected based on their reproductive history and functional tests. Both groups of mares were evaluated during three consecutive cycles: Cycle 1, untreated cycle (control); Cycle 2, AI with dead semen; Cycle 3, AI with dead semen and immunomodulation. Endometrial biopsies were taken during the three cycles as follows: Cycle 1—at estrus, when follicles ≥35 mm and at diestrus (7 ± 1 days after ovulation); Cycle 2—at estrus 24 h post-AI, and at diestrus; Cycle 3—at estrus 24 h after treatment with a *Mycobacterium phlei* cell-wall extract (MCWE) and AI, and at diestrus. The mRNA transcription (mRNAT) of IL-8 and IL-10 were determined by real-time PCR. Image analysis of immunonhistochemistry slides was performed using digital software (Image-Pro Plus v 5.0; Media Cybernetics); the percentage of stained area was determined for Mayor Histocompatibility Complex II (MHC-II), polymorphonuclear leukocytes (PMN) and T lymphocytes (TL) on each tissue section. In Cycle 1, SM had significantly higher MHC-II, TL, PMN and IL-8 than RM during estrus ($P < 0.006$, $P < 0.0005$, $P < 0.05$, respectively), while transcription of IL-10 was significantly lower than in RM ($P < 0.0001$). During diestrus, SM had higher levels of TL, PMN and IL-8 than RM ($P < 0.0001$). After AI (Cycle 2), SM had higher levels of IL-8 and lower levels of IL-10 than RM at estrus and no differences were detected for MHC-II, TL and PMN positive cells. During diestrus in the same cycle, all the immune parameters were higher in SM mares ($P < 0.005$, $P < 0.0004$, $P < 0.0001$, $P < 0.02$, respectively). When MCWE was applied at the time of AI (Cycle 3), SM expressed significant higher levels of IL-10 24 h after treatment ($P < 0.005$), which were also higher than in the control Cycle 2 or after AI (Cycle 2). However, no significant differences were detected for MHC-II, lymphocytes-PMN or IL-8 between SM and RM during diestrus in Cycle 3. This study showed that SM had higher levels of all immune parameters except IL-10 than RM during Cycle 1. After AI (Cycle 2), the inflammatory condition persisted in SM but not RM mares until day 7 post-ovulation. Following treatment with MCWE at the time of AI (Cycle 3) uterine immunological changes in SM resulted in an endometrial immune environment similar to that found in normal RM.


*Keywords:* Endometritis; Uterus; Immunomodulation; Mares

## 1. Introduction

In mares, endometrial tissue has all the components of mucosal-associated lymphoid tissue (Watson and Thomson, 1996). Dynamic populations of leucocytes are not only a way of tissue remodeling and/or defense mechanisms in the reproductive tract, but production of polypeptides such as cytokines and chemokines are also well recognized defense mechanisms. These act as immune ''hormones'' in response to sperm challenge or endocrine changes during the oestrous cycle (Tremellen et al., 1998). Both endometrial and immune cells

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express surface markers of the Major Histocompatibility Complex II (MHC-II) (Watson and Dixon, 1993). When endometrial tissue receives a stimulus such as semen or bacteria, a rapid inflammatory process begins with neutrophil migration and fluid accumulation with the presence of inflammatory mediators such as plasma proteins, complement, inducible nitric oxide synthase (iNOS), nitric oxide and prostaglandins E and F 2α (Watson, 1989; Troedsson et al., 1993; Alghamdi et al., 2005). Breeding in normal, resistant mares (RM) results in a transient neutrophilic endometritis that typically resolves within 48 h. However, in susceptible mares (SM), a persistent post-breeding endometritis (PPBE) develops, resulting in persistent accumulation of inflammatory fluid within the uterus, which reduces fertility and results in serious economic losses. In a previous study, we documented a significant induction of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6 and TNF-α in SM post-breeding that did not occur in RM. We have also demonstrated that administration of an immunomodulator to SM prior to artificial insemination (AI) results in a significant decrease in IL-1β mRNA expression, showing a modulation of the inflammatory process (Fumuso et al., 2003). We propose that immunomodulators may be useful to control PPBE in SM. In cattle with confirmed endometritis, intrauterine infusion of 100 μg of *Escherichia coli* LPS (immunomodulator agent) on the day of estrus resulted in a 100% increase in the total intraluminal leukocyte count and a significant increase in pregnancy rates during the following cycle (Singh et al., 2000). In 2003, Zerbe et al. demonstrated that recombinant human interleukin 8 (rhIL-8) was effective in attracting polymorphonuclear leucocytes (PMN) and efficiently induced transmigration into the uterus within 6 h following administration in cattle and horses (Zerbe et al., 2003). Mares, however, showed a more profound response, with at least 15 times more neutrophils compared with cows. The objective of this study was to characterize immune responses (IL-8, IL-10, MHC-II, T lymphocytes [TL] and PMN) in SM and RM with and without artificial insemination (AI) and immunomodulation.

## 2. Materials and methods

### 2.1. Selection of mares

Sixteen mares were selected from a herd of 51 mares aged 6–16 years, diagnosed not pregnant and with good body and vulvar scores based on their capacity to clear an intra-uterine inoculation with $10^6$ CFU of *Streptococcus zooepidemicus*. Two groups of eight resistant (RM) and eight susceptible mares (SM) were constituted based on the results obtained by ultrasonographyc evidence of intra-uterine fluid, endometrial cytology and bacteriology 72 h after the inoculation. A value of "1" was assigned to each positive result (fluid, cytology, bacteriology). Mares with a final score of 0 or 1 were considered resistant and those final score of 2 or 3 were considered susceptible. In addition, the ability of these mares to become pregnant after two cycles in natural breeding with fertile stallions was assessed. A summary of the pertinent traits exhibited by the mares that entered the study is given in Table 1. Pregnancy rates were 100% for RM (8/8 RM) and 0% for SM (0/8 SM). Abortion was induced with PGF2α in those mares that became pregnant after natural breeding at least 5 months before the trial.

#### 2.1.1. S. zooepidemicus inoculation

The *S. zooepidemicus* inoculum was performed according to Cadario et al. (1999) with minor modifications. Briefly, a genital virulent strain of *S.*

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

| Mares | Exfoliative cytology[a] | Culture result[b] | Uterine fluid[c] | Pregnancy test[d] |
|---|---|---|---|---|
| Resistant mares | | | | |
| 1 | Negative | Negative | 0 | + |
| 2 | Negative | Negative | 1 | + |
| 3 | Negative | Negative | 1 | + |
| 4 | Negative | Negative | 1 | + |
| 5 | Negative | Negative | 0 | + |
| 6 | Negative | Negative | 0 | + |
| 7 | Negative | Negative | 1 | + |
| 8 | Negative | Negative | 0 | + |
| Susceptible mares | | | | |
| 9 | Positive | Positive | 1 | − |
| 10 | Negative | Positive | 1 | − |
| 11 | Negative | Positive | 1 | − |
| 12 | Positive | Positive | 1 | − |
| 13 | Positive | Positive | 2 | − |
| 14 | Positive | Positive | 4 | − |
| 15 | Positive | Positive | 1 | − |
| 16 | Positive | Negative | 2 | − |

[a] Exfoliative cytology: "Positive" and "Negative" indicates >12 or <12 polymorphonuclear leukocytes per microscopic field at 400×, respectively.

[b] Culture results were given as "Negative" when no bacteria/yeast grew after culturing the endometrial swab content.

[c] Presence of fluid was ultrasonographically registered and scored as described by McKinnon et al. (1988). The results marked as a–c were obtained after experimental inoculation test with *Streptococcus zooepidemicus*.

[d] Pregnancy test result before entering the trial after two cycles with fertile stallions: "+" means pregnant and "−", not pregnant.

*zooepidemicus* was grown overnight in Brain Heart Infusion Broth (Merck, Argentina) at 37 °C, washed twice in PBS, brought to $1 \times 10^9$ mL$^{-1}$ and stored at −70 °C until inoculation. Before inoculation, this culture was thawed and 0.1 mL was diluted to 10 mL in PBS (to give a $1 \times 10^7$ mL$^{-1}$ suspension). Finally, 5 mL of this suspension was brought to 50 mL with PBS to obtain a $1 \times 10^6$ mL$^{-1}$ suspension. While mares were in estrus, 50 mL of this solution was inoculated into the uterus through a plastic pipette. Clearance of *S. zooepidemicus* was evaluated at 72 h post-inoculation by ultrasonographic examination of the uterus for luminal fluid, endometrial culture for bacteria and endometrial cytology.

#### 2.1.2. Bacteriology

Endometrial samples were collected using guarded Culturvet swabs (Culturvet, Argentina) and immediately placed in Amies' transport medium (Oxoid, London, UK). Samples were studied using conventional bacteriology testing procedures.

#### 2.1.3. Endometrial cytology

Cytology samples were collected using guarded protective Culturvet swabs, smeared on glass slides, dried at room temperature and stained with Giemsa stain (15 in. Biopur, Argentina). PMN were counted in 20 fields (400×) and a mean number per field was calculated. Mares with mean PMN counts >12 were considered to have inflammation, corresponding to a degree 3–4 in a scale according to Miragaya et al. (1997) and Vásquez-López et al. (2000).

#### 2.1.4. Fertility test

Mares were confirmed to be in the ovulatory season and cycling regularly by a series of rectal palpations and ultrasonographic examinations over a 2-month period prior to the experiment. Once the 16 mares were detected cycling, they were placed with a fertile stallion on pasture for natural breeding for a period of 7 weeks (March to April). Two weeks after the end of the breeding period, the mares were examined for pregnancy by ultrasonography (5-MHz linear array, real time, Portable Scanner 100 vet, Pie Medical Equip. BV, Maastricht, Netherlands).

### 2.2. Experimental design

Analysis of variance using PROC GLM with SASV8 for double split-plot experimental design was used to compare resistant versus susceptible mares. Since the data were not normally distributed, an exponential transformation was applied using medians and quartile ranges rather than means and standard deviation. Three consecutive cycles with different treatments were conducted utilizing the same groups of susceptible and resistant mares. Data analyses (described below) followed identical procedures. Data were represented by group mRNA transcription values for each of the two cytokines studied and percentage of stained area for cells positive to MHC-II, TL and PMN.

#### 2.2.1. Cycle 1: Untreated cycle

Two endometrial biopsy samples were taken from each mare during estrus using a biopsy punch (Alligator jaw instrument, Pilling, PA, USA). Mares were determined to be in estrus when transrectal palpation and ultrasonography showed a relaxed cervix accompanied by a lack of uterine tone, the presence of endometrial edema and a dominant ovarian follicle (≥35 mm). The day of ovulation was determined ultrasonically and recorded, and a second set of two endometrial samples were taken 7 ± 1 days post-ovulation (diestrus). One biopsy sample was immediately frozen and stored in liquid nitrogen until determination of IL-8, IL-10, mRNA transcription by real-time PCR (RT-PCR); the other biopsy was placed in Bouin solution for 12 h and then in 70% alcohol until processed for immunohistochemical detection of MHC-II+, PMN and TL.

#### 2.2.2. Cycle 2: Artificial insemination (AI)

Dead sperm was used to study the inflammatory effect of semen deposition in the uterus, avoiding the otherwise superimposed effects of conception. Semen was collected from four fertile stallions using a Missouri artificial vagina. Semen samples were pooled and diluted in Kenney's extender without antibiotics. Final AI dose was adjusted to $5 \times 10^8$ mL$^{-1}$ spermatozoa with progressive motility. Semen was killed by carrying out two rounds of freezing and thawing and finally storing the sample at −20 °C. Progressive motility was confirmed to be 0% following this treatment. When mares were detected in estrus (as determined in Cycle 1) they were artificially inseminated with this semen preparation. Biopsy samples were taken 24 ± 8 h after AI. The day of ovulation was determined ultrasonically and recorded. The second biopsy samples (in diestrus) were taken on day 7 ± 1 post-ovulation.

#### 2.2.3. Cycle 3: AI and immunomodulation

This was similar to Cycle 2 except that an immunomodulator was administered to the mares at the time of AI. An emulsion of cell-wall extracts,

purified from *Mycobacterium phlei* (Equimune IV™, Bioniche Animal Health, USA Inc.; MCWE), and marketed for the treatment of equine respiratory infectious complex, was administered intravenously in a single dose of 1.5 mg. Endometrial biopsies were taken at 24 ± 8 h after AI and MCWE treatment. The day of ovulation was determined ultrasonically and the second set of endometrial samples was taken at day 7 ± 1 post-ovulation (diestrus).

### 2.2.4. RNA isolation, DNAse treatment of RNA samples and cDNA synthesis

Endometrial tissue was thawed at room temperature in a dry incubator for 10 min. From each sample, 50 mg of tissue was weighed and put into 1 mL of stabilization reagent. The samples were disrupted and homogenized for 2 min using a tissue grinder (Mixer Mill MM 300, Qiagen, CA) at 20 Hz or MHz. Total RNA extraction was performed using the RNeasy Mini Kit (Qiagen); according to manufacture's instructions. 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), 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) and 1 μL of Moloney murine leukemia virus reverse transcriptase (200 U/μL). 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.

### 2.2.5. Real-time PCR

Gene specific primers and internal oligonucleotide probes for equine IL-8, IL-10 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) used were as 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, Foster City, CA). 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. Serial dilutions of cDNA from 24 h Concanavalin A-stimulated equine PBMCs were used to generate a standard curve for each gene of interest. Each sample 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. To account for variation in the amount and quality of starting material, all the results were normalized to G3PDH expression. The threshold cycle values for each gene were compared to its respective standard curve to generate a relative transcript concentration.

### 2.2.6. Immunohistochemistry

Peroxidase–antiperoxidase immunohistochemical reaction to detect MHC-II and PMN-TL positive cells in endometrial biopsies was used according to Sternberger et al. (1970). Monoclonal primary antibodies, developed in mouse were used. For MHC-II, anti-human HLA-DR Alpha Chain (M0746, DAKO®), that cross react with equine MHC-II expressing cells, diluted 1/10 was used (Kalsow et al., 1998). The WS 66, clone CVS 6 antibody, diluted 1/5 was used to mark PMNs and TL. This his antibody did not differentiate between both cell types. Positive reaction in paraffin-embedded cuts was previously described by Kalsow et al. (1998, 1999). A second anti-mouse antibody developed in rabbit (Anti-Mouse IgG M-7023, SIGMA®) diluted 1/25 in Tris Carragina Triton solution was used. Peroxidase–antiperoxidase complex developed in mouse (P2416SIGMA®) diluted 1/250 was used. Control sections were included in which the first antibody was replaced by normal horse serum; sections of lymph nodes were included as positive controls.

### 2.2.7. Morphometric evaluation

Image analysis was performed using digital software (Image-Pro Plus v 5.0; Media Cybernetics). The percentage of stained area was determined in five fields with a magnification of 400×. Immunoreactivity was quantified using an immunohistochemical scoring system that corresponds to an image analysis-based system, numeric data obtained from the image analysis were exported for statistical analysis according to Section 2.2.

Table 2
Baseline immunological parameters in endometrial tissue of RM and SM during estrus and diestrus in Cycle 1

| Estrus | | | | Diestrus | | |
|---|---|---|---|---|---|---|
| Parameter | RM | SM | *P*-value | RM | SM | *P*-value |
| MHC-II | 4.4 (2.1–11.8) | 24.1 (13–27.3) | 0.006 | 0.5 (0.1–4.3) | 0.8 (0.3–10.8) | 0.28 |
| TL, PMN | 7.6 (4.5–12.6) | 20.9 (16.0–28.8) | 0.0005 | 4.4 (1.8–4.7) | 16.6 (12.7–27.9) | 0.0001 |
| IL-8 | 69.3 (11.4–145.8) | 20101.2 (18,197–36,707) | 0.05 | 10.1 (6.4–30.9) | 82.2 (44.8–1464) | 0.0001 |
| IL-10 | 1667.0 (1233–3420) | 5.2 (3.4–102.1) | 0.0001 | 2.7 (1.9–6.3) | 1163.0 (967–1535) | <0.0001 |

MHC-II and TL, PMN were expressed as the percentage of stained area determined in an average of five fields with a magnification of 400×. Numeric data obtained from the image were exported for statistical analysis. The values represent median (range) relative mRNA expression within each group ($n = 8$).

## 3. Results

### 3.1. Immunological parameters in the control cycle (Cycle 1)

Baseline immunological parameters during estrus and diestrus for SM and RM are shown in Table 2. During estrus, SM had significantly higher levels of MHC-II, TL, PMN (Figs. 1 and 2) and IL-8 than RM ($P < 0.006$, $P < 0.0005$, $P < 0.05$, respectively), while IL-10 mRNA expression was significantly lower than in RM ($P < 0.0001$). During diestrus, SM had significantly higher levels of TL, PMN, IL-8 and IL-10 than RM ($P < 0.0001$).

### 3.2. Effect of artificial insemination (Cycle 2)

In endometrial samples taken 24 h after AI, RM showed significantly lower expression of IL-8 and higher expression of IL-10 than SM ($P < 0.0009$ and $P < 0.01$). TL, PMN and MHC-II did not differ significantly between the two groups of mares. However, during diestrus, MHC-II, TL, PMN and IL-8 were significantly higher in SM than in RM ($P < 0.005$, $P < 0.0004$, $P < 0.0001$ and $P < 0.02$). Differences in IL-10 mRNA expression between SM and RM during diestrus were not statistically significant (Table 3).

### 3.3. Effects of immunomodulator (Cycle 3)

In the endometrial samples collected 24 h after mares were inseminated and treated with the *M. phlei* cell-wall extract, the levels of MHC-II, TL, PMN and IL-8 expression between RM and SM were not significantly different. However, expression of IL-10 was significantly higher in SM than in RM ($P < 0.005$). During diestrus, all the immunological parameters studied did not differ between RM and SM (Table 4). Significant statistical differences between susceptible and resistant mares for all the immunological parameters analyzed are summarized in Table 5.

## 4. Discussion

This study describes the dynamics of MHC-II-expressing cells, TL, PMN, IL-8 and IL-10 expression

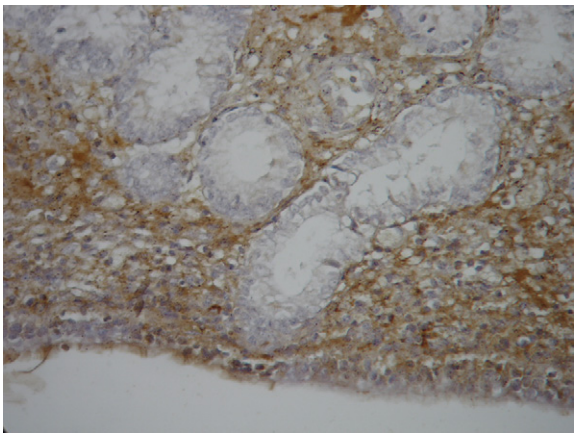

Fig. 1. Cells expressing MHC-II marked with M0746, DAKO® (40×).

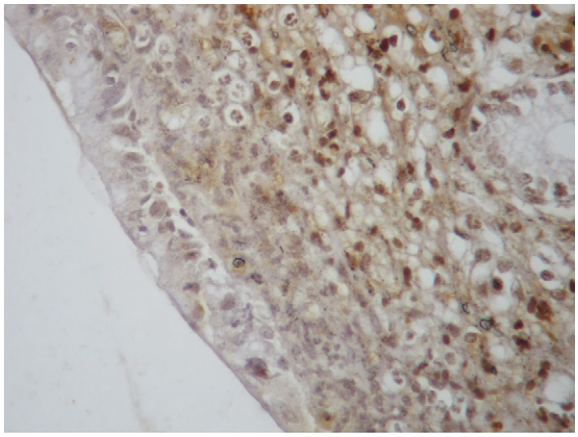

Fig. 2. Lymphocytes and PMN marked with WS 66, clon CVS 6 (40×).

Table 3
Immunological parameters in endometrial tissue of RM and SM 24 h after AI and during diestrus (Cycle 2)

| Parameter | Estrus | | | Diestrus | | |
|---|---|---|---|---|---|---|
| | Resistant | Susceptible | *P*-value | Resistant | Susceptible | *P*-value |
| MHC-II | 17.2 (3.8–27.5) | 18.1 (12.6–24.1) | 0.5 | 1.0 (0.1–2.8) | 15.1 (3.2–21.3) | 0.005 |
| TL, PMN | 23.4 (4.4–30.5) | 26.3 (14.9–40.1) | 0.3 | 6.3 (1.9–9.6) | 25.8 (21.1–33.4) | 0.0004 |
| IL-8 | 168.8 (114–1905) | 14859.0 (6497–16,437) | 0.0009 | 4.1 (4.1–6.8) | 832.2 (392–8075) | 0.0001 |
| IL-10 | 22.5 (11.8–456) | 4.5 (3.2–6.6) | 0.01 | 2.1 (1.1–2.4) | 20.0 (5.5–214.4) | 0.02 |

MHC-II and TL, PMN were expressed as the percentage of stained area determined in an average of five fields with a magnification of 400×. Numeric data obtained from the image were exported for statistical analysis. The values represent median (range) relative mRNA expression within each group ($n = 8$).

Table 4
Immunological parameters in endometrial tissue of RM and SM 24 h after AI plus immunomodulation treatment and in diestrus (Cycle 3)

| Parameter | Estrus | | | Diestrus | | |
|---|---|---|---|---|---|---|
| | Resistant | Susceptible | *P*-value | Resistant | Susceptible | *P*-value |
| MHC-II | 5.8 (5.3–13.6) | 13.0 (7.1–27.7) | 0.5 | 1.0 (0.3–1.4) | 0.4 (0.1–2.6) | 0.24 |
| TL, PMN | 16.1 (8.3–34.3) | 23.2 (12.4–33.1) | 0.5 | 4.6 (2.9–5.0) | 8.2 (3.8–10.9) | 0.09 |
| IL-8 | 173.8 (24.4–10,512) | 23460.0 (650–46,718) | 0.28 | 16.9 (10.3–25.4) | 13.8 (4.0–31.5) | 0.23 |
| IL-10 | 6.5 (3.7–12.1) | 1941.0 (607–7859) | 0.005 | 5.5 (1.7–8.3) | 4.7 (2.1–7.0) | 0.92 |

MHC-II and TL, PMN were expressed as the percentage of stained area determined in an average of five fields with a magnification of 400×. Numeric data obtained from the image were exported for statistical analysis. The values represent median (range) relative mRNA expression within each group ($n = 8$).

in resistant and susceptible mares during estrus and diestrus and under different conditions. To our knowledge this is the first study showing mRNA transcription of IL-8, a chemokine playing a key role in neutrophil chemotaxis, and T cell (Miller and Krangel, 1992), and IL-10, an important anti-inflammatory cytokine, in the endometrium of mares. The effect of AI on cells expressing MHC-II was also analyzed, apparently for the first time. In the untreated cycle (Cycle 1), SM had a different immune profile than RM. Susceptible mares had higher numbers of PMNs, positive MHC-II cells and higher IL-8 mRNA expression during estrus, which is consistent with an inflamed endometrium. Therefore, this study confirms key differences in some basic immunological parameters between SM and RM that could explain why SM maintains an inflamed endometrial status. The constantly high IL-8 expression in the endometrial tissue of SM throughout the estrous cycle may in part explain the continuous inflammatory response seen in these mares. Interleukin-8 belongs to the chemokine family and is responsible for chemotactic migration and activation of neutrophils and other cell types (such as monocytes, lymphocytes, basophils and eosinophils) at sites of inflammation (Miller and Krangel, 1992; Stoeckle and Barker, 1990). The main inflammatory impact of IL-8 lies in its chemotactic effect on neutrophils and its ability to stimulate granulocyte activity. In addition, IL-8 is involved in neutrophil recruitment by up-regulating cell-surface adhesion molecule expression (such as endothelial leukocyte adhesion molecule, ELAM-1, and intracellular adhesion molecule, ICAM-1), thereby enhancing neutrophil adherence to endothelial cells (Warren, 1990) and facilitating diapedesis through vessel walls. Thus, IL-8 mediates the recruitment and activation of neutrophils in inflamed tissue (Huber et al., 1991). Uterine immunity involves both innate and adaptive mechanisms: antibody opsonization of bacteria, phagocytosis of microorganisms and foreign bodies (including spermatozoa), and physical clearance enhanced by uterine contractility (Troedsson et al.,

Table 5
Comparison of SM and RM over three cycles

| Parameter | Cycle 1: Control | | Cycle 2: AI | | Cycle 3: AI + MCWE | |
|---|---|---|---|---|---|---|
| | Estrus | Diestrus | Estrus | Diestrus | Estrus | Diestrus |
| MHC-II | ↑ | ND | ND | ↑ | ND | ND |
| TL, PMN | ↑ | ↑ | ND | ↑ | ND | ND |
| IL-8 | ↑ | ↑ | ↑ | ↑ | ND | ND |
| IL-10 | ↓ | ↓ | ↓ | ↑ | ↑ | ND |

TL: T lymphocytes; PMN: polymorphonuclear cells, ND: no significant differences, ↑: SM significantly higher than RM, ↓: SM significantly lower than RM.

1990; Troedsson, 1997, 1999, 1995; Watson et al., 1987; LeBlanc et al., 1991). A number of studies have been conducted in order to detect differences in immune parameters between resistant and susceptible mares. In a study by Hansen and Asbury (1987), no defect in phagocytosis was found as a primary dysfunction of neutrophils in SM. Although phagocytosis of yeast blastospores by blood neutrophils from RM and SM was similar, uterine neutrophils from SM showed decreased phagocytic activity compared to uterine neutrophils from RM (Asbury and Hansen, 1987; Watson et al., 1987; Watson and Thomson, 1996). Immunoglobulin production also was studied and it was shown to be slightly higher in SM than in RM (Asbury et al., 1980; Mitchell et al., 1982). The efficiency of uterine defense mechanisms in the mare does not seem to depend solely on humoral factors such as immunoglobulins. Defects involving other components such as complement and other unidentified components of the defense system may contribute to failure of the uterus to clear infection (Asbury et al., 1980; Waelchli and Winder, 1991). Neutrophils accumulate mostly within the lumen of the uterus, and are rarely seen in the lamina propia, except during acute inflammation or estrus. While the presence of PMNs during inflammation is brief, macrophages can remain in inflamed tissue for months (MacKay, 2000); for this reason, mononuclear infiltration of the lamina propia is associated with repeated or chronic infections in SM (Causey, 2006). Macrophages, antigen-activated T cells, endothelial and epithelial cells, and even neutrophils produce IL-8 which may explain the high levels of this cytokine and subsequently the more abundant PMN cells and lymphocytes in SM. Although, the antibody used cannot differentiate between PMNs and T cells, it was demonstrated that in mares susceptible to endometritis, both types of cells could be increased (Troedsson, 1997; Watson and Thomson, 1996). MHC-II is a protein expressed on the surface of immune and non-immune cells and is involved in antigen presentation. Based on the results observed in Cycle 1, it appears that an increased number of infiltrated macrophages in the endometrial tissue of SM could lead to increased IL-8 mRNA expression creating a vicious circle and maintaining the inflammatory condition over time. The anti-inflammatory effect of IL-10, prohibiting human monocytes (at mRNA level) from producing IL-1 alpha, IL-1 beta, IL-6, IL-8, TNF alpha, granulocyte-macrophage colony stimulating (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) following activation has been demonstrated by Khatri and Caligiuri (1998). In addition, MHC-II expression is down-regulated by IL-10 (Khatri and Caligiuri, 1998). During estrus in Cycle 1, we found that IL-10 was lower in SM than in RM, which could contribute to the maintenance of the inflamed state in SM because of the lack of an anti-inflammatory stimulus. During diestrus of Cycle 1, SM maintained higher levels of PMN, TL and IL-8. Although SM showed higher expression of IL-10 mRNA, it appeared to be insufficient to control the inflammation. During Cycle 2, we evaluated the effect of semen as an inflammatory stimulus. Twenty-four hours after AI, no differences for PMN, TL, and MHC-II expression were detected between SM and RM. It is very likely both groups of mares reacted to the AI in a similar manner with migration of PMN and other inflammatory cells into the endometrial tissue. However, differences in cytokines profiles were still evident: SM had higher levels of IL-8 and lower levels of IL-10 than RM.

During diestrus Cycle 2, RM had a lower number of immune cells present in the endometrium, which would indicate that the inflammatory process was already resolved by day 7. In contrast, SM had high levels of all immune parameters studied which would indicate that the inflammatory process created by the AI continued 7–8 days after ovulation. These results agree with those presented by others about the inability of SM to restore the uterine environment after coitus or AI (Troedsson, 1999; Watson, 2000). If we assume that RM are a model for the normal inflammatory response after AI, the cytokine of SM after AI appears altered (higher IL-8, lower IL-10) and could explain the persistence of the inflammatory response during diestrus. This persistent cytokine pattern consisting of higher pro-inflammatory and lower anti-inflammatory cytokine levels observed in the endometrium of SM in estrus and diestrus (Cycle 1), and with and without insemination (Cycles 1 and 2) may explain the low reproductive performance of these mares.

In humans, it has been shown (Srivastava et al., 1996; Kelly et al., 1997) that seminal plasma induces anti- and pro-inflammatory immune responses in the endometrium due to IL-10 and IL-8, respectively, which may act in combination, initially to promote sperm survival and then to facilitate sperm removal and endometrial clearance. It has been shown that human seminal plasma contains both IL-10 and IL-8 (Denison et al., 1999). Mares subject to AI with frozen semen exhibit endometritis, due mainly to intact or damaged spermatozoa, which produce a similar inflammatory reaction to that produced in humans (Kotilainen et al., 1994). Troedsson et al. (1993) demonstrated that the persistent inflammation in SM is not due to defective immunoglobulin G (IgG), IgA or complement concentrations. In addition, primary defects

in neutrophil phagocytosis or opsonization could not be demonstrated (Asbury and Hansen, 1987; Brown et al., 1985). Uterine lymphatic drainage appears impaired in SM and the delay in uterine clearance may be related to a pendulous uterus and stretching of the broad ligament (LeBlanc et al., 1995, 1998). A depressed frequency, duration and intensity of myometrial contractions following intrauterine inoculation of *S. zooepidemicus* in SM has also been demonstrated by Troedsson et al. (1993). A recent study suggests a possible role of nitric oxide (NO) in delaying uterine clearance in SM, mediated by smooth muscle relaxation (Alghamdi et al., 2005). It is known that inflammation results in synthesis and release of prostaglandins and NO (Wheeler et al., 1997; Jabbour and Sales, 2004). Considering that IL-10 is a powerful NO inhibitor (Ferlazzo et al., 2003), the lower transcription of IL-10 observed in SM in the present study could have led to increased levels of NO and an inefficient clearance mechanism by inhibiting smooth muscle contraction of the uterus.

In Cycle 3, the endometrial expression of the pro-inflammatory cytokine IL-8 24 h after AI and immunomodulation was reduced in SM to values similar to those found in RM. During diestrus, no differences were detected between SM and RM in any of the immune parameters studied, except for IL-10 which was over-expressed in SM. Therefore, based on these results, it can be assumed that administration of MCWE at the time of endometrial challenge induced changes in the uterine immune system of SM that helped to re-establish a normal endometrial environment.

The combination of AI as inflammatory stimulus plus the pathogen-associated molecular patterns from MCWE probably produced a dramatic increase of IL-10 in SM, powerful enough to control IL-8 and to decrease MHC-II positive cells, PMN and TL during diestrus. It is known that immunomodulators like MCWE contain active compounds that bind to surface receptors on macrophages and dendritic cells, resulting in cell activation and subsequent cytokine induction (Underhill et al., 1999). The cell-wall preparation of *Mycobacterium* spp. contains potent pathogen-associated molecular patterns such as lipoarabinomannan (LAM), trehalose dimycolate (TDM) and mycolic acid (MA). These compounds are known to trigger the expression of several cytokines including IL-6, TNF-α, IL-1β, IL-10 and IL-12 (Indrigo et al., 2003). Another effect of LAM is maturation of phagosome arrest (Korf et al., 2005), whereas TDM, in particular, promotes prolonged survival and mediates trafficking events of macrophages (Korf et al., 2005). In addition, mycolic acid, upon stimulation of the cells, induces antibacterial and cellular immune defenses (Korf et al., 2005). Moreover, macrophages treated with mycolic acid and TDM secreted IL-6, IL-10 and TNF-α upon secondary exposure to inflammatory triggers (Korf et al., 2005). In the present study, treatment with *M. phlei* cell-wall extract at the time of AI triggered a uterine immune response that modified the endometrial environment of the SM. The active agents and/or the mechanisms of action of the immunomodulator that triggered the effects found in this study in the SM are still not known.

This study cannot explain if the low levels of IL-10 in SM are due to a prolonged exposure to mitogen-activated protein kinase phosphatase, a protein that negatively regulates the synthesis of IL-10 or to non-activated macrophages (Chi et al., 2006). Elevated expression of IL-8 throughout the cycle and low expression of IL-10 in the endometrial tissue of SM appear to be major factors that may explain the reduced capacity of these animals to resolve the post-breeding endometritis. Additionally, it seems likely that IL-10 plays a key role in regulating the mechanism of action of the other immune parameters in the mare uterine environment.

Summarizing, the different levels in expression of endometrial IL-8, IL-10 and MHC-II and PMN-T cells, observed between RM and SM during the untreated cycle were mostly maintained following AI and disappeared after treatment with MCWE. It appears that treating susceptible mares with MCWE can assist in restoring homeostatic uterine immune mechanisms thereby controlling post-breeding endometritis through a powerful IL-10 stimulation. The significance of this immune modulation observed in SM needs to be further investigated in terms of pregnancy rates.

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## References

Alghamdi, A., Fosster, D., Carlson, C., Troedsson, M., 2005. Nitric oxide levels and nitric oxide synthase expression in uterine samples from mares susceptible and resistant to persistent breeding-induced endometritis. Am. J. Reprod. Immunol. 53, 230–237.

Asbury, A.C., Halliwell, R.E.W., Foster, G.W., Longino, S.J., 1980. Immunoglobulins in uterine secretions of mares with differing resistance to endometritis. Theriogenology 14, 299–308.

Asbury, A.C., Hansen, P.J., 1987. Effects of susceptibility of mares to endometritis and stage of cycle on phagocytic activity of uterine-derived neutrophils. J. Reprod. Fertil. 35 (Suppl), 311–316.

Brown, A.E., Hansen, P.J., Asbury, A.C., 1985. Opsonization of bacteria by uterine secretions of cyclic mares. Am. J. Reprod. Immunol. Microbiol. 9 (4), 119–123.

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.

Causey, R.C., 2006. Making sense of equine uterine infections: the many faces of physical clearance. Vet. J. 72 (3), 405–421.

Chi, H., Barry, S.P., Roth, R.J., Wu, J.J., Jones, E.A., Bennett, A.M., Flavell, R.A., 2006. Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. PNAS 103 (7), 2274–2279.

Denison, F.C., Grant, V.E., Calder, A.A., Kelly, R.W., 1999. Seminal plasma components stimulate interleukin-8 and interleukin-10 release. Mol. Hum. Reprod. 5, 220–226.

Ferlazzo, V., D'Agostino, P., Milano, S., Caruso, R., Feo, S., Cillari, E., Parente, L., 2003. Anti-inflammatory effects of annexin-1: stimulation of IL-10 release and inhibition of nitric oxide synthesis. Int. Immunopharmacol. 3, 1363–1369.

Fumuso, E., Giguère, S., Wade, J., Rogan, D., Videla-Dorna, I., Bowden, R., 2003. Endometrial IL-1 β, IL-6 and TNF-α, mRNA expression in mares resistant or susceptible to post-breeding endometritis; effects of estrous cycle, artificial insemination and immunomodulation. Vet. Immunol. Immunopathol. 96, 23–41.

Garton, N.J., Gilleron, M., Brando, T., Dan, H.H., Giguere, 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. 277 (35), 31722–31733.

Hansen, P.J., Asbury, A.C., 1987. Opsonins of Streptococcus in uterine flushings of mares susceptible and resistant to endometritis: control of secretion and partial characterization. Am. J. Vet. Res. 48 (4), 646–650.

Huber, A.R., Kunkel, S.L., Todd, R.F., Weiss, S.J., 1991. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science 254, 99–102.

Indrigo, J., Hunter, R.L., Actor, J.K., 2003. Cord factor trehalose 6,6′-dimycolate (TDM) mediates trafficking events during mycobacterial infection of murine macrophages. Microbiology 149 (8), 2049–2059.

Jabbour, H.N., Sales, K.J., 2004. Prostaglandin receptor signalling and function in human endometrial pathology. Trends Endocrinol. Metab. 15, 398–404.

Kalsow, C., Albrecht, T., Steinberg, N., Lunn, D., 1998. Antibody selection for immunohistochemical survey of equine tissue. J. Comp. Pathol. 119, 467–472.

Kalsow, C., Dubielzig, R., Dwyer, A., 1999. Immunopathology of pineal glands from horses with uveitis. Invest. Ophthalmol. Vis. Sci. 40 (7), 1611–1615.

Khatri, V.P., Caligiuri, M.A., 1998. Interleukin 10 and its receptor. In: Delves, P.J., Roit, I.M. (Eds.), Encyclopedia of Immunology, second ed. Academic Press, San Diego, USA, pp. 1475–1478.

Kelly, R.W., Carr, G.G., Critchley, H.O., 1997. A cytokine switch induced by human seminal plasma: an immune modulation with implications for sexually transmitted disease. Hum. Reprod. 12 (4), 677–681.

Korf, J., Stoltz, A., Verschoor, J., De Baetselier, P., Grooten, J., 2005. The Mycobacterium tuberculosis cell wall component mycolic acid elicits pathogen-associated host innate immune responses. Eur. J. Immunnol. 35, 890–900.

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

LeBlanc, M., Ward, L., Tran, T., Widders, P., 1991. Identification and opsonic activity of immunoglobulins recognizing *Streptococcus zooepidemicus* antigens in uterine fluids of mares. J. Reprod. Fertil. 44 (S), 289–296.

LeBlanc, M., Johnson, R., Calderwood Mays, M., Valderrama, C., 1995. Lymphatic clearance of india ink in reproductively normal mares and mares susceptible to endometritis. Biol. Reprod. Mono. 1, 501–506.

LeBlanc, M.M., Neuwirth, L., Jones, L., Cage, C., Mauragis, D., 1998. Differences in uterine position of reproductively normal mares and those with delayed uterine clearance detected by scintigraphy. Theriogenology 50 (1), 49–54.

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

McKinnon, A.O., Squires, E.L., Harrison, L.A., Blach, E.L., Shideler, R.K., 1988. Ultrasonographic 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.

Miller, M.D., Krangel, M.S., 1992. Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. Crit. Rev. Immunol. 12, 17–46.

Miragaya, M.H., Woods, G.L., Losinno, L., 1997. Endometritis, salpingitis and fertilisation rates after mating mares with a history of intrauterine luminal fluid accumulation. Equine Vet. J. 25 (Suppl.), 109–112.

Mitchell, G., Liu, I.K., Perryman, L.E., Stabenfeldt, G.H., Hughes, J.P., 1982. Preferential production and secretion of immunoglobulins by the equine endometrium-a mucosal immune system. J. Reprod. Fertil. 32 (Suppl.), 161–168.

Singh, J., Sidhu, S.S., Dhaliwal, G.S., Pangaonkar, G.R., Nanda, A.S., Grewal, A.S., 2000. Effectiveness of lipopolysaccharide as an intrauterine immunomodulator in curing bacterial endometritis in repeat breeding cross-bred cows. Anim. Reprod. Sci. 59, 159–166.

Srivastava, M.D., Lippes, J., Srivastava, B.I., 1996. Cytokines of the human reproductive tract. Am. J. Reprod. Immunol. 36 (September (3)), 157–166.

Sternberger, L.A., Hardy, P.H., Cuculis, J.J., Meyer, H.G., 1970. The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J. Histochem. Cytochem. 18 (May (5)), 315–333.

Stoeckle, M.Y., Barker, K.A., 1990. Two burgeoning families of platelet factor 4-related proteins: mediators of the inflammatory response. New Biol. 2, 313–323.

Tremellen, K.P., Seamark, R.F., Robertson, S.A., 1998. Seminal transforming growth factor beta1 stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus. Biol. Reprod. 58 (5), 1217–1225.

Troedsson, M., Concha, C., Einarsson, S., Holmberg, O., 1990. A preliminary study of uterine derived polymorphonuclear cell function in mares with chronic uterine infections. Acta Vet. Scand. 31, 187–192.

Troedsson, M., Liu, I., Thurmond, M., 1993. Immunoglobulin (IgG and IgA) and complement (C3) concentrations in uterine secretions following an intrauterine challenge of *Streptococcus zooepidemicus* in mares susceptible to versus resistant to chronic uterine infection. Biol. Reprod. 49, 502–506.

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.

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., 2000. Manual de citología Endometrial en yeguas. Rev. Col. Cienc. Pec. 13S (S), 33.

Waelchli, R.O., Winder, N.C., 1991 Nov. Mononuclear cell infiltration of the equine endometrium: immunohistochemical studies. Equine Vet. J. 23 (November (6)), 470–474.

Warren, J.S., 1990. Interleukins and tumor necrosis factor in inflammation. Crit. Rev. Clin. Lab. Sci. 28, 37–59.

Watson, E., Stokes, C., Bourne, F., 1987. Cellular and humoral mechanisms in mares susceptible and resistant to persistent edometritis. Vet. Immunol. Immunopathol. 16, 107–121.

Watson, E.D., 1989. Release of immunoreactive arachidonate metabolites by equine endometrium in vitro. Am. J. Vet. Res. 50 (8), 1207–1209.

Watson, E.D., Dixon, C.E., 1993. An immunohistological study of MHC class II expression and T lymphocytes in the endometrium of the mare. Equine Vet. J. 25 (2), 120–124.

Watson, E., Thomson, R., 1996. Lymphocyte subsets in the endometrium of genitally normal mares susceptible to endometritis. Equine Vet. J. 28, 106–110.

Watson, E., July 2000. Post-breeding endometritis in the mare. Proc. 14th Internat. Congress Animal Reproduction. Elsevier, Stockholm, pp. 221–232.

Wheeler, M.A., Smith, S.D., Garcia-Cardena, G., Nathan, C.F., Weiss, R.M., Sessa, W.C., 1997. Bacterial infection induces nitric oxide synthase in human neutrophils. J. Clin. Invest. 99, 110–116.

Zerbe, H., Schuberth, H.J., Engelke, F., Frank, J., Klug, E., Leibold, W., 2003. Development and comparison of in vivo and in vitro models for endometritis in cows and mares. Theriogenology 60 (2), 209–223.