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Effect of immunomodulatory therapy on the endometrial inflammatory response to induced infectious endometritis in susceptible mares

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Abstract

The objective of the present study was to evaluate the effect of immunomodulatory therapy (glucocorticoids (GC) and mycobacterium cell wall extract (MCWE)) on the endometrial gene expression of inflammatory cytokines in susceptible mares with induced infectious endometritis. Endometrial gene expression of pro- and anti-inflammatory cytokines; interleukin (*IL)-1β*, *IL-6, IL-8, IL-10*, tumor necrosis factor (TNF)-α, *IL-1* receptor antagonist (ra), acute phase protein (APP) serum amyloid A (SAA) and clinical parameters were evaluated. Five mares were classified as susceptible to persistent endometritis based on their endometrial histopathology and ability to clear an induced uterine inflammation. To investigate the effect of immunomodulatory therapy, the mares were inoculated with 10^5 colony forming units (CFU) *Escherichia coli* in three consecutive estrus cycles in a modified cross-over study design. Thus, each mare served as its own control and the treatment type was performed in randomized order. The effect of treatment with MCWE (1.5 mg Settle IV), dexamethasone (0.1 mg per kg IV) or no treatment was investigated. All mares were free from uterine inflammation before each *E. coli* inoculation. Endometrial biopsies were recovered 3, 24 and 72 h post inoculation. Relative gene-expression analyses were performed by quantitative reverse transcriptase PCR (qRT-PCR). Endometrial gene expression of inflammatory cytokines was modulated by administration of GC. Expression of proinflammatory cytokines (*IL-1β, IL-6, IL-8*) and *SAA* was significantly lower in the GC treated group late in the study period (72 h) compared to "no treatment" and MCWE treatment. Increased expression of the anti-inflammatory cytokine *IL-10* was observed 3 and 24 h after *E. coli* infusion and GC treatment. A significant decrease of *SAA* expression was observed after MCWE treatment compared to "no treatment". MCWE and GC treatment had a significant effect on the clearance of uterine pathogens and number of mares retaining fluid after *E. coli* infusion. The results of the current investigation suggest that GC is capable of effectively modulating the innate immune response to induced infectious endometritis in susceptible mares.

Keywords: Infectious endometritis; Immunomodulatory therapy; Inflammatory cytokines; Serum amyloid A

1. Introduction

Transient breeding-induced endometritis is a normal event immediately following breeding, and the inflammatory response is necessary for the effective removal of debris, bacteria and excess spermatozoa from the

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uterine lumen [1]. In the healthy uterus, inflammation is resolved well before the embryo descends from the oviduct into the uterine lumen 5 to 6 days after ovulation and conception [2]. A sterile and non-inflamed uterine environment is necessary for survival of the early embryo and maintenance of pregnancy [3]. Mares can be classified as resistant or susceptible to persistent endometritis based on their ability to clear uterine inflammation and infection [4]. A resistant mare is typically capable of clearing infectious endometritis within 48 h, whereas a susceptible mare will remain infected beyond 96 h [5]. Impaired and reduced myometrial activity will lead to intrauterine fluid accumulation [6] and will, together with dysfunctional opsonization, result in impaired phagocytosis of pathogens [7,8]. These factors seem to be major contributors to the pathogenesis of susceptibility to persistent endometritis.

An innate immune response will be activated by the presence of bacteria and semen within the uterine lumen [9–13], and is primarily initiated through activation of blood monocytes, dendritic cells and tissue macrophages [14,15]. Inflammatory cytokines modulate the acute phase response that involves potent systemic and local effects, and function to minimize tissue damage and promote repair processes, and thereby rapidly restore normal physiological function by host homeostatic mechanisms [15]. Increased endometrial gene expression of proinflammatory cytokines has been demonstrated in mares with sperm-induced endometritis [11,12,16]; in response to artificial insemination (AI) with seminal plasma, semen extender and phosphate buffered saline (PBS) [16], and in mares with experimentally induced *E. coli* endometritis [9,17]. It has been demonstrated that susceptible mares exhibit a sustained endometrial inflammatory response to intrauterine inoculation of *E. coli* compared to resistant mares [17]. Traditionally, mares with persistent endometritis (susceptible mares) are treated with uterine lavage, antibiotics and ecbolic drugs to clear uterine inflammation and infection [18,19]. Few studies have investigated the use of non-specific immunomodulatory therapy in mares susceptible to persistent endometritis [12,20,21]. Over the past 30 years, mycobacterium cell wall skeletons (MCWS) have been used for anticancer therapy because of their ability to stimulate the immune system, including induction of cytokine synthesis by immune cells [22]. Mycobacterium cell wall extract (MCWE) from *Mycobacterium phlei* cell walls has previously been suggested to modulate expression of some cytokines in susceptible mares after induction of uterine inflammation (AI with killed semen) [12,23] and to improve clearance of uterine infection and inflammation in susceptible mares experimentally infected with *Streptococcus equi* subsp. *zooepidemicus* [21].

Glucocorticoid (GC) therapy is a common medication to manage inflammatory diseases because of potent antiinflammatory and immunosuppressive properties. In mares with multiple factors that predispose them to persistent endometritis, administration of GC has been demonstrated to increase pregnancy rates compared to control mares [20,24]. Although these few studies have focused on the effect of immunomodulatory therapy in susceptible mares, there are no reports relating GC therapy to the kinetics of the inflammatory response and presence of pathogens in the uterine lumen.

The aim of the present study was to evaluate the effect of immunotherapy (GC and MCWE) on endometrial gene expression of inflammatory cytokines, *SAA*, bacteriologic and cytologic examinations of uterine samples and intrauterine fluid accumulation in susceptible mares with induced infectious endometritis.

2. Materials and methods

2.1. Selection of mares

In total, 48 non-pregnant mares were screened for susceptibility to persistent endometritis as described by Christoffersen, et al. [17]. In summary, a histopathological evaluation of the endometrium according to the Kenney scale [25] and the mares' ability to clear induced uterine inflammation was evaluated. Mares with a grade IIb or III endometrium [25], a decreased ability to clear intrauterine inflammation ($>$ 2 polymorphonuclear neutrophils (PMN) per 5 fields at $\times$400 magnification), and intrauterine fluid 96 h after AI with 10^9 killed spermatozoa solubilized in milk based extender, were selected for the study. Five mares of mixed breeds and a mean age of 20 yrs (range 12–25 yrs) were selected and used in the present study. All mares were maintained at the Department of Veterinary Science's Maine Chance Farm, University of Kentucky, Lexington, KY, USA. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

2.2. E. coli inoculation and collection of uterine samples

To investigate the effect of immunomodulatory therapy, the mares were inoculated with *E. coli* in three consecutive estrus cycles in a modified cross-over study design. In the first estrus cycle, all mares were inocu-

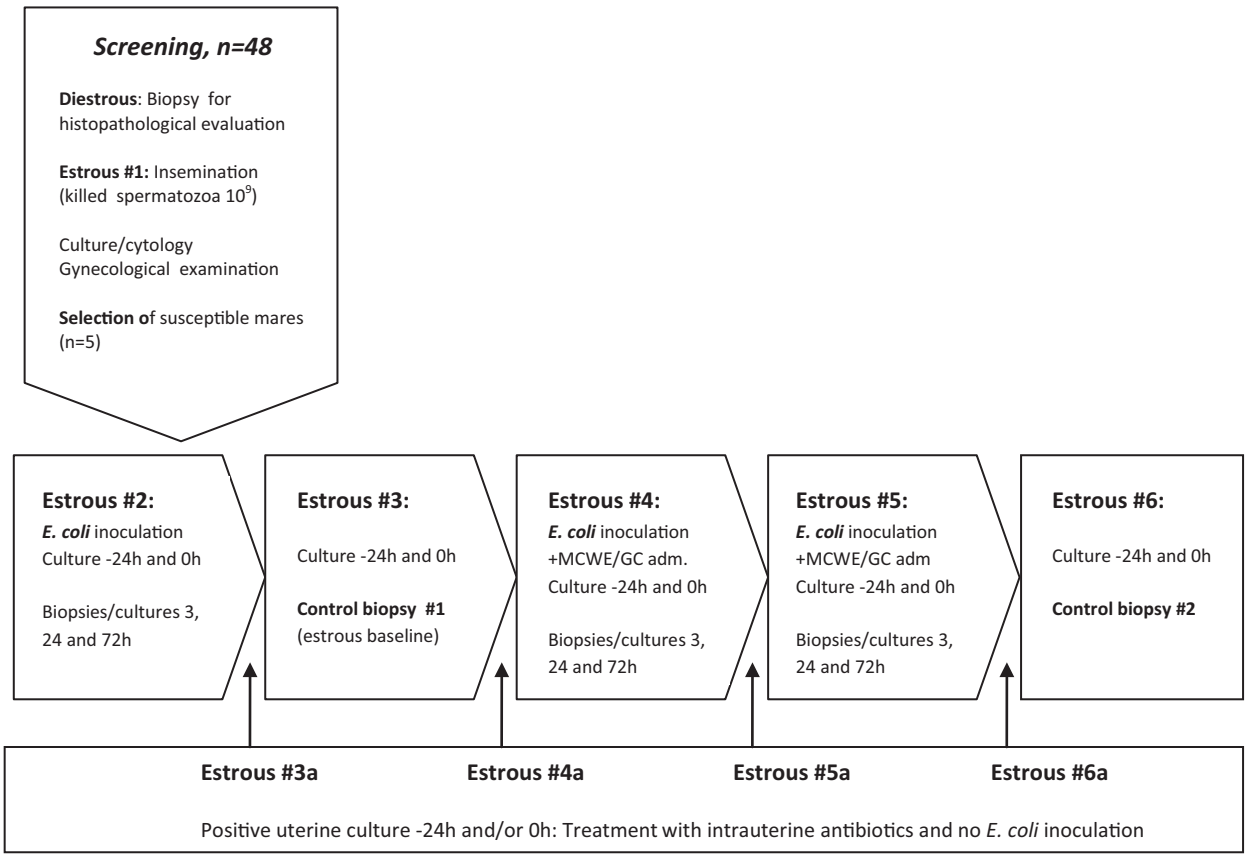


Fig. 1. Flow chart depicting the steps and tests used for selecting susceptible mares for the study, and the timeline for *E. coli* inoculations, administration of immunomodulatory therapy, and collection of uterine samples.

lated with *E. coli* and no immunomodulatory therapy was administered. In the following cycle, control biopsies were obtained, and in the two subsequent estrus cycles, the mares were inoculated with *E. coli* and treated with GC and MCWE in a randomized order (Fig. 1).

Mares were examined daily for follicular development, intrauterine fluid, development of uterine edema and uterine and cervical tone. When presence of a dominant follicle (> 25 mm), uterine edema and decreased uterine and cervical tone was detected, a uterine swab sample (Minitube of America, Verona, WI, USA) was collected. *E. coli* was infused when a follicle larger than 30 mm was observed (24–48 h later). The double guarded uterine swab method was used because it was considered least sensitive to contamination of bacteria from the vaginal flora compared to, e.g., a low volume uterine lavage. Immediately before inoculation, mares were prepared as for AI, and a uterine swab sample was collected (0 h) for bacterial culture and cytology to verify a sterile and non-inflamed uterine environment at time of inoculation. A total of 10^5 colony forming units (CFU) of *E. coli* in 10 ml of PBS (pH 7.4) was infused into the uterus via a sterile insemination catheter (Butler Schein Animal Health, Dublin, OH, USA) of each mare in each estrus cycle. In the two following estrus cycles, challenges were performed by intrauterine infusion of 10^5 CFU of *E. coli* and random assignment to the two different treatments: 1) 0.1 mg per kg dexamethasone (Butler Schein Animal Health) IV at the time of *E. coli* infusion, 2) 1.5 mg Settle (Bioniche Animal Health, Athens, GA, USA) IV 24 h before *E. coli* infusion (manufacturer recommends administration 24 h before breeding). The dose of dexamethasone (0.1 mg/kg IV) has previously been reported as a safe dose for treatment of post breeding endometritis [20]. A previous study has evaluated the route of administration of Settle, and found intravenous and intrauterine administration as effective in clearance of inoculated *S. zooepidemicus* [21]. Mares with positive uterine cultures after *E. coli* inoculation and the last sample collection at 72 h (*E. coli* or other pathogens)

were treated for three to five days during estrous with intrauterine antibiotics according to sensitivity testing and intrauterine lavage and oxytocin (10 IU IM b.i.d.) either in the inoculation cycle or the subsequent cycle. Only mares free of infection and inflammation evaluated by the uterine swab sample obtained 24 to 48 h before inoculation, were inoculated with *E. coli*. The *E. coli* strain (241) was originally isolated from a mare with infectious endometritis, and previously used in an experimental endometritis model in mares [9,17].

Transrectal ultrasonography of the reproductive organs was performed 0, 3, 24, 48 and 72 h after *E. coli* inoculation for detection of intrauterine fluid and ovary status. More than 2 cm depth of intrauterine fluid was recorded as fluid retention (measured by a single measurement within the uterine body and/or horns). Uterine swab samples and endometrial biopsies were collected using an alligator jaw biopsy punch (Equivet; Kruuse, A/S, Langeskov, Denmark) introduced into the uterus through a sterile speculum (Equivet) 3, 24 and 72 h after *E. coli* infusion. The mares received 2500 IU of human chorionic gonadotropin (Chorulon; Intervet, Millsboro, DE, USA) IV as an ovulating-inducing agent when a follicle >35 mm and pronounced uterine edema was present.

2.3. Control biopsies

In the estrus following the first and last *E. coli* inoculation, uterine swab samples were collected from the mares to test for bacterial growth and uterine inflammation. If the swabs were sterile and had negative cytology, control biopsies were collected 24 h later. If the mares had growth of uterine pathogens and/or positive cytology, they were treated with intrauterine lavage and antimicrobials according to sensitivity testing, and a control biopsy was collected in the following estrus (providing negative cytology and no growth of pathogens from a new uterine swab). All mares had no growth of pathogens and negative cytology from a uterine swab sample at the time point for obtaining the control biopsy. The control biopsy collected in the estrus cycle following the initial *E. coli* infusion was used as "estrus baseline level" and gene expression levels after *E. coli* infusions were expressed relative to this baseline (n-fold change to estrus baseline level). The control biopsy collected in the estrus cycle following the last *E. coli* infusion was collected to investigate if gene expression levels of inflammatory cytokines returned to estrus baseline levels following several *E. coli* infusions and immunomodulatory therapy.

2.4. Preparation of E. coli inocula

E. coli kept at −80 °C was streaked on a blood agar plate (5% horse blood) and incubated for 24 h at 37 °C. A single colony was transferred to 2 ml of sterile Brain Heart Infusion broth (Fischer Scientific, Pittsburgh, PA, USA) and incubated overnight at 37 °C. A serial dilution of the overnight broth was performed using sterile PBS to 10^6 CFU per mL and then diluted in 9 ml of sterile PBS to a final concentration of 10^5 CFU per inoculum. The inocula were kept on ice until use (maximum 2 h).

2.5. Bacterial examination and exfoliative cytology of endometrial swabs and biopsies

Immediately after sampling, endometrial biopsies were divided into two pieces with a sterile scalpel. One part of the biopsy was dissected into small pieces (1–2 mm) and stored in RNA later (Ambion, Austin, TX, USA) at 4 °C for 24 h, followed by storage at −20 °C until further processing. The other part of the biopsy and the uterine swab were streaked on a blood agar (5% horse blood) and incubated aerobically for 24 h at 37 °C. Bacterial growth was identified according to colony morphology, Hancock stain-morphology, hemolysis and catalase and potassium hydroxide (3% KOH) tests. Colonies were counted on blood agar plates and scored: no growth/sterile: < 5 CFU; mild growth: 5 to 10 CFU; moderate growth: 11 to 50 CFU; and heavy growth: >50 CFU. Culture results were recorded as *E. coli*, β-hemolytic streptococci, other uterine pathogens or no growth. When more than three different isolates were present, the culture was recorded as contamination. Following plating for culture, the biopsies and swabs were smeared on glass slides which were dried at room temperature and stained with Diff-Quick (Fisher Scientific), and evaluated by light microscopy (×400 magnification). Cytologic classification of the uterine biopsies and swabs was based on the number of PMNs present per 200 endometrial cells examined [26]. The PMNs were counted and scored: no inflammation, 0–1 PMN; mild inflammation 2 PMNs; moderate inflammation, 3 to 4 PMNs; and severe inflammation, ≥ 5 PMNs.

2.6. Quantitative RT-PCR analyses

Total RNA was isolated from 60 mg of endometrial tissue stored in RNA later using 650 μl TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. A total RNA isolation kit (Promega, Mannheim, Germany), including DNase treatment was

Table 1
Primer sequences used for qRT-PCR.

Target (gene)	Primer sequence (5′–3′)	Product size Bp	GenBank accession number/primer source
SAA F	CCT GGG CTG CTA AAG TCA TC	169	[27]
SAA R	AGG CCA TGA GGT CTG AAG TG		
TNF-α F	GGC CCA GAC ACT CAG ATC AT	73	[9]
TNF-α R	TTG GGG GTT TGC TAC AAC AT		
IL-1β F	CAG TCT TCA GTG CTC AGG TTT CTG	84	[9]
IL-1β R	CAT TGC CGC TGC AGT AAG T		
IL-10 F	GCT GGA GGA CTT TAA GGG TTA C	76	[9]
IL-10 R	CAT CAC CTC CTC CAG GTA AAA		
IL-8 F	CTT TCT GCA GCT CTG TGT GAA G	189	[9]
IL-8 R	GCA GAC CTC AGC TCC GTT GAC		
β-actin F	CGT GGG CCG CCC TAG GCA CCA	243	AF035774.1
β-actin R	TTG GCC TTA GGG TTC AGG GGG G		
IL-6 F	GGA TGC TTC CAA TCT GGG TTC AAT	65	[28]
IL-6 R	TCC GAA AGA CCA GTG GTG ATT TT		
IL-1ra F	ACA AAT GTG GCT CCT CCA AG	88	NM_001082525
IL-1ra R	TTT CAG AGC GTC AGA AGT GC		

used for clean-up of the extracted RNA. Isolated RNA was quantified via spectrophotometry using a NanoDrop ND-1000 (Agilent Technologies, Palo Alto, CA, USA). All samples had 260/280 ratio of 1.95 or higher and 260/230 ratio of 2.0 or higher and were used for further analysis. 1000 ng of the RNA samples was reverse transcribed using an RT-PCR kit (Promega), oligo-dT (Promega) and random primers (R&D Systems, Minneapolis, MN, USA). The total volume of each reaction was 25 μl. Reactions were incubated at 25 °C for 10 min, heated at 42 °C for 60 min, heated at 95 °C for 5 min, then cooled to 4 °C and stored at −20 °C until qRT-PCR analysis.

The mRNA expression of *IL-1β, IL-1ra, IL-6, IL-8, IL-10, TNF-α*, and *SAA* in endometrial tissue was measured by qRT-PCR (Table 1).

All primers were commercially synthesized (Invitrogen). The qRT-PCR was completed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: 95 °C for 10 min; 45 cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C for 30 s; 55 to 95 °C for dissociation. The qRT-PCR reactions were performed in 384-well plates with a final volume of 20 μl per reaction. Each reaction contained a diluted (10×) cDNA sample (4 μl), 10 μl SYBR Green, 2 μl of each primer (forward and reverse, 10 μM) and 2 μl ddH_2O. A calibrator (pool of all cDNA samples) and a no-template control (RNase-free water) were included on every qRT-PCR plate. Samples were done in duplicates. Efficiency of amplification for each primer was monitored through the analysis of serial dilutions (10-fold). The melting curves of the amplified PCR products were obtained for confirmation of specific amplification. The product sizes of specific products were verified on a 1% agarose gel. A pool of all endometrial samples served as the calibrator, and was added as internal control during each qRT-PCR analysis.

All gene amplifications were normalized to the reference gene *β-actin*, which previously has been described as the most stable reference gene in a panel of potential reference genes in equine endometrial tissue in experimental endometritis models [9,17].

2.7. Data analysis and statistics

Cycle threshold (Ct) values were obtained through the auto Ct function. Following efficiency correction, the mean threshold cycle (C_T) was calculated and then normalized to the reference gene using delta (Δ) C_T. The calibrator was used to carry out an additional normalization step in order to account for differences in amplification dynamics between PCR reactions between different PCR reaction plates. Changes in relative expression were calculated using the $2^{-\Delta\Delta Ct}$ method [29]. The specific transcripts are presented as n-fold change relative to estrus baseline levels (control biopsy). Outliers were defined as relative gene expression levels differing more than 2 x standard deviation, and were excluded from further data analyses.

The effect of intrauterine infusion of *E. coli* and immunomodulatory therapy on repeated measurements of mRNA expression in endometrial tissue and the cytologic response was statistically analyzed using a repeated measures analysis of variance procedure in SAS (PROC MIXED). A first order autoregres-

sive covariance structure was defined to take into account significant autocorrelation between measurements within mares. Differences in least squares mean estimates from the repeated measurement analyses were used to identify time points where the analyzed marker increased/decreased significantly. Bonferroni's multiple comparison procedure was used to control for Type I errors.

The effects of intrauterine infusion of *E. coli* and GC and MCWE treatment on the presence of intrauterine fluid, bacterial growth of *E. coli, S. zooepidemicus* and other pathogens were statistically analyzed using linear logistic regression (PROC GENMOD) in SAS. A logit transformation of data was used to describe the relationship between the outcome and the explanatory variable. A generalized score test (Wald's test) was used in the type 3 analysis, and significant differences between the time points for sample collection were identified by using least square means. Goodness-of-fit tests were performed to control the model of analyses of a dichotomous outcome.

All values are presented as means ± standard error of the means (SEM). Assumptions were checked on residual plots and tested for normality. Initial inspection of the data revealed that serum amyloid A (SAA) and cytokine mRNA expression varied markedly between individuals. Because variances were not homogeneous, $2^{-\Delta\Delta Ct}$ values were log transformed, and geometrical least square means statistically compared. All statistical calculations were made with the software SAS 9.2 (SAS Institute, Cary, NC, USA). Graphs were made using the software GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The level of significance was set to $P \leq 0.05$.

3. Results

3.1. Clinical and gynecologic examination

All mares were free from intrauterine fluid at the time of inoculation with *E. coli*. All five mares developed intrauterine fluid accumulation 24 h after inoculation with *E. coli* ("no treatment") and four of these had intrauterine fluid at the last biopsy collection at 72 h. The GC and MCWE treatment lowered the number of mares retaining fluid post inoculation. Only one mare had intrauterine fluid accumulation after *E. coli* inoculation and treatment with GC and MCWE. There was also a tendency to uterine growth of *S. zooepidemicus* having an effect on the fluid accumulation in the mares after *E. coli* inoculation ($P = 0.06$).

Mare	Treat	0h	3h	24h	72h
A	NT[a]	ng[d]	ng	+coli	+ coli
A	GC[b]	ng	ng	n g	ng
A	MCWE[c]	ng	ng	n g	ng
B	NT	ng	ng	+ coli	ng
B	GC	ng	+coli	n g	ng
B	MCWE	ng	ng	n g	ng
C	NT	ng	+ coli	+++ Sez[e]	+++ Sez
C	GC	ng	+coli	n g	ng
C	MCWE	ng	ng	n g	ng
D	NT	ng	ng	+++ Sez	+++ Sez
D	GC	ng	ng	+Sez	+++Sez
D	MCWE	ng	+coli	+++Sez	+++Sez
E	NT	ng	+ Sez	++coli/++Sez	++Sez
E	GC	ng	++coli	n g	ng
E	MCWE	ng	ng	n g	ng

Fig. 2. Bacterial growth from uterine swabs and biopsies before (0 h) and 3, 24 and 72 h after intrauterine infusion of *E. coli* and treatment with immune-modulators. [a]No treatment, [b]treatment with 0.1 mg per kg dexamethasone IV at the time for *E. coli* inoculation, [c]treatment with 1.5 mg MCWE IV 24 h before inoculation, [d]no growth, [e]*S. zooepidemicus*, + mild growth, ++ moderate growth, +++ heavy growth.

3.2. Microbiology

Sterile uterine cultures were obtained from all five mares at the time of *E. coli* inoculation. All mares cultured positive for *E. coli, S. zooepidemicus* or both at 3 or 24 h after intrauterine inoculation of *E. coli* in the "no treatment" cycle (Fig. 2). At the last sample collection (72 h) in the "no treatment" cycle three mares had moderate to heavy growth of *S. zooepidemicus*, one mare mild growth of *E. coli* and from one mare a sterile culture was obtained. Following MCWE treatment one mare had mild growth of *E. coli* after *E. coli* inoculation. Only one mare was positive for *S. zooepidemicus* after *E. coli* infusion and GC and MCWE treatment, respectively. Immunomodulatory treatment had no significant effect on the number of mares cultured positive for *E. coli*.

3.3. Exfoliative cytology

All mares had moderate to severe endometrial neutrophilia immediately (3 h) and severe neutrophilia at 24 and 72 h after *E. coli* inoculation. The cytology scores did not differ between control and treatment cycles (data not shown).

3.4. Endometrial gene expression

A significant increased gene expression of *IL-1β* and *IL-6* was observed immediately (3 h) after *E. coli* in-

oculation compared to estrus baseline despite treatment with GC and MCWE. Expression of *SAA* was significantly higher 3 and 24 h after *E. coli* inoculation ("no treatment") compared to estrus baseline levels. Endometrial mRNA transcripts of *IL-1β* and *IL-6* were significantly higher 3 h post inoculation compared to later in the study period (24 and 72 h) (Fig. 3a, b).

3.5. Effect of immunomodulatory therapy on endometrial gene expression

The GC treatment had a significant effect on the endometrial gene expression of pro- and anti-inflammatory cytokines and *SAA* when compared to expression levels after "no treatment" and MCWE treatment.

The gene expression of the proinflammatory cytokine *IL-1β* was lower 3 h (5-fold, P = 0.05) and 72 h (10-fold, P = 0.01) after GC treatment compared to "no treatment". No difference in gene expression between MCWE and "no treatment" and GC treatment was demonstrated (Fig. 3a). Expression of the proinflammatory *IL-6* was higher immediately (3 h) after GC treatment (4-fold, P = 0.05) compared to "no treatment", whereas a lower gene expression was observed for *IL-6* late in the study period (72 h) (10-fold, P = 0.03) when compared to the "no treatment" cycle. The MCWE treatment did not change the expression of *IL-6* compared to GC or "no treatment" (Fig. 3b). Expression of *IL-8* was lower 72 h after CG treatment compared to "no treatment" (7-fold, P = 0.05) and MCWE treatment (23-fold, P = 0.05) (Fig. 3c). No significant changes between the treatments were observed at other time points. No significant differences in gene expression of *TNF-α* were observed after *E. coli* infusion despite treatment with immunomodulatory therapy (Fig. 3d). Expression of *SAA* was lower 3 h (5-fold, P = 0.02) and 24 h (5-fold, 0.03) after GC treatment; and 24 h after MCWE treatment (11-fold, P = 0.03) compared to "no treatment" (Fig. 3e).

A higher expression of *IL-10* was observed 3 h after GC treatment compared to "no treatment" (8-fold, P = 0.01) and at 3 h (5-fold, P = 0.03) and 24 h (4-fold, P = 0.05) compared to MCWE treatment (Fig. 3f). Endometrial mRNA transcripts of *IL-1ra* were decreased 3 h after GC treatment compared to MCWE treatment (11-fold, P = 0.05). No differences in expression were observed between the treatments at other time points (Fig. 3g).

The relationship between pro- and anti-inflammatory cytokines *IL-1β:IL-1ra* was lower 72 h after *E. coli* inoculation and GC treatment compared to "no treatment" (5-fold, P = 0.03). No significant change in the *IL-1β:IL-1ra* ratio was observed after MCWE treatment compared to "no treatment" or GC treatment (Fig. 3h).

3.6. Gene expression compared to control level

A control biopsy was collected in an estrus cycle after the last *E. coli* inoculation; the mares were confirmed free of infection and inflammation (exfoliative cytology) at the time of biopsy collection. Gene expression of *IL-1β* was lower in the control biopsy compared to the expression at 72 h "no treatment" (26-fold, P = 0.02) and MCWE treatment (8-fold, P = 0.05) (Fig. 3a). Expression of *IL-6* (11-fold, P = 0.03), *SAA* (30-fold, P = 0.05) and *IL-1β:IL-1ra* (6-fold, P = 0.05) was significantly lower in the estrus cycle after the last *E. coli* infusion compared to 72 h after *E. coli* infusion and no immunomodulatory therapy (Fig. 3b, e, h).

4. Discussion

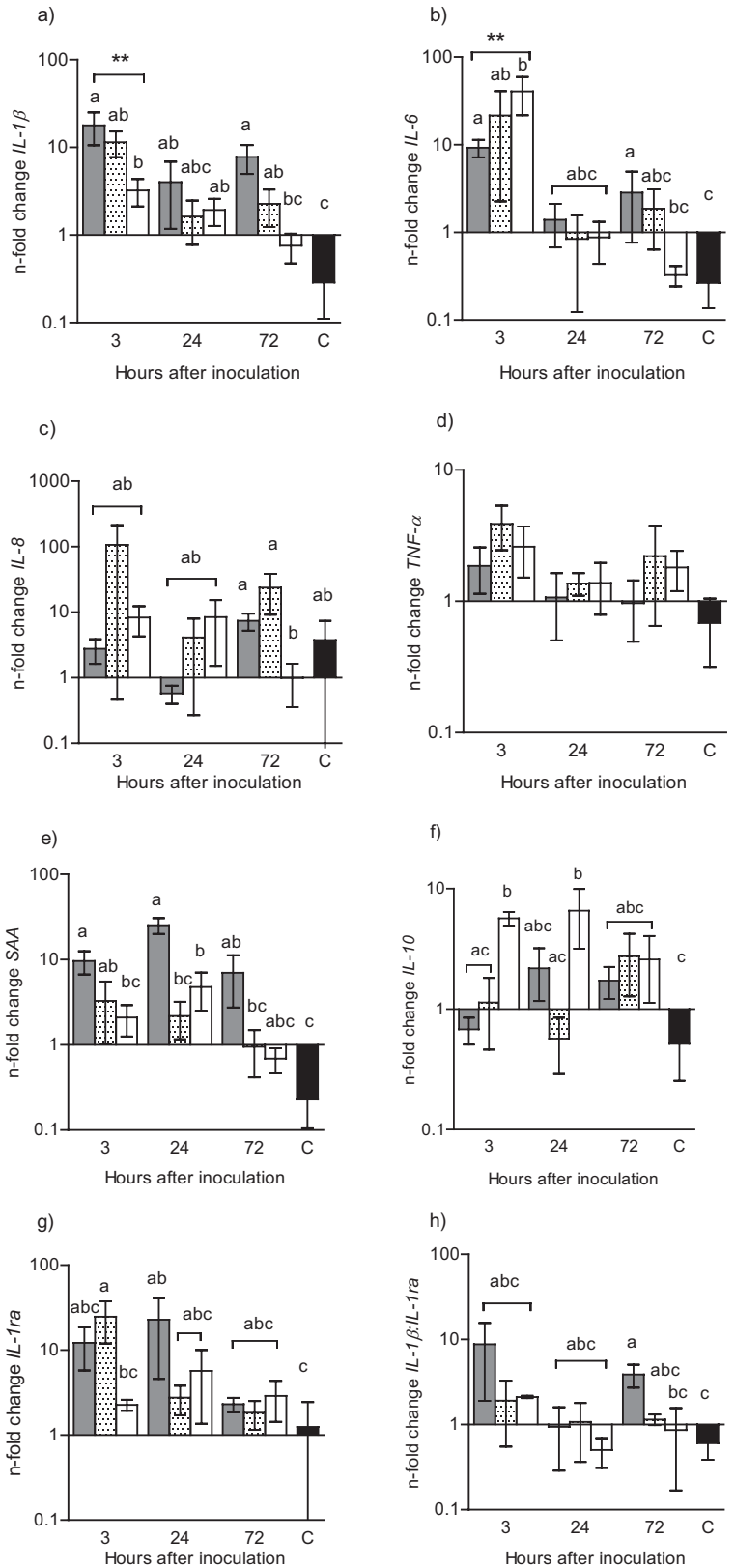
The present study demonstrates diverse and marked effects of immunomodulatory therapy on the endometrial expression of inflammatory cytokines and *SAA* and clinical symptoms of uterine infection in susceptible mares.

4.1. Effect of immunomodulatory therapy on endometrial gene expression of cytokines and SAA

All mares had a significant increase in gene expression of the proinflammatory cytokines *IL-1β* and *IL-6* immediately (3 h) after *E. coli* inoculation despite treatments compared to 24 and 72 h. Treatment with GC or MCWE had no effect on *TNF-α* expression in the susceptible mares in the present study. In a previous study it was demonstrated that susceptible mares failed to upregulate endometrial expression of *TNF-α* in response to experimentally induced *E. coli* endometritis compared to resistant mares, which may indicate a "leak" in the first line of defense against uterine pathogens [17]. Endometrial gene expression of *IL-1β, IL-6, SAA* and the ratio of *IL-1β:IL-1ra* was significantly increased 72 h after *E. coli* inoculation and no immunomodulatory therapy compared to the estrus cycle after the last *E. coli* inoculation. This finding indicates a sustained endometrial inflammatory response at 72 h.

4.2. Effect of GC on the endometrial gene expression of cytokines and SAA

Treatment with GC at the time of *E. coli* infusion had a significant modulating effect on endometrial ex-


a)
n-fold change IL-1β
Hours after inoculation
b)
n-fold change IL-6
Hours after inoculation
c)
n-fold change IL-8
Hours after inoculation
d)
n-fold change TNF-α
Hours after inoculation
e)
n-fold change SAA
Hours after inoculation
f)
n-fold change IL-10
Hours after inoculation
g)
n-fold change IL-1ra
Hours after inoculation
h)
n-fold change IL-1β/IL-1ra
Hours after inoculation
3
24
72
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pression of cytokines (*IL-1β, IL-1ra, IL-6, IL-8, IL-10*) and *SAA* in susceptible mares. A significant decreased expression of *IL-1β* immediately (3 h) after infusion and late (72 h) in the study period was observed. The inhibitory effect of GC on the production and release of numerous proinflammatory cytokines (e.g., IL-1α, IL-1β, IL-6, IL-8, TNF is well documented [30–33]. The inhibitory effect of GC is primarily carried out at the GC receptor (GR) level [34]. The ligand bound GR can interact with signaling pathways e.g., components of the Toll like receptor (TLR) signaling complex, and thereby modulate proinflammatory gene expression [35]. Interactions on the gene expression may also occur directly in the nucleus [36]. The significant decrease in *SAA* expression in endometrial cells 3 and 24 h after *E. coli* infusion and GC treatment may be a response to the decreased *IL-1β* expression observed after GC treatment, since the IL-1 type cytokines IL-1β and TNF-α are the major stimulating cytokines increasing the SAA synthesis [37,38]. However, no change in endometrial expression of *TNF-α* after GC treatment was observed. Expression of *SAA* was also significantly decreased 24 h after *E. coli* infusion and MCWE treatment.

Surprisingly, a significant increased expression of *IL-6* was demonstrated at 3 h, whereas a significant decrease was observed 24 and 72 h after *E. coli* inoculation and GC treatment compared to "no treatment" and MCWE treatment. The apparent synergistic effect of GC and proinflammatory cytokines IL-1 and IL-6 has previously been demonstrated in hepatic cells, where GC strongly potentiated the IL-1β and IL-6 induced acute phase response [15]. The significantly lower *IL-6* expression after GC treatment compared to "no treatment" may have been induced by GC's inhibitory effect on the production and release of IL-6 [30]. Expression of *IL-8* was lower 72 h after GC treatment compared to "no treatment" and MCWE treatment. IL-8 is a potent chemo-attractant and is responsible for the transepithelial migration of PMNs into the tissue [39]. In resistant mares, a rapid increase in *IL-8* expression has been demonstrated initially (3 h) after AI with killed semen or inoculation of *E. coli*, whereas susceptible mares showed an increased *IL-8* expression that correlated to the severity of neutrophilia after bacterial infusion as well as after AI [12,17]. The high expression of *IL-8* in susceptible mares correlated to growth of uterine pathogens [17]. In the present study, no correlation between the severity of neutrophilia and expression of *IL-8* could be established. Suppression of IL-8 in response to GC treatment has previously been described [40], and it is most likely that the GC treatment is responsible for the lowered *IL-8* expression observed in the present study.

The initial pro-inflammatory response is controlled by intrinsic anti-inflammatory cytokines IL-10 [41], IL-1ra [42], IL-4 [43], IL-13 [44,45], and by stimulation of the hypothalamic-pituitary-adrenal axis (HPA) [46]. In the present study, we investigated the endometrial expression of *IL-10* and *IL-1ra*, and found a significantly higher expression of *IL-10*, 3 and 24 h after GC treatment compared to "no treatment" and MCWE-treatment. GC enhances phagocytotic activity of macrophages and monocytes by upregulating the scavenger receptor CD163 and inducing expression of the anti-inflammatory IL-10 production and release [47,48]. This mechanism may be responsible for the increased *IL-10* expression observed in the present study. IL-10 acts as a potent general anti-inflammatory effector by reducing transcription of pro-inflammatory cytokines by monocytes and macrophages [49,50]. This may have played an important role in modulation of the pro-inflammatory response observed after GC treatment. Although a previous study demonstrated that GC can stimulate *IL-1ra* expression in a human airway epithelial cell line [51], we found a non-significant decrease in *IL-1ra* expression 3 h after *E. coli* infusion and GC treatment.

It has been demonstrated that susceptible mares show sustained high endometrial gene expression levels of inflammatory cytokines in response to intrauterine *E. coli* infusion compared to resistant mares [17]. Release of inflammatory cytokines from sentinel cells is a part of the innate immune response, and they take part in the initiation, regulation and resolution of inflammation [41,52–55]. The GC treatment significantly modulated the endometrial gene expression of pro- and anti-inflammatory cytokines after intrauterine *E. coli* infusion

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Fig. 3. The mRNA transcripts of endometrial a) *IL-1β*, b) *IL-6*, c) *IL-8*, d) *TNF-α*, e) *SAA*, f) *IL-10*, g) *IL-1ra* and h) *IL-1β: IL-1ra* in mares after intrauterine infusion of *E. coli* and GC- and MCWE-treatment. Gene expressions are normalized to β-actin and displayed as n-fold change to estrous baseline level (mean ± sem). Different letters indicate significant differences ($P < 0.05$) between "no treatment" (gray bars), MCWE-treatment (spotted bars), GC-treatment (white bars) and gene expression levels in an estrous cycle after the last *E. coli* inoculation (black bars). Asterisks indicate significant differences in gene expression levels 3 h after *E. coli* inoculation compared to other time points. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

with increased expression of anti-inflammatory *IL-10* initially after *E. coli* inoculation and decreased expressions of pro-inflammatory cytokines late in the study period (72 h).

4.3. Effect of MCWE treatment on the endometrial gene expression

Administration of MCWE 24 h before *E. coli* inoculation did not modulate the endometrial inflammatory response significantly, which is in contrast to previous challenge studies by Fumuso, et al. [11,12]. In the present study, only endometrial expression of *SAA* was significantly lower 24 h after *E. coli* infusion compared to the "no treatment" cycle. A lower gene expression of *IL-1β* was observed 24 and 72 h after *E. coli* inoculation compared to immediately after inoculation (3 h), however the change in *IL-1β* expression was not significantly different from the "no treatment" cycle. No significant changes in expression of pro- and anti-inflammatory cytokines were observed. Cell wall-DNA (MCC) and MCWS from a variety of mycobacteria, including *Mycobacteriump phlei* has been shown to possess immunomodulatory activity, including muramyl dipeptide (basic unit of peptidoglycan) [56], trehalose dimycolate [57] and LAM [58]. An immunomodulatory effect of MCWE on endometrial cytokine expression in susceptible mares with induced endometrial inflammation by AI with dead spermatozoa has previously been demonstrated [11,12]. Administration of MCWE at the time of insemination significantly decreased expression levels of *IL-1β* and increased *IL-10* level 24 h after AI [11,12]. The decreased expression of *IL-1β*, 24 h after AI and MCWE treatment is in contrast to the induction of proinflammatory cytokines previously demonstrated after administration of MCWE or MCC. The immunomodulatory effects of the MCWE component LAM are mediated in part by selective induction of the cytokines, IL-1, IL-6, IL-8, IL-10 and TNF-α [58]. A possible explanation for discrepancies between outcomes of studies evaluating the effect of MCWE is the criteria by which mares are selected as susceptible to persistent endometritis. It is important to determine that mares are free from inflammation before bacterial inoculation, because chronic inflammation may change cytokine mRNA profiles at the baseline level, therefore altering gene expression data analyzed using a relative quantification method. The model used in this experiment utilizes strict criteria and, therefore, is appropriate for the study.

4.4. Microbiology

Few *E. coli* colonies could be isolated from few mares (5 mares in 15 inoculation cycles) immediately (3 h) after inoculation, whereas three out of five mares became culture positive for *S. zooepidemicus* after *E. coli* infusion and "no treatment". All mares cleared the infused *E. coli* except for one mare that had mild growth ($<$ 10 cfu) 72 h after inoculation. The rapid uterine clearance of inoculated pathogens during estrous is in accordance with findings after intrauterine inoculation of a moderate dose of *S. zooepidemicus* in estrous mares [59,60]. The relatively low inoculation dose and the cycle stage may explain why the susceptible mares in the present study were capable of effective clearance of the inoculated pathogens. In most inoculation studies, the gram positive *S. zooepidemicus* has been used as intrauterine inoculums and often in a higher dose (10^8–10^9 CFU) which could explain why susceptible mares in these studies remained infected [61,62]. It can also be speculated that a difference in uterine clearance of different pathogens exists. A study by Burleson, and coworkers demonstrated that intrauterine fluid and severe neutrophilia of cytology specimen were more common when β-hemolytic streptococci was isolated than when *E. coli* was found in mares with endometritis [63]. In the present study, one mare had positive cultures for *E. coli* by the end of the study period. All five mares, however, had intrauterine fluid accumulation 24 h after *E. coli* inoculation (no treatment) and four of these had intrauterine fluid and positive cytology at the last sample collected at 72 h indicating a prolonged inflammatory response, although no *E. coli* could be isolated.

All mares with growth of uterine pathogens at the end of the study period (four out of five mares after *E. coli* and no treatment; one mare after *E. coli* and GC treatment; and one mare after *E. coli* and MCWE treatment), were treated for three to five days with intrauterine lavage and intrauterine antimicrobials. All mares were confirmed free of infection and inflammation at the time for the subsequent *E. coli* inoculation.

In chronically infected mares, *S. zooepidemicus* may reside deep within the endometrium [64], and recent findings suggest that *S. zooepidemicus* is capable of establishing a persistent infection which can be activated by inducing endometrial inflammation [17]. We recently demonstrated a significant increased endometrial gene expression of *IL-1β* and *IL-1ra* in susceptible mares with heavy growth of *S. zooepidemicus*, suggesting that an altered inflammatory response most likely

may be influenced by the presence of uterine pathogens in the susceptible mare [17]. Further investigations are, however, required to determine the exact role of different pathogens on the endometrial inflammatory response. Only one mare had uterine growth of *S. zooepidemicus* after *E. coli* inoculation and treatment with GC and MCWE. The GC stimulate expression of the mannose receptor (MR) or the scavenger receptor CD163, promoting clearance of microorganisms, dead cell bodies and antigens [65,66], and may be responsible for the elimination of intrauterine pathogens in the mares after GC treatment. The MCWE stimulates the induction of proinflammatory cytokines and chemokines [58] and act as a chemo-attractant for PMNs causing an influx of PMNs to the inflamed tissue [67]. Even though no differences in gene expression of proinflammatory cytokines were observed after *E. coli* infusion and MCWE treatment, an up-regulated immune response by other mechanisms in response to MCWE treatment may be responsible for the clearance of uterine pathogens.

Mares with growth of *S. zooepidemicus* were treated with luminal penicillin before reinoculation and treatment with GC and MCWE. Recent work by our group suggests that *S. zooepidemicus* can use dormancy to increase their chances of survival. Dormant bacteria are not resistant to antimicrobials, but can still survive antimicrobial treatment because of a low metabolic turnover (Petersen, et al., unpublished data). Based on these new findings, the penicillin used for intrauterine treatment of *S. zooepidemicus* only had an effect on the *S. zooepidemicus* in the uterine lumen, and not on the bacteria residing within the endometrium. Therefore, it is most likely the immune modulatory treatment which had an effect on the clearance of uterine pathogens. How GC and MCWE are capable of inhibiting the inflammation-induced activation of the *S. zooepidemicus* localized deep within the endometrium is not known, and further investigations are required.

4.5. Intrauterine fluid accumulation

Presence of *S. zooepidemicus* also correlated to the fluid accumulation after *E. coli* inoculation. Fewer mares had intrauterine fluid accumulation after *E. coli* infusion and GC and MCWE treatment compared to "no treatment", which may be related to the reduced number of mares having uterine growth of *S. zooepidemicus* after immunomodulatory therapy. Uterine infections caused by *S. zooepidemicus* are often characterized by intrauterine fluid retention [63].

The pathogenesis of delayed uterine clearance is multifactorial, but reduced myometrial activity/contractility has been demonstrated to be one of the key factors [5]. Nitric oxide (NO) is released from sentinel cells in response to microbial products and mediates smooth muscle relaxation [68,69]. Alghamdi, et al. [70] showed that susceptible mares had significantly higher uterine levels of NO and its inducible synthase (iNOS) in response to AI compared to resistant mares. The susceptible mares in the present study may have had high uterine levels of NO and iNOS in response to *E. coli* inoculation and subsequent growth of *S. zooepidemicus* in the "no treatment" cycle causing decreased myometrial contractions, leading to intrauterine fluid accumulation. The GC suppression of iNOS causing decreased NO concentration has been demonstrated in epithelial and endothelial cells in a variety of organs [71–74] via the inhibitory effect of GC on iNOS inducible cytokines (IL-1β, TNF-α, IFN-γ) [75]. In the present study, GC administered to susceptible mares at the time of *E. coli* infusion decreased the endometrial expression of *IL-1*β, which may have inhibited iNOS expression. The modulating effect of GC on uterine fluid retention has also been demonstrated in brood mares after AI [20]. The significant fewer mares with uterine fluid after MCWE treatment may simply be due to the reduced number of mares with growth of uterine pathogens. Further investigations are required to evaluate the exact effect of immunomodulatory therapy on NO and iNOS and fluid retention in mares with infectious endometritis.

4.6. Exfoliative cytology

The PMN is the major infiltrating cell during acute inflammation because of capacity of rolling, adhesion, activation and transmigration through the blood vessels [76]. In the present study, all mares had positive cytology 72 h after *E. coli* inoculation and no significant change in cytology score was observed after GC and MCWE treatment, respectively. These results are in accordance to cytology scores obtained 12 h after AI and GC treatment at breeding time in broodmares [20] and 24 h after AI with dead spermatozoa and MCWE treatment in susceptible mares [12]. An inoculation study with intrauterine infusion of *S. zooepidemicus* and MCWE treatment 48 h after inoculation in susceptible mares showed a reduced numbers of mares positive on uterine cytology 3 to 6 days post inoculation compared to a control group. The negative cytology after MCWE treatment correlated to uterine clearance of the inoculated *S. zooepidemicus* [21]. The contradicting findings in numbers of PMN in mares in differ-

ent studies evaluating the immunomodulatory effect of MCWE may be due to the different time points for obtaining cytology samples and different pathogens inoculated. In the present study, the mares were able to clear the inoculated *E. coli*, and only one mare had intrauterine growth of *S. zooepidemicus* after GC and MCWE treatment. No correlation between presence of uterine pathogens and cytology score could be demonstrated. The mycolic acid of MCWE acts as a chemoattractant for monocytes and PMN [67], and an increased number of PMN in the uterine lumen initially after bacterial infusion and MCWE treatment was expected.

5. Conclusion

In conclusion, the results of the current investigation demonstrated that GC is capable of effectively modulating the innate immune response to induced infectious endometritis in susceptible mares with decreased gene expressions of the pro-inflammatory cytokines, *IL-1β, IL-6, IL-8* and *SAA*, and stimulation of an anti-inflammatory response (*IL-10*) after administration. The relationship between pro- and anti-inflammatory responses was normalized compared to bacterial infusion without treatment indicating a well-balanced endometrial immunologic response to infectious endometritis. Treatment with MCWE did not modulate the gene expression of inflammatory cytokines, however a modulating effect on intrauterine fluid accumulations and the clearance of uterine pathogens in the mares was observed. The effect of MCWE is therefore likely mediated through a system not assessed in the current investigation. The immunomodulatory therapy had a significant effect on the numbers of mares infected with *S. zooepidemicus* after *E. coli* infusion. How GC and MCWE can inhibit the activation of persistent *S. zooepidemicus* residing deeply in the endometrium is not known, and further investigations are required to explain potential mechanism. Based on the results from the present study, further investigations to determine the exact role of persistent uterine infections with *S. zooepidemicus* in susceptible mares are also required.

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