Equine Veterinary Journal

Equine Veterinary Journal ISSN 0425-1644
DOI: 10.1111/evj.12266

The effect of treatment with immune modulators on endometrial cytokine expression in mares susceptible to persistent breeding-induced endometritis

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Summary

Reasons for performing study: Research has shown that 6 h after breeding is a critical time during the uterine innate immune response, and the failure to respond appropriately will result in persistent breeding-induced endometritis. This presents a potential opportunity to modulate the course of inflammation towards a timely resolution.
Objectives: To evaluate the effects of immune modulation on endometrial mRNA expression of inflammatory genes in susceptible mares 6 h after breeding. The hypothesis was that immune modulation alters endometrial cytokine expression in susceptible mares.
Study design: A randomised controlled study to evaluate the effects of mycobacterial cell wall extract and dexamethasone on endometrial gene expression after insemination in 6 mares susceptible to persistent breeding-induced endometritis.
Methods: Six susceptible mares were selected based on their uterine inflammatory response to insemination. Mares were inseminated during 3 oestrous cycles with 1×10^9 nonviable spermatozoa and 1) no additional treatment (control), or in combination with 2) dexamethasone (50 mg i.v.) at the time of insemination, or 3) with mycobacterial cell wall extract (1.5 ml i.v.) administered 24 h prior to insemination. Mares received one treatment per cycle in randomised order, and each mare served as her own control. Endometrial biopsies were collected 6 h after breeding, and quantitative polymerase chain reaction analysis for interleukin (IL)1β, IL6, interferon γ, IL10 and IL1RA was performed. Relative quantification values reported fold changes in mRNA expression from the control. Data were analysed using an ANOVA and significance was set at $P<0.05$.
Results: Expression of IL1β mRNA was lower after treatment with dexamethasone ($P<0.001$) and mycobacterial cell wall extract ($P<0.05$) when compared with control. No differences were detected in the mRNA expression of the other cytokines after any of the treatments.
Conclusions: Treatment with immune modulators alters endometrial mRNA expression of IL1β after insemination. A better understanding of the mechanisms of immune modulation in the equine uterus can help to improve treatments for persistent breeding-induced endometritis.

Keywords: horse; endometritis; uterine inflammation; endometrium; equine uteus

Introduction

Transient uterine inflammation in response to breeding is a physiological reaction that aids in the clearance of excess spermatozoa and debris. While the inflammation normally resolves itself in a timely manner, persistent breeding-induced endometritis (PBIE) can interfere with pregnancy.

The inflammatory response is a complex process involving many signalling cascades, and cytokines have a significant role in the recognition of pathogens and recruitment of inflammatory cells [1,2]. Among these cytokines, the interleukin (IL) 1 family is critical in the development and control of inflammation. Interleukin 1β is a potent proinflammatory cytokine released at the onset of inflammation, which triggers an upregulation in the transcription of other proinflammatory cytokines, leading to the activation and recruitment of inflammatory cells [3,4]. Many cells, including epithelial cells, produce IL1β in response to pathogens. Another important proinflammatory cytokine involved with both the innate and adaptive inflammatory response is interferon gamma (IFNγ). In addition to its antimicrobial properties, IFNγ aids in the migration of inflammatory cells through vessel walls [5]. Additionally, the release of IFNγ leads to the upregulation of inducible nitric oxide synthase, an enzyme that produces nitric oxide, which is increased in the uterine secretions of susceptible mares after breeding [6,7].

Cytokine signalling and the innate immune response during inflammation is well documented in other species and organ systems, however there are fewer studies focusing on these inflammatory pathways in the mare's reproductive tract. Previous work has detected a differential expression of endometrial cytokine mRNA in susceptible and resistant mares 24 h after insemination [8–10]. The inflammatory cytokine response to spermatozoa within the first 24 h after insemination was recently investigated, and it was concluded that a critical time in the development of PBIE occurs around 6 h after breeding [11].

Uterine lavage and the use of ecbolic agents are common preventative and symptomatic treatments to assist with uterine clearance of inflammatory debris. Additional therapeutic approaches in conjunction with traditional treatments in cases where these treatments are ineffective include the use of immunomodulators. Glucocorticoids act to depress the response of proinflammatory cytokines, chemokines and other proteins associated with inflammation such as inducible nitric oxide synthase, Cyclo-oxygenase-2 and adhesion molecules [12,13]. In addition to depressing the proinflammatory response, glucocorticoids upregulate the anti-inflammatory cytokines such as IL10 and IL1RA [12]. Dexamethasone blocks the translational and post translational production of IL1 [14], and is used as a treatment for endometritis in mares, although the mechanisms of action in the equine uterus have not been determined. Pregnancy rates were improved in problem mares when treated with 5 consecutive treatments of prednisolone acetate [15]. Furthermore, oral administration of 200 mg of dexamethasone twice daily for 5 days before breeding improved pregnancy rates in subfertile mares [16]. Bucca *et al*. [17] reported a reduction in clinical signs of PBIE with i.v. administration of dexamethasone at the time of breeding, and concluded that a single dose of 50 mg dexamethasone is effective for the treatment of PBIE in mares with at least 3 risk factors for the condition [17]. In contrast to the immune suppressing actions of glucocorticoids, mycobacterial cell wall extract (MCWE) is thought to stimulate the immune response to reduce persistent inflammation. In mares, treatment with MCWE decreased the incidence of endometritis after challenge with *Streptococcus zooepidemicus* [18], and increased pregnancy rates in mares bred during their foal heat cycle [19]. In addition, MCWE was shown to decrease levels of proinflammatory cytokines in susceptible mares towards levels of resistant mares 24 h after breeding [8,10].

Although the efficacy of immune modulators has been investigated, data on the mechanism of action for these drugs as a treatment for PBIE are sparse. The specific aim of this experiment was to evaluate the effects

of dexamethasone and MCWE on the expression of inflammatory cytokine mRNA in mares susceptible to PBIE at 6 h after insemination with 1×10^9 spermatozoa in 30 ml of milk-based semen extender (Equi-Pro)[a], with a hypothesis that these treatments will alter cytokine expression when compared with nontreated susceptible mares.

Materials and methods

Animals

Mares of mixed breeds and breeding history, aged 15–26 years, were kept on pasture supplemented with grain and hay, and were provided with water and salt *ad libitum*. Semen was collected from 3 stallions with similar housing conditions to that of the mares.

Preparation of sperm for insemination

Previous research demonstrated that spermatozoa cause an endometrial inflammatory response, while seminal plasma contains proteins that depress the immune response [11,20]. For this experiment, semen from 3 stallions was pooled, then the seminal plasma was removed using centrifugation ($1000 \times \boldsymbol{g}$ for 10 min), and the pellet was resuspended in 30 ml of milk-based semen extender (Equi-Pro[a]) in aliquots of 1×10^9 spermatozoa. Spermatozoa were rendered nonviable by at least 2 freeze (-20°C)/thaw (room temperature) cycles of the aliquots. This method of processing standardised each dose of inseminate, thereby minimising variability in inflammatory stimulus that may be attributed to differences in stallion, semen viability or seminal plasma proteins.

General experimental procedure

Mares were examined for susceptibility to PBIE based on endometrial histology and the results of a spermatozoa challenge as previously described [11,21,22]. Endometrial biopsies (approximately 300 mg of tissue) were acquired during dioestrus using an alligator jaw biopsy instrument, fixed in 10% formalin, sectioned at 5 μm and stained with haematoxylin and eosin. Each biopsy was examined for periglandular fibrosis, inflammatory cells, glandular distribution and lymphatic lacunae, then graded according to Kenney and Doig [23]. Mares with scores of IIB or III were considered as potentially susceptible to PBIE. After selection as potentially susceptible to PBIE, the reproductive tracts of the mares were observed regularly using transrectal ultrasonography, and the mares were inseminated with nonviable spermatozoa (prepared as described above) during oestrus, which was characterised by the presence of uterine oedema, a relaxed cervix, and at least one follicle 35 mm or larger in diameter in the absence of a *corpus luteum*. Immediately prior to insemination, mares were evaluated for the presence of intrauterine inflammatory cells using either a cytobrush[a], or low volume lavage to obtain samples (both are accepted methods for collecting uterine cells), and for intrauterine bacterial growth using a double-guarded swab[a] to obtain samples. Inflammation was defined as more than 2 neutrophils/5 fields at 400× magnification. Only mares with negative cytology and culture prior to insemination were considered for the experiment; if mares were positive for cytology or culture at the time of insemination, they were treated with intrauterine lavage as needed and intrauterine antibiotics daily for 5 days (according to sensitivity testing), and insemination was attempted again during the following cycle. During the course of the experiment, antibiotic treatment was required 3 times (one mare had a positive culture for one cycle, and one mare had a positive culture for 2 nonconsecutive cycles).

Potentially susceptible mares were evaluated 96 h after insemination and were confirmed as susceptible if they had: 1) positive cytology with either positive or negative culture; and 2) uterine fluid retention. All mares not meeting the criteria for classification as susceptible were excluded from the study. A total of 6 susceptible mares (age 15–24 years) were identified and used for the experiment.

Treatments

Oestrus was induced through administration of prostaglandin (Lutalyse 7.5 mg[b]) after Day 5 post ovulation over 3 subsequent cycles (the average number of days between treatments was 22 ± 12 days). Once oestrus was detected, mares were evaluated for endometrial cytological and bacterial findings. Mares with negative cytology and uterine culture results were administered one of the following treatments: 1) insemination with nonviable spermatozoa when a follicle ≥35 mm and uterine oedema were present, 2) 50 mg dexamethasone i.v and insemination with nonviable spermatozoa when a follicle ≥35 mm and uterine oedema were present, and 3) 1.5 ml MCWE i.v. when a follicle ≥30 mm and uterine oedema were present, and insemination with nonviable spermatozoa 24 h after treatment. All mares received each treatment in randomised order, and the vital signs and digital pulse after treatment with dexamethasone were monitored several times within the first 24 h (although one dose of 50 mg i.v. has been shown to be safe [17]).

Sample collection: endometrial tissue

Endometrial biopsies were collected 6 h after breeding, and stored in RNAlater[c] overnight at 4°C, then moved to -20°C for storage until further processing.

Quantitative polymerase chain reaction analysis

Total RNA was extracted using TRIzol® Reagent[d], precipitated using sodium acetate and isopropanol, resuspended in ddH_2O and DNAse treated with a commercially available kit (DNA-free™[c]), then analysed for quality and quantity using a NanoDrop spectrophotometer[e]. A 1.5 μg sample of RNA[f] in 41.5 μl ddH_2O was reverse transcribed in a reaction using AMV Reverse Transcriptase (0.5 μl;10 u/μl), 5 × RT Buffer[f] (16 μl), RNAsin[f] (1 μl; 40 u/μl), MgCl[f] (16 μl; 25 mmol/l), dNTP[f] (4 μl;10 mmol/l), and Oligo(dT) Primer[f] (1 μl; 500 μg/ml). Samples were incubated at 42°C for 60 min, then 95°C for 5 min. cDNA was diluted 1:1 with ddH_2O, and quantitative polymerase chain reaction (PCR) for each sample was performed using 4.5 μl of cDNA, 5 μl of Sensimix II[g] and 0.5 μl of a custom primer/probe set[c] (Table 1). Primer/probe sets were sequenced and compared with the equine genome, and bioinformatic analysis was performed by Applied Biosystems to ensure specificity. Reactions were performed in duplicate using the 7900HT Fast Real-Time PCR System[c], and were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. Polymerase chain reaction efficiencies were calculated using LinRegPCR (version 7.0). Beta-actin was used as the reference gene, as it is stably expressed in equine endometrial tissue and has served as the reference gene in previous studies [11,22]. Results are expressed as mean relative quantification values, which were calculated using the $2^{-\Delta\Delta CT}$ method [24],

TABLE 1: Primer probe set sequences for the detection of equine mRNA. Primer probe sets were designed using assays-by-design (Applied Biosystems[c])

Cytokine	Forward primer sequence	Reverse primer sequence	Probe sequence
Eqβ-actin	CTGGACTTCGAGCAGGAGATG	CGTCGGGCAGCTCGTA	CCGCGGCCTCCAGCT
EqIL1β	CCGACACCAGTGACATGATGA	ATCCTCCTCAAAGAACAGGTCATTC	ATTGCCGCTGCAGTAAG
EqIL6	GGATGCTTCCAATCTGGGTTCAAT	TCCGAAAGACCAGTGGTGATTTT	ATCAGGCAGGTCTCCTG
EqIL10	ATGCCCCAGGCTGAGAAC	CGGAGGGTCTTCAGCTTTTCC	CCAGACATCAAGGAGCACG
EqIL1RA	AGTTGCTGGATACTTGCAAGAATCA	GAGTCCCAGGAATAGAGCATCAG	CATCTATCTTCTCTTGTAATTTA
EqINFγ	AGCAGCACCAGCAAGCT	TTTGCGCTGGACCTTCAGA	ATTGAGATTCCGGTAAATG

with the calibrator as the mean cycle threshold (ΔC_T) value of the control samples.

Quantitative PCR data were analysed using SigmaStat[h] with a one-way repeated measures ANOVA. Prior to analysis, data were log10 transformed for normal distribution when needed, and Kruskal–Wallis one-way ANOVA on Ranks was used for data that were not normally distributed after transformation. There were 90 data points: 6 mares × 5 cytokines × 3 treatments = 90 points. One point was removed because the PCR efficiency was low, leaving 89 points. Outliers were defined as ± 2 s.d. from the mean and were removed from statistical analyses (4 of 89 total data points; 4.5%). Comparisons were made between treatments for the susceptible mares, and significance was set to $P<0.05$.

Results

Of the 6 mares used for this study, one developed a bacterial infection that required treatment, causing a one-cycle break between treatment cycles. None of the mares showed adverse reactions to the treatments. Expression of IL1β was lower after treatment with dexamethasone ($P<0.001$) and MCWE ($P = 0.04$) when compared with control (Fig 1a). There was a tendency of an overall effect of treatment on IFNγ mRNA expression ($P = 0.079$; Fig 1b). There was no effect of treatment on mRNA expression of IL1RA, IL6 or IL10 with either treatment (Figs 1c–e).

Discussion

Although MCWE is commonly used to stimulate the immune response for the reduction and treatment of disease [25–27], the level of the proinflammatory cytokine IL1β mRNA in the present study was decreased, not elevated, after treatment with MCWE. Given that previous research demonstrated an upregulation of proinflammatory cytokines occurring in cells 4–8 h after *in vitro* stimulation [28], it is possible that this increase occurred earlier than the time when biopsies were collected for this study. Uterine samples were collected 6 h after breeding, which was actually 30 h after treatment with MCWE. While speculative, a proposed mechanism is as follows: IL1β may have been decreased due to the MCWE priming the local immune response (thereby altering the cytokine response after the breeding challenge), and a decrease in IL1β could lead to a decreased proinflammatory response and a normal resolution of inflammation. The reduction of IL1β observed in this study may be a reflection of the ultimate effect of MCWE after inflammatory challenge, rather than a reflection of the immediate effect of MCWE on the endometrium. Previous studies investigating the effect of MCWE on endometrial inflammation after breeding also demonstrated a decrease in inflammation after treatment, but as with this study, those studies observed inflammation 30–48 h after administration of the drug [7,10]. A study investigating the precise timing of the effects of this treatment is warranted.

Although no effect of dexamethasone was observed on the anti-inflammatory cytokines in this study, IL1β mRNA expression was decreased after administration of dexamethasone. This is in agreement with another study that found that dexamethasone decreased IL1β mRNA expression in susceptible mares with experimentally induced bacterial endometritis [22].

During a functional inflammatory response, there is a balance of the pro- and anti-inflammatory components [1]. Anti-inflammatory cytokines modulate the pro-inflammatory response, which if overactive, can lead to tissue damage [29,30]. In a previous study, although no differences were observed between resistant and susceptible mares after breeding, susceptible mares did have a different pattern of mRNA expression of the proinflammatory cytokines (including IL1β), with a slower resolution back to normal. Additionally, compared with resistant mares, susceptible mares had a decreased anti-inflammatory response 6 h after breeding, which may account for the differences in the patterns of proinflammatory cytokines. Furthermore, polymorphonuclear leucocyte counts were increased at all time points in susceptible mares after breeding, whereas resistant mares did not have a significant increase at any time point investigated [11]. Finally, susceptible mares had an increase in nitric oxide production and accumulation after breeding compared with resistant mares, which has been hypothesised to play a role in decreased myoelectrical activity and uterine clearance [6,7]. Increased polymorphonuclear leucocytes and fluid accumulation are indicative of uterine inflammation, which may result in part due to a dysfunctional ability to balance the inflammatory response. Although this study did not detect an alteration in the anti-inflammatory response with either treatment 6 h after breeding, the decrease in IL1β mRNA expression may reflect the mechanisms that led to decreased clinical signs observed after treatment with immunomodulators in other studies [31].

In this study, treatment had no effect on the anti-inflammatory cytokines 6 h after breeding, but did have a suppressive effect on proinflammatory cytokine response. Interestingly, when investigating mRNA expression of these cytokines after bacterial-induced endometritis in susceptible mares, expression of IL6 and IL10 was higher 3 h after treatment with dexamethasone when compared with untreated controls [31]. Although this study did not investigate the inflammatory response 3 h after breeding, it is possible that during this time the cytokines would have been differentially expressed, potentially leading to the decrease in IL1β observed 6 h after breeding.

Of the 89 data points in this study, 4 were removed as outliers for this experiment, and all 4 points came from the same mare. Susceptible mares are by nature 'abnormal', so the authors elected to include the mare with the outlier data points, however, if she were excluded from the study due to the fact that all outlier data points came from her, the results for IFNγ would have been different. When looking at the IFNγ mRNA expression, the relative quantification value of the control sample from that mare was much higher than the other samples in that group, leading to a greater s.e.m. for the control samples. However, the data point was within the range of inclusion as defined by the study, and therefore was included in the data for IFNγ. When including the data point, the data were not normally distributed (even after data transformation) and were analysed nonparametrically, to yield a P value of 0.079 for the effect of treatment. However, if the mare had been excluded from the study, the data would have been normally distributed, and dexamethasone would have decreased mRNA expression of IFNγ ($P = 0.02$).

Seminal plasma was removed in this study to remove variability due to seminal plasma proteins. The model of using spermatozoa is similar to the practice of removing most of the seminal plasma during processing for freezing spermatozoa in preparation for artificial insemination in practice; however, studying the effect of these immunomodulators in the presence of whole semen would be valuable for industries prohibiting artificial insemination. Additionally, the formula for the extender used in this study is proprietary; however, studying the effects of different components of the extender on uterine inflammation would be valuable.

Conclusion

The cascades of inflammatory signals are complex and intertwined, with many points for regulation throughout. Treatment with immune modulation therapy has proven effective clinically in other studies; yet a complete understanding of the mechanisms behind these treatments remains elusive. The results from this study show that these treatments may act to change the inflammatory cascade early on in the inflammatory process, especially at the level of IL1β. Continued investigations into the mechanisms of these treatments have the potential to yield valuable information so that treatments can be improved in time, dosage and frequency.

Authors' declaration of interests

Dr Squires is a paid consultant for Bioniche (the company that makes MCWE).

Ethical animal research

The experiment was approved by the University of Kentucky's IACUC (protocol numbers 2009-0455 and 2009-0602).

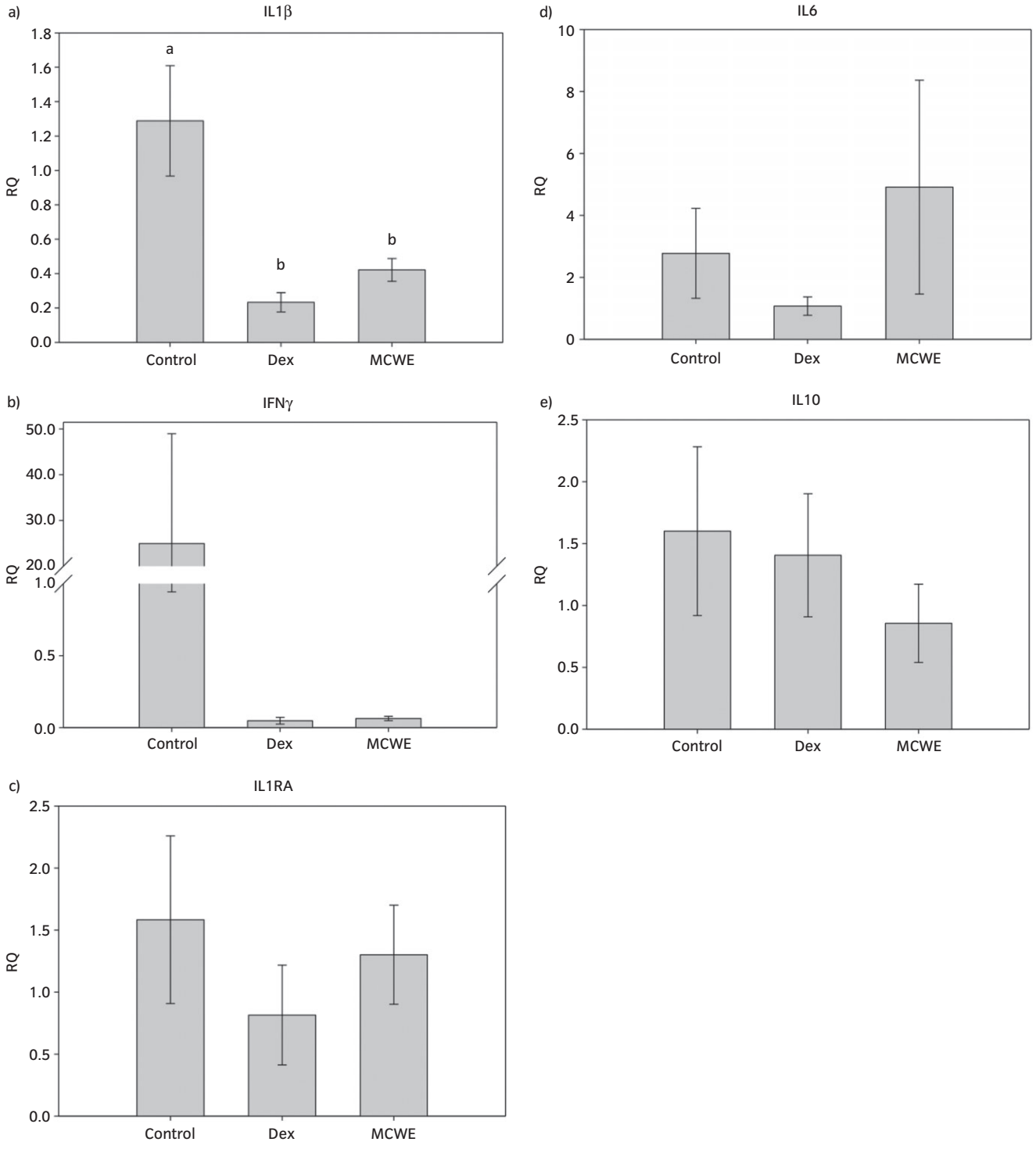

Fig 1: Endometrial mRNA expression (relative quantification: RQ) of inflammatory cytokines in susceptible mares 6 h after breeding with dexamethasone (DEX) or mycobacterial cell wall extract (MCWE). Treatments are compared with untreated susceptible mares 6 h after breeding. a) Interleukin (IL)1B; b) interferon (IFN)γ; c) ILRA; d) IL6; e) IL10. Error bars represent s.e.m. and differing letters indicate a significant difference in mRNA expression in treatment vs. controls ($P<0.05$).

Sources of funding

This work was supported by funds from the Gluck Fellowship in the Department of Veterinary Science, the Janet H. Koller Endowment for Equine Research, and Bioniche Life Sciences Inc.

Acknowledgements

The authors thank Mr Lynn Ennis, Mr Kevin Gallagher and the University of Kentucky's Maine Chance Farm for the management and care of the horses, and the University of Kentucky Veterinary

Diagnostic Laboratory for the use of their equipment and sample processing.

Authorship

E.M. Woodward participated in experimental design, sample collection and processing, data analysis/interpretation and writing of the manuscript. M. Christoffersen participated in the experimental design and writing of the manuscript. D. Horohov participated in the writing of the manuscript and provided lab equipment. E.L. Squires participated in the writing of the manuscript and provided the MCWE. M.H.T. Troedsson participated in experimental design, data interpretation, writing of the manuscript and provided laboratory space and horses.

Manufacturers' addresses

[a]Minitube of America, Verona, Wisconsin, USA.
[b]Pfizer, New York, New York, USA.
[c]Applied Biosystems, Carlsbad, California, USA.
[d]Invitrogen, Carlsbad, California, USA.
[e]Thermo Scientific, Wilmington, Delaware, USA.
[f]Promega, Wisconsin, USA.
[g]Bioline, Taunton, Massachusetts, USA.
[h]Systat Software Inc., San Jose, California, USA.

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