Endometrial inflammatory markers of the early immune response in mares susceptible or resistant to persistent breeding-induced endometritis

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Abstract

Transient endometritis after breeding is necessary for clearance of bacteria and spermatozoa; however, in a subpopulation of mares, the inflammation fails to resolve in a timely fashion. The objective of this study was to describe the uterine inflammatory response in mares susceptible or resistant to persistent breeding-induced endometritis (PBIE) during the first 24 h after induction of uterine inflammation. Twelve mares were classified as susceptible ($n=6$) or resistant ($n=6$) to PBIE. Mares were inseminated over five estrous cycles and endometrial biopsies were collected at one time point per cycle before (0) and 2, 6, 12, and 24 h after insemination. qPCR analysis for *IL1B*, *IL6*, *IL8*, *IFNG*, *TNF (TNFA)*, *IL10*, and *IL1RN* was performed, and endometrial inflammatory cells were counted for each sample. Relative quantification values reported fold changes in mRNA expression from 0 h values. A general pattern of expression post insemination was observed in both groups of mares. Cytokine mRNA increased at 2 h, peaked between 2 and 12 h, and then decreased. Differences were detected between groups of mares 6 h after challenge; resistant mares had higher mRNA expression of *IL6*, *IL1RN*, and *IL10* than susceptible mares. Susceptible mares had an increased number of polymorphonuclear neutrophils in the endometrium 2 and 12 h after breeding when compared with resistant mares. These findings describe an inherent difference in the initial immune response to insemination and may help explain the transient nature of inflammation in resistant mares, whereas susceptible mares develop a persistent inflammation.

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Introduction

A transient uterine inflammation in response to breeding (semen and contaminating bacteria) is a normal physiological reaction that aids in the clearance of excess semen and debris from the uterus. The conceptus arrives into the uterus around 5.5–6.5 days after fertilization (Oguri & Tsutsumi 1972, Betteridge *et al.* 1982, Freeman *et al.* 1991), and in horses and other species, uterine inflammation was found to be harmful to the conceptus and interfere with pregnancy (Adams *et al.* 1987, Reilas *et al.* 2000, Hill & Gilbert 2008). Therefore, it is important that breeding-induced endometritis be resolved before the arrival of the conceptus into the uterus. While uterine inflammation normally resolves within 24–48 h, some mares develop a persistent endometritis, which has been cited as a leading reproductive health concern in equine veterinary practice (Traub-Dargatz *et al.* 1991). Susceptibility to the failure of uterine clearance of a bacterial challenge

has been associated with impaired myoelectrical activity in response to inflammation (Troedsson *et al.* 1993a); however, the underlying mechanisms responsible remain poorly understood.

The inflammatory process involves chemokine-initiated recruitment of polymorphonuclear neutrophils (PMNs) and other inflammatory cells to the area of insult, leading to the removal of pathogens and resolution of inflammation (Parham 2005). Understanding the mechanisms of uterine inflammation is important when developing new protocols and treatments for persistent breeding-induced endometritis (PBIE). Fumuso *et al.* investigated endometrial cytokine mRNA expression 24 h after artificial insemination in susceptible and resistant mares. The group detected an increase in the pro-inflammatory cytokines interleukin-1 β (*IL1B*), *IL6*, and tumor necrosis factor α (*TNF (TNFA)*) in all mares after insemination and found that mares susceptible to PBIE had higher basal levels of mRNA expression of the cytokines when compared with resistant mares

(Fumuso *et al.* 2003). The same group subsequently found increased mRNA expression of the neutrophil chemoattractant *IL8* and lower expression of the inflammatory modulating cytokine *IL10* in susceptible mares compared with resistant mares 24 h after insemination (Fumuso *et al.* 2006, 2007). However, in contrast to Fumuso *et al.* other researchers were unable to find a significant *IL8* response 24 h after insemination in reproductively normal pony mares (Nash *et al.* 2010). Endometrial mRNA expression of *TNFA*, *IL1B*, *IL8*, and *IL10* were found to be upregulated in response to an i.u. infusion with a high dose of *Escherichia coli* as early as 3 h after challenge in normal diestrous mares (Christoffersen *et al.* 2010). Uterine clearance begins as early as 30 min after infusion with radiocolloid (LeBlanc *et al.* 1994), and myoelectrical patterns in susceptible and resistant mares differ as early as 6 h after bacterial inoculation into the uterus (Troedsson *et al.* 1993a). Given these findings, it is possible that the critical time frame for cytokine mRNA expression as it relates to susceptibility to PBIE occurs much earlier than 24 h (as previously investigated). Knowledge of the time frame of events and inflammatory cascades after breeding is critical for understanding of the development

of PBIE. Therefore, the objective of this study was to profile pro- and anti-inflammatory cytokine mRNA expression patterns and the numbers of endometrial PMNs in the equine endometrium at several time points within the first 24 h after a spermatozoa challenge. We hypothesized that i) there is a change in the endometrial inflammatory cytokine mRNA expression and endometrial PMN infiltration in mares at varying time points up to 24 h after insemination and ii) that mares susceptible to PBIE have a different profile of cytokine expression and endometrial PMN infiltration than mares resistant to PBIE within the first 24 h of breeding.

Results

Cytokine mRNA expression

IL1B mRNA expression was increased at 2 and 6 h for both groups of mares after insemination ($P \leq 0.01$). No differences were observed between susceptible and resistant mares at any time point (Fig. 1a).

IL6 mRNA expression was increased at all time points in both groups of mares after insemination ($P \leq 0.047$). In addition, susceptible mares had a higher level of

Figure 1 (a, b, c, d, e, f and g) Mean endometrial cytokine mRNA expression before (0) and 2, 6, 12, 24 h after insemination with killed spermatozoa in susceptible (S; dark bars) and resistant (R; light bars) mares. RQ values describe fold changes in each mare compared to the Δ CT value of resistant mares at 0 h. Data presented in graphs are the raw RQ values, although data were log10 or square root transformed for statistical analysis. Error bars report S.E.M. Comparisons were made within groups (S or R) at each time point and different letters over the bars (capital letters for susceptible; lowercase for resistant) within each group represent significant differences. Comparisons were also made between groups at each time point, and significant differences are represented by an asterisk (*). Significance set to $P < 0.05$.

expression at 2 h when compared with 24 h ($P=0.033$) and resistant mares had an increase at 6 h when compared with 12 and 24 h ($P\leq 0.002$). Resistant mares had a higher level of expression at 6 h when compared with susceptible mares ($P=0.01$) (Fig. 1b).

IL8 mRNA expression in resistant mares was increased at 2 and 6 h after insemination ($P<0.04$) followed by a decrease from 6 to 12 h ($P=0.042$). In susceptible mares, there was a significant rise in expression at 2 h after insemination ($P=0.005$). There were no differences detected in expression between susceptible and resistant mares (Fig. 1c).

Interferon gamma (*IFNG*) mRNA expression in resistant mares was increased at 2 and 6 h after insemination ($P\leq 0.001$). Susceptible mares had increased expression at 6 h compared with pre-insemination levels, 12, and 24 h ($P\leq 0.046$). There were no differences between susceptible and resistant mares; however, the patterns of *IFNG* mRNA expression differed between the two groups shortly after insemination. Although both groups exhibited a response, the resistant mares had increased expression at 2 h, whereas mRNA expression in the susceptible group did not increase until 6 h after insemination (Fig. 1d).

TNFA mRNA expression in susceptible mares did not differ at any time point. In resistant mares, there was an increase in expression at 2 h compared with the pre-insemination, 12, and 24 h time points ($P\leq 0.032$). Expression decreased at 12 h from 2 to 6 h ($P\leq 0.034$), and at 24 h, was lower than pre-insemination levels ($P=0.046$). There were no differences between groups (Fig. 1e).

IL10 mRNA expression in resistant mares was upregulated at 6 h compared with all time points ($P\leq 0.001$), and at 2 h compared with pre-insemination levels and 24 h ($P\leq 0.045$). Susceptible mares showed an increase at 6 h compared with pre-insemination levels and 24 h ($P\leq 0.027$). Resistant mares had higher expression than susceptible mares at 6 h ($P=0.021$) (Fig. 1f).

IL1RN mRNA expression peaked at 6 h in resistant mares ($P<0.001$), with a marked increase from 2 to 6 h ($P<0.001$) and a decrease from 6 to 12 h ($P<0.001$). Expression returned to pre-insemination levels by 24 h. Susceptible mares had increased expression at all time points after insemination ($P\leq 0.017$), with the highest levels observed at 6 and 12 h. Resistant mares had higher mRNA expression at 6 h than susceptible mares ($P=0.013$), and susceptible mares had higher expression at 12 h than resistant mares ($P=0.017$) (Fig. 1g).

The mRNA expression patterns for the pro-inflammatory cytokines *IL1B*, *IL8*, *IFNG*, and *TNFA* were similar in both groups of mares, with an initial increase at 2 and 6 h, followed by a decrease toward pre-insemination levels. Although there were no differences between susceptible and resistant mares at any time point for these cytokines, there were subtle variations in the rise toward the peak and the return to pre-insemination level expression. All mares returned to pre-insemination levels

of mRNA expression of *IL1B* and *IL8* by 12 h. However, resistant mares had a significant decrease in expression from 6 to 12 h, while the susceptible mares had a less rapid decline in expression to pre-insemination levels compared with the resistant mares.

There were distinct differences in the patterns of expression between resistant and susceptible mares for *IL6* and the inflammatory modulating cytokines *IL1RN* and *IL10*. mRNA expression for all three cytokines was decreased in the susceptible mares at 6 h when compared with the resistant mares, and susceptible mares had a higher expression of *IL1RN* at 12 h when compared with the resistant mares. An outline connecting the mean expression at each time point for the three cytokines shows that the resistant mares have an expression profile with a sharp peak at 6 h, while the lines for the susceptible mares resemble more of a hump, with slower increases to and decreases from the peak.

Endometrial inflammatory cells

Susceptible mares had an increase in the number of PMNs in the endometrial tissue at all time points after breeding ($P\leq 0.012$) (Fig. 2). There were no significant increases in PMN infiltration at any time point for the resistant mares; however, it should be noted that there was a tendency for an increase 6 h after breeding compared with the pre-insemination ($P=0.054$) and 2 ($P=0.056$) h time points (Fig. 2). Susceptible mares had a higher infiltration of PMNs 2 ($P=0.010$) and 12 ($P=0.031$) h after breeding compared with resistant mares (Fig. 2).

Discussion

Pro-inflammatory cytokines initiate inflammation, and once produced, a cascade of inflammatory events occurs (Kuby 1992, Parham 2005). A timely response to pathogens is important for an effective resolution of inflammation and preservation of tissue, and *IFNG* is important in the initiation of inflammation (Schroder *et al.* 2004). The delayed rise in *IFNG* mRNA in the susceptible mares observed in this study may reflect a delayed inflammatory response to spermatozoa compared with resistant mares. Furthermore, the differences observed in the mRNA expression patterns of the pro-inflammatory cytokines suggest that susceptible mares have a slower return to pre-insemination levels, possibly contributing to the prolonged clinical signs observed in the clinical setting (Zent *et al.* 1998).

As with the other pro-inflammatory cytokines, mRNA expression for *IL6* increased in response to challenge in both groups. *IL6* was the only pro-inflammatory cytokine with a significant difference between the two groups, with resistant mares having increased mRNA expression compared with susceptible mares at 6 h (which is similar

Figure 2 Mean endometrial PMN infiltration before (0) and 2, 6, 12, and 24 h after insemination with killed spermatozoa in susceptible (S; dark bars) and resistant (R; light bars) mares. Error bars report S.E.M. Comparisons were made within groups (S or R) at each time point and different letters over the bars (capital letters for susceptible; lowercase for resistant) within each group represent significant differences. Comparisons were also made between groups at each time point, and significant differences are represented by an asterisk (*). Significance set to $P < 0.05$.

to the findings of the inflammatory modulating cytokines *IL10* and *IL1RN*). While *IL6* is considered a pro-inflammatory cytokine, it has protective roles in early acute inflammation and shock through the modulation of other pro-inflammatory cytokines (Barton & Jackson 1993, Xing *et al.* 1998, Diao & Kohanawa 2005) and the induction of inflammatory modulating cytokines, such as *IL1RN* (Tilg *et al.* 1994). In addition to the resolution of acute inflammation, *IL6* and its receptor has been suggested to have implications in the transition from innate to acquired immunity and the persistence of chronic inflammation (Kishimoto *et al.* 1995, Atreya *et al.* 2000, Jones 2005). It is possible that the increase in *IL6* mRNA 6 h after insemination in resistant mares compared with susceptible mares may reflect initial inflammatory modulating roles of *IL6* (similar to those of *IL10* and *IL1RN*) during this time (Tilg *et al.* 1994). Treatment with the anti-inflammatory dexamethasone in susceptible mares has been demonstrated to increase endometrial *IL6* mRNA expression 3 h after inoculation with *E. coli* when compared with susceptible mares inoculated without dexamethasone treatment (Christoffersen *et al.* 2012a). The same group observed an increase in mRNA expression of the immune modulating cytokine *IL10* and a decrease in the pro-inflammatory cytokine *IL1B* 3 h after treatment. These findings further support the idea that *IL6* may be initially involved with an immune modulating response to challenge, as the initial mRNA response of *IL6* to dexamethasone and *E. coli* was similar to that of the anti-inflammatory *IL10* and opposite to that of the pro-inflammatory *IL1B*. In addition, the elevated levels of *IL6* mRNA observed in untreated susceptible mares inoculated with *E. coli* at 72 h is similar to the mRNA response of the *IL1B* and opposite to *IL10* (Christoffersen *et al.* 2012b), which may reflect

the pro-inflammatory actions of *IL6* associated with inflammation more persistent in nature.

There is a delicate balance between the pro- and anti-inflammatory response; pro-inflammatory cytokines act to initiate and increase the response, while anti-inflammatory cytokines modulate the pro-inflammatory cytokines (Parham 2005, Hackett *et al.* 2008). *IL10* is essential for the modulation of the immune response, and deficiencies in *IL10* can lead to tissue damage (Cyktor & Turner 2011). In addition, the *IL1* system is involved with both pathologic states as well as normal reproductive function (Takehara *et al.* 1994, Van der Hoek *et al.* 1998, Martoriati *et al.* 2003, Gerard *et al.* 2004). *IL1RN* mediates *IL1*, which, if overactive, can damage tissue (Gerard *et al.* 2004). In this study, susceptible mares had a less defined immune modulating response than resistant mares, suggesting that these mares were less able to respond to and initiate the modulation of inflammation.

The cytokine cascade initiates inflammatory cell recruitment, and it is possible that an increase in cytokine expression could be related to the increase in endometrial PMNs observed in this study. The findings that PMN counts in this study were observed to be highest 6 h after breeding in susceptible mares are consistent with *in vitro* and *in vivo* studies that found that PMN migration peaked 6 h after *IL8* induction (Zerbe *et al.* 2003). Interestingly, in this study, susceptible mares had an increase in *IL8* mRNA 2 h after breeding, whereas resistant mares had an increase at both 2 and 6 h after breeding. However, the resistant mares had no significant increase in endometrial PMN infiltration, while the susceptible mares had increases in endometrial PMNs at all time points compared with pre-insemination counts. It is unclear why *IL8* mRNA expression did not

directly correlate with PMN infiltration. A possible explanation is that resistant mares have a differential posttranscriptional regulation of *IL8* compared with susceptible mares. Also, the migration of PMNs to the uterine lumen may be hindered in the susceptible mares because the endometria of susceptible mares have more degenerative changes, and PMNs may have trouble moving through the fibrotic endometrial tissue. In addition, the uterine contractions in response to inflammation may aid in the movement of PMNs into the uterine lumen, and the migration of the PMNs may be slower in susceptible mares, as they have reduced myometrial activity (Troedsson *et al.* 1993a, LeBlanc *et al.* 1994). The increase in endometrial PMNs in susceptible mares may reflect longer persistence of PMN accumulation rather than differences in cellular responses and may be more a symptom than cause of prolonged inflammation. These explanations are speculative and warrant further investigation. An increase in PMNs or other inflammatory cells is reflective of an inflammatory response, and regardless of the source of cytokines, the differences in cytokine expression observed between the two groups of mares illustrate differences occurring on an mRNA level during the resolution of inflammation.

These data support previous research that illustrated a local immune response in response to intrauterine challenge with dead spermatozoa (Fumuso *et al.* 2007), and the conclusions that susceptible mares are less capable of clearing the uterine inflammation than resistant mares (Hughes & Loy 1969, Troedsson *et al.* 1993b, Fumuso *et al.* 2006, Woodward *et al.* 2012). However, the results conflict with those from other studies as to which cytokines differ and at which time points (Fumuso *et al.* 2003, 2006). A possible explanation for discrepancies between studies is the criteria by which mares are selected as resistant vs susceptible to PBIE. It is important to determine that mares are free of inflammation before breeding (via uterine culture and cytology) because chronic inflammation may shift cytokine mRNA profiles before insemination, therefore altering results whether data are to be analyzed using a relative quantification method (as may have been the case in the later studies). A critical factor for classification of mares is to ensure that the criteria for susceptibility and resistance are stringent enough to represent the truly resistant or susceptible mare, as many mares fall into an intermediate category. The model used in this experiment uses such strict criteria and therefore is appropriate for the study of PBIE. In addition, these data demonstrate that the time point of 24 h may be too late to see a response with some cytokines in normal mares, which could explain inconsistencies with this experiment and previously published works (Fumuso *et al.* 2006, Nash *et al.* 2010). For example, if normal mares are meant to resolve inflammation within 48 h, it is plausible that at 24 h after challenge, neutrophils are no longer being actively recruited, and although neutrophils are likely to still be

present in the uterus (Nash *et al.* 2010), *IL8* may not be produced at elevated levels during this time.

The findings that susceptible mares have an altered initial inflammatory response on a gene expression level support other data describing differences in the ability to clear inflammation between resistant and susceptible mares after bacterial challenge (Troedsson & Liu 1991, Troedsson *et al.* 1993a, LeBlanc *et al.* 1994). Although the clinical signs differentiating resistant and susceptible mares are observed several days after insemination, to better define the pathogenesis of PBIE it is important to elucidate the timing of the uterine immune response of the resistant mare compared to the susceptible mare at a molecular level. It is possible that the actual peaks of cytokine expression may fall at a time point other than the ones observed in this study. However, when considering the five time points investigated, our data suggest that around 6 h after insemination may be a critical time in developing susceptibility, as this time point was both the peak of expression observed in this study for many of the cytokines and the time point at which susceptible and resistant mares differed in their mRNA expression of *IL6*, *IL10*, and *IL1RN*. Furthermore, a failure to resolve breeding-induced inflammation in a timely fashion may be due in part to a failure to mobilize inflammatory (both pro- and anti-inflammatory) cytokines during the early inflammatory period, which could contribute to a delayed resolution of inflammation in susceptible mares.

It is unknown whether there was an effect of stallion on inflammatory response. Spermatozoa have been shown to be the component in the ejaculate that causes inflammation, with seminal plasma modulating the response (Troedsson *et al.* 2005, Fiala *et al.* 2007). To account for any differences between the protein composition of the seminal plasma or viability counts between stallions, the seminal plasma was removed and all spermatozoa were killed before insemination. In conclusion, the results from this study suggest that around 6 h after insemination may be a critical time in the establishment of the mechanism for uterine response and clearance. It is possible that the altered local cytokine response observed in susceptible mares could lead to an impaired ability to clear inflammation, as the cytokine response to inflammation is the first step in recognition and removal of foreign material. With an increased understanding of the cytokine pathways shortly after breeding, research can continue in elucidating the mechanisms in later stages of inflammation, and eventually, treatment strategies can be improved upon.

Materials and Methods

Animals

Ninety-two mares (*Equus caballus*) of mixed breeds, age (2–27 years old), and foaling history were kept on pasture

supplemented with grain and hay and were provided with water and salt *ad libitum*. Three stallions were maintained under similar housing conditions to that of the mares. The experiment was approved by the University of Kentucky's Institutional Animal Care and Use Committee (protocol numbers 2009-0455 and 2009-0602).

Preparation of sperm for insemination

Semen was regularly collected from the stallions and used as needed throughout the experiment. Freeze-killed spermatozoa were prepared by removing the seminal plasma from ejaculates using centrifugation (2000 *g* for 10 min), and resuspending the spermatozoa pellet in milk-based extender (EquiPro, Minitube, Verona, WI, USA) in aliquots of 1×10^9 spermatozoa in 30 ml extender followed by at least two freeze (-20°C)/thaw cycles of the aliquots to kill the spermatozoa.

General experimental procedure for the selection of mares

Mares were examined for susceptibility to PBIE based on endometrial histology and the results of a spermatozoa challenge as described previously (Woodward *et al.* 2012). Endometrial biopsies (~ 300 mg of tissue) were obtained during diestrus using an alligator jaw biopsy instrument, fixed in 10% formalin, sectioned at $5\ \mu\text{m}$, and stained with hematoxylin and eosin. Each biopsy was examined for periglandular fibrosis, inflammatory cells, glandular distribution, and lymphatic lacunae and then graded according to Kenney & Doig (1986). Mares with scores of I or IIA were considered potentially resistant to PBIE ($n=27$), while those with scores of IIB or III were potentially susceptible to PBIE ($n=65$) (Troedsson *et al.* 1993b, Woodward *et al.* 2012).

After selection as potentially resistant or susceptible to PBIE, the mares' reproductive tracts were observed regularly for signs of estrus and pending ovulation using transrectal ultrasonography. Estrus was defined by the presence of uterine edema, a relaxed cervix, and at least one follicle 35 mm or larger in diameter in the absence of a corpus luteum. Once estrus was detected, mares were evaluated for the presence of intrauterine inflammatory cells using a cytobrush (Minitube) or low-volume lavage to obtain samples and for intrauterine bacteria using a double-guarded swab (Minitube) to obtain samples and then were subsequently inseminated with freeze-killed spermatozoa (prepared as earlier). For the cytological evaluations, a sample positive for inflammation was defined as more than two

neutrophils per five fields at $\times 400$ magnification. Swab samples were cultured on blood agar, then incubated at 37°C for 24 h, and evaluated for bacterial growth. Mares were challenged with an i.u. inoculation of 1×10^9 freeze-killed stallion spermatozoa in 30 ml of milk-based semen extender (Minitube). Only mares with negative cytology and no bacterial growth before insemination were considered for the experiment.

Potentially susceptible mares were evaluated 96 h after insemination and were confirmed as susceptible if they had i) positive cytology and ii) uterine fluid retention. Potentially resistant mares were assessed 48 h after breeding, and those with i) negative cytology and culture in addition to ii) no uterine fluid retention were confirmed as resistant mares (Troedsson & Liu 1991, Alghamdi *et al.* 2005). All mares not meeting the criteria for classification as susceptible or resistant were considered as intermediate and excluded from the study. From the herd of 92 mares, a total of six susceptible and six resistant mares were identified and used in this study.

Treatments

Over five subsequent cycles, estrus was induced after day 5 of diestrus by i.m. administration of prostaglandin $\text{F}_{2\alpha}$ (Lutalyse 7.5 mg, Pfizer, New York, NY, USA). Once estrus was detected, mares were evaluated for cytological and bacterial findings (as described earlier) and subsequently inseminated with 1×10^9 dead spermatozoa (as described earlier). Only estrous cycles with negative cytology and culture were used for the experiment; if mares were positive for cytology or culture at the time of insemination, they were treated and inseminated again during the following cycle (this happened four times throughout the experiment).

Two uterine biopsies were collected at one time point per cycle in randomized order. Time points were 0 (before insemination), 2, 6, 12, and 24 h after insemination. The tissue from the first biopsy was either snap frozen in liquid nitrogen and stored at -80°C until further processing or stored in RNeasy (Applied Biosystems) overnight at 4°C , then moved to -20°C for storage until further processing. Tissue from the second biopsy was fixed in 10% formalin for staining and observation of inflammatory cells.

qPCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen), precipitated using sodium acetate and isopropanol, resuspended

Table 1 Dual hydrolysis primer/probe set sequences for the detection of equine mRNA. Primer/probe sets were designed using Assays-By-Design (Applied Biosystems).

Cytokine	Forward primer sequence	Reverse primer sequence	Probe sequence
EqACTB	CTGGACTTCGAGCAGGAGATG	CGTCGGGCAGCTCGTA	CCGCGGCCTCCAGCT
EqIL1B	CCGACACCAGTGACATGATGA	ATCCTCCTCAAAGAACAGGTCATTC	ATTGCCGCTGCAGTAAG
EqIL6	GGATGCTTCCAATCTGGGTTCAAT	TCCGAAGACCACTGGTGATTTT	ATCAGGCAGGTCTCCTG
EqIL8	GCCGTCTTCTGCTTCTG	CCGAAGCTCTGCAGTAATCTTGAT	CAACCGCAGCTTCAC
EqINFG	AGCAGCACCAGCAAGCT	TTTGCGCTGGACCTTCAGA	ATTGAGATTCCGGTAAATG
EqTNF	TTACCGAATGCCTTCCAGTCAAT	GGGCTACAGGCTTGCTACTT	CCAGACACTCAGATCAT
EqIL10	ATGCCCCAGGCTGAGAAC	CGGAGGGTCTTCAGCTTTTCC	CCAGACATCAAGGAGCAGC
EqIL1RN	AGTTGCTGGATACTTGCAAGAATCA	GAGTCCCAGGAATAGAGCATCAG	CATCTATCTTCTTGTGAATTTA

in ddH₂O, and DNase treated with a commercially available kit (DNA-free, Applied Biosystems) and then analyzed for quality and quantity using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The ranges for the 260/280 and 260/230 ratios were 1.92–2.2 and 1.89–2.4 respectively. RNA (1.5 µg) in 41.5 µl ddH₂O was reverse transcribed in a reaction using AMV Reverse transcriptase (0.5 µl; 10 U/µl), 5×RT buffer (16 µl), RNasin (1 µl; 40 U/µl), MgCl (16 µl; 25 mM), dNTP (4 µl; 10 mM), and Oligo(dT) primer (1 µl; 500 µg/ml) (all reagents from Promega). Samples were incubated at 42 °C for 60 min and then 95 °C for 5 min. cDNA was diluted 1:1 with ddH₂O, and qPCR for each sample was performed using 4.5 µl cDNA, 5 µl Sensimix II (Bioline, Tauton, MA, USA), and 0.5 µl of a custom dual hydrolysis primer/probe set (Applied Biosystems; Table 1). Reactions were performed in duplicate, and using the 7900HT Fast Real-Time PCR System (Applied Biosystems), were incubated at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. PCR efficiencies were calculated using LinRegPCR (version 7.0). β -Actin (*ACTB*) was used as the reference gene, as it was previously determined to be the most stably expressed gene out a panel of four reference genes (*18S* RNA, β -glucuronidase, glyceraldehydes 3-phosphate dehydrogenase, and *ACTB*) (Christoffersen *et al.* 2010). Results are expressed as mean relative quantification values (RQ) that were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001), with the calibrator as the mean cycle threshold (ΔCT) value of the 0 h collections from the resistant mares.

Inflammatory cell count

Biopsies were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin. For each sample, a blinded researcher counted PMNs and lymphocytes from five fields at ×400 magnification and an average number of inflammatory cells per sample were recorded.

Statistical analysis

Data were analyzed using SigmaStat® (Systat Software, Inc., Chicago, IL, USA) with two-way repeated measures ANOVA tests. Data were log₁₀ or square root transformed for normal distribution, and outliers were defined as ± 2 s.d. from the mean and were removed from statistical analyses (five of 406 data points removed). Missing data points were automatically calculated by the software using a general linear model (Sigma Stat User Guide, p. 379). Comparisons were made between groups at each time point and between time points within groups. *Post hoc* analysis was performed using the Holm–Sidak method, and significance was set to $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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