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Immunomodulator use in mares

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Abstract

A delicate balance exists between the immune system and the female reproductive tract, modulating numerous aspects of reproductive health. This includes, but is not limited to, ovulation, breeding-induced uterine inflammation, fertilization, pregnancy maintenance, response to placental infection, and postpartum involution. Additionally, stimulation of innate and adaptive immune systems can assist in clearing pathogens from the reproductive tract, thereby diminishing inflammation and/or infection that may inhibit reproductive health. In this review, the mechanisms through which the immune system modulates reproductive tract will be elucidated and critically evaluated immunomodulators will be discussed.

Keywords: Immunomodulators, persistent breeding-induced endometritis, placentitis

Immune response to breeding

Inflammation and/or infection of the endometrium is the leading cause of subfertility in mares, resulting in persistent breeding-induced endometritis (PBIE), affecting 10 - 15% of the broodmare population.[1] Deposition of sperm, seminal plasma, and/or bacteria in the reproductive tract elicits an inflammatory response that leads to activation of the innate arm of the immune system. This begins with the stimulation of pattern-recognition receptors,[2] induction of the complement system,[3-5] increases in proinflammatory signaling molecules,[6-9] and recruitment of immune cells to the site of inflammation.[5,10-12] Release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from an inflamed endometrium and appearance of activated leukocytes coincide with an increase in myometrial contractility, leading to eventual expulsion of excess fluid and contaminants.[13,14]

In reproductively sound mares that are resistant to PBIE, this inflammation is resolved within 24 - 36 hours, whereas a subset of mares that experience prolonged inflammation are considered susceptible to PBIE.[9,13] This leads to uterine fluid accumulation, extended neutrophilia within the uterine lumen, and persistent inflammation for > 96 hours, potentially impeding embryo viability upon migration from the oviduct. Although both susceptible and resistant mares experience this activation of the innate immune response, susceptible mares fail to mount the antiinflammatory response required to mitigate the proinflammatory signaling, and this is speculated to be the primary cause of persistent inflammation in these mares. The critical period for the immune response is within 6 hours after insemination.[8] In this period, resistant mares experienced an upregulation of various antiinflammatory and immunomodulatory cytokines, including interleukin (IL)-1 receptor antagonist, IL-10, and IL-6.[8] In contrast, susceptible mares failed to upregulate these immunomodulating cytokines in this critical period, leading to prolonged inflammation. This was specifically noted in increased expression of proinflammatory cytokines (IL-1β and IL-8), both contributing to chemotaxis of various immune cells to sites of inflammation, and specifically in recruitment of polymorphonuclear neutrophils (PMN).[15] Therefore, increased expression of these cytokines coincides with the pronounced neutrophilia noted in susceptible mares. Additionally, susceptible mares accumulated excessive nitric oxide (NO) in the uterus after breeding.[14] This has also been noted via an upregulation of mRNA expression for inducible nitric oxide synthase (iNOS) in the endometrium of susceptible mares after breeding.[16] Accumulation of NO in susceptible mares is believed to cause impaired myometrial contractions, resulting in delayed uterine clearance of inflammation from the uterus.[17,18] Differences in immune response between susceptible and resistant mares are summarized (Figure 1).

Several therapeutics are utilized for the management of PBIE, with many targeting the resolution of this persistent inflammation. Deemed immunomodulators, these therapeutics modify the immune response or the function of the immune system through a variety of pathways, including stimulation or suppression of signaling molecules, recruitment of essential immune cell types, or activation of other aspects of immunity, including antigen recognition and processing. Critically evaluated immunomodulators available for PBIE treatment are reviewed.

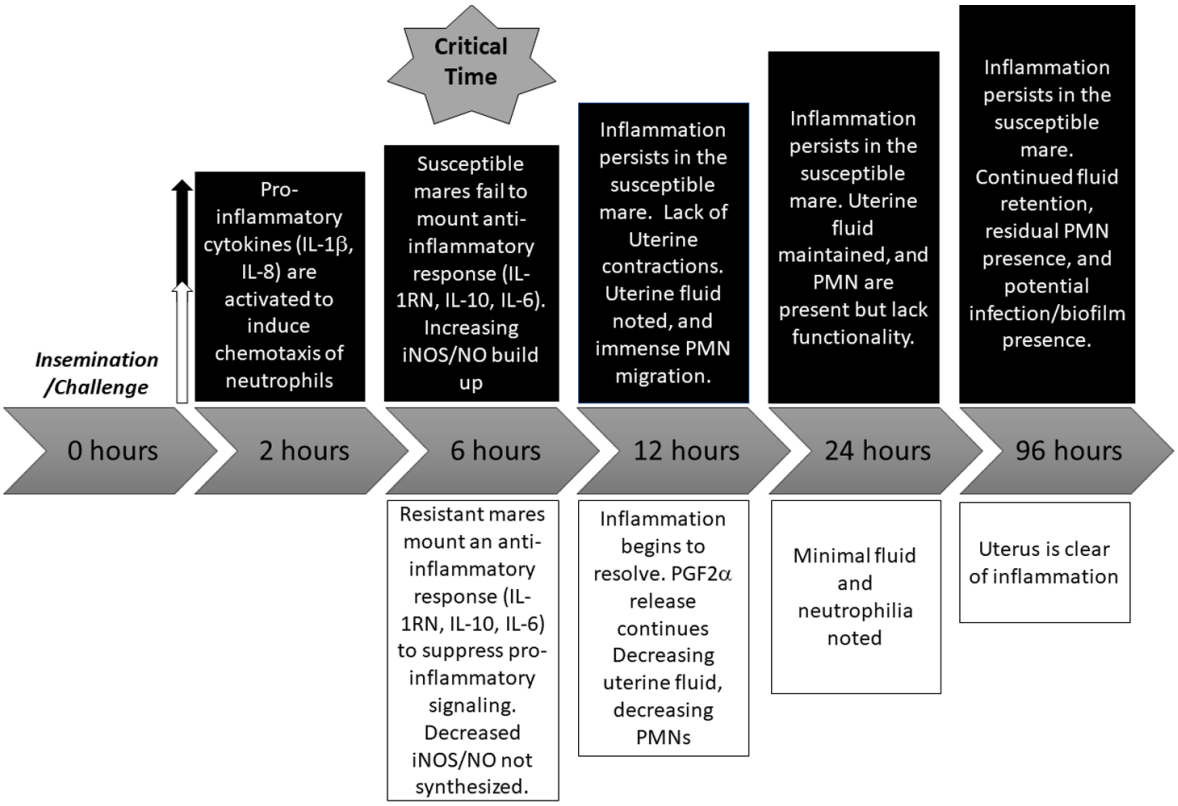

Figure 1. Immune response to breeding in resistant and susceptible mares to persistent breeding-induced endometritis

Immunomodulation of persistent endometritis

Selection of an immunomodulator for the treatment of PBIE must be considered carefully, as proinflammatory signaling is necessary for the clearance of excess sperm, debris, and pathogen recognition, whereas antiinflammatory signaling is crucial for a timely resolution of this inflammation. Pivotal studies on the immune response to breeding indicate that activation of proinflammatory pathways occurs as rapidly as 30 minutes after deposition of semen and is necessary for the chemotaxis of PMNs into the uterine lumen for proper degradation of nonviable sperm and bacteria.[5,8,19] Therefore, antiinflammatory or immunomodulating therapeutics either prior to breeding or immediately after breeding may impede a mare's essential innate immune response. Consequently, results from studies must be interpreted critically regarding the time and route of treatment and observed downstream effects.

Nonsteroidal antiinflammatory drugs

Nonsteroidal antiinflammatories (NSAIDs) are routinely used with the intent to diminish persistent inflammation noted in susceptible mares. Most NSAIDs inhibit cyclooxygenases (COX-1 and COX-2), thereby decreasing production of prostaglandins. Since the primary activity of $PGF_{2\alpha}$ is activation of myometrial contractility, inhibition of this lipid can lead to insufficient uterine contractions and luminal fluid accumulation if mares are not concurrently treated with an ecbolic to stimulate uterine contractions. Intravenous flunixin meglumine (1.1 mg/kg, 2 hours after insemination) treatment, substantially increased the number of PMNs in the uterine lumen at 8 hours; however, they were decreased at 25 hours after insemination.[20]

In contrast, select COX-2 inhibitors (function solely through the inhibition of COX-2 with no effect on COX-1) had a positive effect on uterine clearance after breeding. When treated orally once in the periovulatory period, firocoxib (Equioxx®, 0.2 mg/kg) decreased the severity of neutrophilia. Additionally, firocoxib treatment did not impact ovulation, embryo collection, or embryo mobility.[21,22] Vedaprofen, an additional selective COX-2 inhibitor, increased fertility in PBIE mares.[23] Oral vedaprofen (2 mg/kg) administered twice during the periovulatory period improved pregnancy rates; however, only a limited number of mares were evaluated.[23] It is emphasized that all NSAID treatments of PBIE should be accompanied by ecbolic treatment to stimulate uterine contractions

Glucocorticoids

Glucocorticoids are a class of corticosteroids that modulate the immune system by activating downstream functions of the glucocorticoid receptor. Although various glucocorticoids

are available to the practitioner, only a few have been critically evaluated for their efficacy in treating PBIE. Dexamethasone functioned as an antiinflammatory agent to both sperm and bacterial challenges.[24,25] Additionally, dexamethasone decreased endometrial expression of positive acute phase protein, serum amyloid A (SAA),[26] and similarly prednisolone treatment decreased expression of this protein.[27] Clinically, a single intramuscular treatment of 50 mg dexamethasone at breeding improved pregnancy rates in mares with > 3 risk factors associated with susceptibility to PBIE.[28]

Although limited research has been conducted on the antiinflammatory functions of corticosteroids, a subset was evaluated clinically. Treatment with acetate-9-α-prednisolone (0.1 mg/kg) twice daily also had a positive effect on pregnancy rates in mares with excessive inflammation.[29] Twice daily intramuscular treatment with isoflupredone acetate (Predef® 2X, 10 - 20 mg) 24 hours prior to bacterial challenge in susceptible mares altered the immune response.[27] Responses included reduction in immunoglobulin G (IgG) and increases in positive acute phase proteins' (e.g. haptoglobin) concentrations.

It is noteworthy that various glucocorticoids altered secretion of GnRH and LH, in addition to altering the function of hypothalamic-pituitary-gonadal axis.[30] Additionally, prolonged dexamethasone treatment has been associated with ovulation failure.[31] Therefore, treatment of PBIE should be limited to a single dose of glucocorticoid.

Bacterial extracts

Extracts from nonpathogenic bacteria are utilized for their antiinflammatory, antimicrobial, and anticancer functions in a variety of diseases and disorders. Of these bacterial extracts, only 2 have been evaluated for their efficacy in the treatment of equine PBIE: *Mycobacterium* cell wall fraction (MCWF; Settle®, NovaVive; Canada) and *Propionibacterium acnes* (Eqstim®, Neogen; US). In the research setting, Settle® has shown both antimicrobial and antiinflammatory actions in susceptible mares. Settle® decreased endometrial expressions of IL-1β, a proinflammatory cytokine and increased cytokine IL-10, an antiinflammatory cytokine, after a bacterial challenge with gram positive *Streptococcus equi*.[32] Additionally, Settle® substantially reduced inflammation in several mares that cultured positive for bacteria. These antiinflammatory effects were also noted after a challenge with gram negative *Escherichia coli*, wherein Settle® substantially decreased the endometrial expression of the positive acute phase protein SAA, decreased intrauterine fluid, and also decreased the number of mares that cultured positive for intrauterine bacterial growth.[24] After a challenge with freeze-killed sperm in susceptible mares, Settle® substantially reduced the endometrial expression of proinflammatory cytokines IL-1β and IFNγ.25 Settle® was effective at 1.5 mg dose, either given intravenously or infused into the uterine lumen.[33] Eqstim® treatment of endometritis along with other conventional therapies improved pregnancy rates in mares with clinical endometritis.[1] However, effects of this therapeutic as a sole modality are unknown.

Biologics

During the 1970s, naturally sourced biologics, were evaluated for the treatment of inflammation/infection of uterus. Intrauterine infusion of equine colostrum was the first agent tested.[34] Due to its high immunoglobulin content and potential antimicrobial action, colostrum was collected from mares immediately after parturition and 120 ml infused into the uterus of subfertile mares. Mares returned to normal cyclicity and uterine lumen had various immunoglobulins (IgG, IgM, and IgE). However, intrauterine deficiency in IgG and IgA in susceptible mares was not likely the cause of susceptibility to persistent endometritis.[4]

A key factor believed to distinguish resistant from susceptible mares is the innate immune system's ability to degrade and digest bacteria and excess sperm from the uterine lumen. The addition of blood serum substantially increased opsonization potential in both resistant and susceptible mares, indicating an antimicrobial effect of blood plasma. Uterine-derived PMNs from susceptible mares were able to migrate toward a chemical gradient and phagocytosis was fully functional if a healthy environment was provided.[35] However, phagocytosis was impaired in uterine secretions from susceptible mares. It was concluded that dysfunctions of PMNs from susceptible mares were due to a poor environment rather than the PMNs of susceptible mares. Nevertheless, it was suggested that addition of fresh blood-derived leucocytes to autologous plasma prior to post-breeding intrauterine infusions can improve pregnancy rates in barren mares.[36] There was clinical improvement (24/29 mares) and pregnancy rate (15/29 mares) in mares experiencing uterine inflammation after intrauterine infusion of plasma.[37] However, it was ineffective in the treatment of either lymphocytic or infectious endometritis.38 These findings were further supported by the observation that intrauterine treatment with autologous blood plasma in susceptible mares was less effective than antibiotics, ecbolics, or uterine lavage in mares with acute infectious endometritis.[39] It is noteworthy that there was a subgroup of individual susceptible mares that responded adequately to autologous plasma treatment.

Decades later, plasma treatment protocols were modified with intrauterine treatment of platelet-rich plasma (PRP). A concentrate of PRP protein derived from whole blood, contained several growth factors and cytokines released from the intracytoplasmic granules when cells are activated.[40] PRP is utilized in several tissues and disorders for its antiinflammatory, antimicrobial, and angiogenic properties, including joint disease, soft tissue injury, and skin wounds.[41-43] PRP functioned as an antiinflammatory agent when infused within the uterine lumen by decreasing the expression of proinflammatory cytokines (COX-2, IL-1β, and TNF).[44-45] Although a small number of mares were investigated, this study demonstrated an increase in fertility rates in susceptible mares receiving PRP, without a detected change in intrauterine

fluid. Furthermore, time of treatment (24 hours preinsemination or 4 hours postinsemination) had similar effects. Although PRP did not increase antiinflammatory cytokine expression, it decreased mRNA expression of proinflammatory cytokines.[46] Clinically, intrauterine PRP treatment at insemination decreased the amount of intrauterine fluid and improved pregnancy rates, indicating that time of treatment may cause variable outcomes.[47]

Intrauterine infusion of lactoferrin, expanded our understanding of its immunomodulation. Lactoferrin, present in high concentrations in both milk and seminal plasma, has been described as both antiinflammatory and antimicrobial in a variety of species.[48-52] However, its bactericidal properties have not been elucidated in the horse. In a periovulatory susceptible mare, 150 µg/ml human recombinant lactoferrin (Re-LF) treatment, suppressed endometrial expression of pleiotropic TNF.[52] Lactoferrin treatment along with artificial insemination in healthy resistant mares also substantially decreased expression of various proinflammatory cytokines (including IL-1β and IL-8).[53] In susceptible mares, Re-LF treatment, 6 hours after insemination, decreased endometrial expression of proinflammatory IFNγ and simultaneously increased expression of antiinflammatory IL-1RN.[51] Additionally, Re-LF substantially decreased the number of neutrophils in the uterine lumen. Further work is necessary to assess bactericidal properties of Re-LF and its efficacy in treatment of infectious endometritis.

Mesenchymal stem cells (MSCs) have also been investigated for their antiinflammatory and regenerative effects.[54] For the past 2 decades, MSCs have been utilized in a variety of diseases/disorders in humans; however, research in their capabilities for horses is more limited. Intrauterine infusion of bone marrow-derived MSCs at 24 hours pre-insemination in normal mares decreased the number of neutrophils in the uterine lumen in addition to increasing the expression of antiinflammatory IL-1RN.[55] Antiinflammatory effects of MSCs in the treatment of endometritis were compared between adipose tissue and endometrium.[56] Both adipose tissue and endometrial-derived MSCs decreased concentrations of pleiotropic IL-6 and TNF in the luminal fluid. Within the endometrium, adipose-derived MSCs significantly decreased expression of proinflammatory IL-1a and IL-8 in addition to IL-6 and TNF. In contrast, endometrial-derived MSCs significantly decreased proinflammatory IL-8 and simultaneously increased endometrial expression of antiinflammatory IL-10. No cellular infiltration within the endometrium was noted 24 hours following infusion of MSCs had no cellular infiltration, although there was a substantial decrease in luminal neutrophilia.[57] This study concluded that MSCs are a safe and effective antiinflammatory option for the uterine lumen without invading or implanting within the endometrium.

Immune response to pregnancy

The immune system of pregnant females exists in a delicate balance between protection and recognition, as it must both tolerate the semi-allogeneic fetus while still defending the body from pathogens that can impede the health of both mother and fetus. It is now understood that this phenomenon is due to fluctuating adaptive immune system, specifically effector and regulatory T lymphocytes.[58] These immune cells are both crucial for pregnancy maintenance and proper embryo development, and also essential for responses to pathogen detection.[59-61] However, many of the signals necessary for pathogen recognition and elimination are similar to the proinflammatory mechanisms preceding parturition, leading to an increased risk of preterm labor or abortion after detection of microbes in the reproductive tract during pregnancy.

Involvement of regulatory T cell (Treg) in pregnancy maintenance is a topic of interest in the development of therapeutic options within this field. In human medicine, several immunomodulators that heighten or mimic Treg function are currently investigated for their ability to support pregnancy in addition to preventing placental infection and/or preterm labor. Tregs are both antiinflammatory and antiapoptotic, and therefore critical in the prevention of effector-type immune responses to paternal antigens present in feto-placental tissues. Limited research has been done in this field in equine reproduction, but it is a topic of interest for future research. We review the therapeutic options currently available for equine practitioners and identify potential future targets.

Immunomodulators for pregnancy maintenance

Immunomodulation for improvement of pregnancy maintenance has been attempted through a variety of avenues, including transplanting lymphocytes from donor stallions, in addition to stimulation of naïve lymphocytes towards Treg development through various cytokines and biologics treatment.[62] Many of these immunomodulators (e.g. recombinant granulocyte-macrophage colony-stimulating factor [Filgrastim]) or intravenous immunoglobulin (plasma) improved success of pregnancy in the human.[62-67]However, the authors are not aware of similar studies in the horse.

In contrast, some effort has been made to determine the use of lymphocyte donation to improve pregnancy maintenance in women and mares. Women experiencing repeated spontaneous abortion during early pregnancy had improved pregnancy success after receiving lymphocyte donation from either the sperm donor or a third-party donor.[68] In 1 meta-analysis, women receiving lymphocytes from the sperm donor had improved pregnancy success by 68%, whereas women that received lymphocytes from a third-party donor improved pregnancy success by 36%. Lymphocyte donation has been attempted in mares.[69] Autologous lymphocyte treatment improved pregnancy rates in Thoroughbred mares that had previously experienced early embryonic loss.[69] In a second study, lymphocytes had no effect on pregnancy outcome. However, neither study had a control group, so the results should be interpreted carefully. Additionally, it is unknown what proportion of this lymphocyte population

were effector or regulatory lymphocytes, and the responses noted could be mitigated or enhanced if these populations were altered. Lower percentage of Tregs during estrus strongly correlated with an increased risk of early embryonic loss,[70] further indicating the crucial role of these cells on pregnancy maintenance.

Immune response to pregnancy-related complications

In women, the balance between recognition of self and detection of pathogen becomes skewed preceding numerous pregnancy-related complications. This alteration in the adaptive immune response is most noted as an increase in activation/recruitment of the proinflammatory Th17 cells alongside a diminished Treg response, leading to rejection of the semi-allogeneic tissue and eventual abortion.[59,71-74]

In mares, the leading cause of infectious abortion is placental infection, and this contributes to 19% of all abortions in North America.[75,76] We recently reported[77] a comparable adaptive immune response to the experimental induction of ascending placentitis to what has been reported in women, with activation of effector functions of the adaptive immune system and suppression of the regulatory arm (Figure 2). This was observed both locally to the feto-placental unit with an increase in Th17-related transcripts, alongside dysregulation of Treg-related transcripts. Additionally, induction of ascending placentitis led to immune alterations in circulation, including an increase in various proinflammatory (IL-2, IFNγ), antiinflammatory (IL-5, IL-10) and pleiotropic cytokines (IL-6, TNF), with IL-6 having the highest fold change of among the cytokines investigated.[78] Further research conducted in our laboratory suggested that the fetus also had an active immune response to placentitis and it was primarily characterized by expression, production, and secretion of both antiinflammatory (IL-10) and pleiotropic (IL-6) cytokines.[79] Pleiotropic IL-6 was recently observed to function through the classical IL-6R signaling pathway during the pathophysiology of ascending placentitis, leading to various downstream antiinflammatory effects, including inhibition of apoptosis and proliferation of epithelial cells.[80] Further work in our laboratory had similar cytokines increase during both ascending and focal mucoid placentitis (the etiology included nocardioform placentitis.[81]) Since all mares investigated within this study produced a viable neonate, additional research is necessary to determine the profile of these cytokines preceding abortion.

Immunomodulation of pregnancy-related complications

A few experimental models exist for induction of pregnancy-related complications, forcing inferences made from the experimental induction of ascending placentitis towards various etiologies in equine reproduction. For ascending placentitis, treatment commonly includes an antimicrobial, progestin (altrenogest; Regu-Mate®), and antiinflammatory (pentoxifylline and/or NSAID) agents.[82] Pentoxifylline is considered both antiinflammatory and anticytokine, due to its dual ability to inhibit the NFkβ pathway while simultaneously acting as both antiTNF and antiIL-6, both of which have been found to elevate during the pathophysiology of ascending placentitis.[83] However, no studies have investigated the role of pentoxifylline on the immune response of the horse. Additionally, although the oral dosage utilized in the present study was 8.8 mg/kg,[84,85] no study has assessed the appropriate dose to use in the horse, with

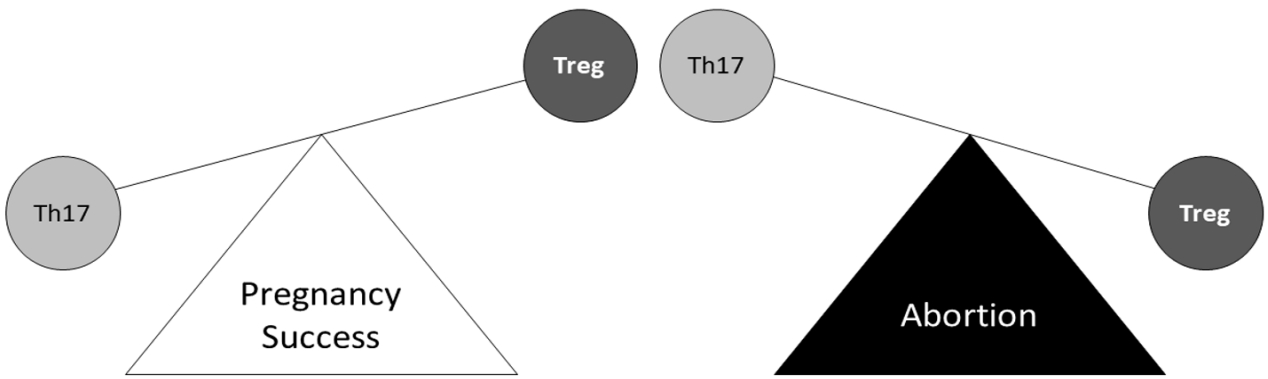

Figure 2. Overview of dysregulation Th17/Treg response after equine placental infection. Equine ascending placentitis begins by detection of extracellular *S. zooepidemicus* that is recognized by an antigen presenting cell. This combination stimulates naïve T cells to mature into Th17 cells through activation of various cytokines (IL-6 and IL-23), cell surface markers (IL-1R1, IL-6R, and IL-21R) and transcriptions factors (STAT3 and IRF4). Additionally, cytokines (TGF-B), cell surface markers (LRRC32/GARP), and transcription factors (FOXp3) that assist in the maturation of Tregs decreased after detection of pathogen, potentially dysregulating Treg production. This imbalance of Th17/Treg maturation precedes abortion in a variety of species and may activate preterm parturition or abortion in the horse after ascending placental infection.

extrapolations being made from other species with differing placentation.

Although other NSAIDs have been evaluated for their efficacy in treatment of placentitis, none has been assessed as a sole modality; therefore, results should be interpreted critically. Clinically, twice daily intravenous flunixin (1 mg/kg) and oral phenylbutazone (2.2 - 4.4 mg/kg), and once daily oral firocoxib (57 mg) are routinely used, albeit with only limited data to support their treatment.[84-88] Flunixin meglumine has been investigated in conjunction with TMS, altrenogest, and estradiol cypionate.[88] While the addition of estradiol cypionate improved pregnancy outcomes, flunixin meglumine as a sole modality had limited positive effects. Firocoxib has also been used along with altrenogest and TMS.[87] Following treatment with firocoxib, a substantial decrease in both IL-1β and IL-6 secretion was observed. As IL-6 has a 4-fold increase following the induction of ascending placentitis, this suppression is intriguing and deserves further work.

Immune response to postpartum involution

Equine pregnancy lasts 340 ± 20 days;[89] therefore, subsequent pregnancy must be established within the first month postpartum to achieve continued commercial success. In the mare, first postpartum estrus occurs from 7 - 16 days after parturition and is referred to as 'foal heat'. This coincides with the process of uterine repair needed after parturition. Uterine involution includes reduction of uterine size, removal of residual debris, contraction of endometrial glands, and elimination of lingering immune cell types. It takes approximately 13 - 15 days and is necessary to ensure a uterine environment that is not hostile to the impending embryo arrival.

Recent research from our laboratory described the role of the immune system in postpartum involution. A significant relationship was noted between the day postpartum and endometrial expression of pleiotropic (IL-6 and GM-CSF) and antiinflammatory cytokines (IL-10).[90] Expression was highest immediately postpartum and then declined as involution progressed. This coincided with a decline in both bacterial growth and neutrophilia, indicating signaling mechanisms required to disallow persistent inflammation from occurring during this physiologic event. Several therapeutics have been attempted to hasten this physiological process to ensure pregnancy success, but with minimal success. We review the use of immunomodulation to alter involution and improve 'foal heat' breeding success.

Immunomodulation of postpartum involution

Parturition enables immense bacterial invasion into the vaginal vault, through the relaxed cervical canal and eventual localization in the uterine lumen and endometrium. This leads to increased neutrophilia and the potential for persistent inflammation/infection that may hinder future fertility postpartum. Intravenous MCWF (1.5 mg ; Settle®) treatment administered 24 hours after parturition improved 'foal heat' breeding success and substantially decreased both bacterial presence and magnitude of neutrophilia.[91] Additional studies (1.5 mg IV MCWF treatment at 24 hours and at 7 days postpartum) increased endometrial expression of proinflammatory cytokines (IL-1β and IFNγ).[90] As these cytokines are essential for the recruitment and chemotaxis of necessary immune cells for bacterial degradation and clearance, it was not surprising that this increase coincided with a reduction in the severity of bacterial growth, in addition to decreasing days required for a clean culture.

Conclusion

Reproductive immunology is constantly advancing our understanding of mare physiology. Continued work is necessary to further elucidate the pathophysiology of several diseases and disorders associated with equine reproduction, including breeding-induced inflammation, immune tolerance of the semi-allogeneic fetus, immune responses to pregnancy-related complications, and immune-mediated response to parturition. As our understanding of this physiology broadens, inferences can be made towards several immunomodulators that may improve these processes, thereby advancing mare reproductive performance.

Conflict of interest

Authors have no affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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