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The phagocytic function of blood-derived polymorphonuclear neutrophils after administration of dexamethasone for the modulation of post-breeding endometritis in the mare

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Summary

Persistent breeding-induced endometritis is a major cause of subfertility in the mare. Most widely applied treatment strategies involve mechanical removal of accumulated uterine fluid via lavage and administration of ecbolic agents such as oxytocin. Recently, corticosteroids administered at the time of breeding have been reported to increase fertility rates in mares susceptible to persistent breeding-induced endometritis by reducing the degree of uterine edema and intraluminal fluid accumulation. Few studies, however, have examined the potential negative effects of corticosteroids in this context, particularly those associated with impaired immunity by altering the function of polymorphonuclear neutrophils (PMNs). The objective of this study was to examine the effect of corticosteroids on the phagocytic ability of PMNs. In this study, six mares, each serving as their own control, were administered 50 mg dexamethasone IV when their ovary contained a 30-35 mm follicle in the presence of uterine edema. A blood sample was obtained before and 24 hours after treatment. The function of PMNs was examined by flow cytometry, measuring the ability of PMNs to bind and phagocytose bacteria in vitro. Results showed no significant difference ($P > 0.05$) in the ability of PMNs to phagocytose bacteria following treatment with dexamethasone versus the control samples. It was concluded that corticosteroids can be administered as adjunctive treatment for the modulation of breeding-induced endometritis, with no significant alteration of PMN function that could predispose the mare to secondary infections. The efficacy of dexamethasone treatment to modulate breeding-induced endometritis was not investigated.

Keywords: corticosteroid, endometritis, phagocytosis, polymorphkernige Neurtophile, equine, reproduction

Die phagozytische Funktion polymorphkerniger Neutrophiler nach Verabreichung von Dexamethason zur Behandlung der Post breeding-Endometritis bei Stuten

Persistierende besamungsinduzierte Endometritis ist eine der wichtigsten Ursachen für Subfertilität bei der Stute. Zu den häufigsten Behandlungsmethoden gehören die Entfernung akkumulierter Flüssigkeit aus dem Uterus durch Uteruslavage sowie die Anwendung von wehenfördernden Wirkstoffen wie Oxytozin. Kürzlich wurde Kortikosteroiden, die zum Zeitpunkt der Besamung appliziert wurden, eine fertilitätsfördernde Wirkung durch Verminderung des Uterusödems und intraluminaler Flüssigkeitsansammlung bei Stuten, die zu persistierender besamungsinduzierter Endometritis neigen, zugesprochen. Dennoch haben nur wenige Studien bisher die potentiell negativen Effekte von Kortikosteroiden in diesem Kontext untersucht, insbesondere die Wirkung bezüglich gestörter Immunität durch Veränderung der Funktion der polymorphkernige Neutrophilen (PMNs). Das Ziel dieser Studie war es, den Effekt von Kortikosteroiden auf die phagozytotische Funktion der PMNs zu untersuchen. Im Versuch wurde sechs Stuten, wobei jede gleichzeitig als eigene Kontrolle diente, 50 mg Dexamethason intravernös appliziert, sobald ein Ovar einen 30-35 mm Follikel besaß und ein Uterusödem vorlag. Vor und 24 Stunden nach der Behandlung wurde je eine Blutprobe genommen. Die Funktion der PMNs wurde per Flowzytometrie bestimmt, wobei die Fähigkeit der PMNs, Bakterien zu binden und zu phagozytieren, in vitro gemessen wurde. Es wurde kein signifikanter Unterschied ($P > 0.05$) bezüglich der Fähigkeit der PMNs, Bakterien zu phagozytieren, zwischen den Proben nach Behandlung mit Dexamethason und den Kontrollen, festgestellt. Aus den Versuchen wurde geschlossen, dass Kortikosteroide als zusätzliche Therapie zur Modifizierung persistierender besamungsinduzierter Endometritis ohne signifikante Veränderung der PMN-Funktion, die zu Sekundärinfektionen prädisponieren könnte, eingesetzt werden können. Die Wirksamkeit der Dexamethasonbehandlung zur Modifizierung besamungsinduzierter Endometritis wurde nicht untersucht.

Schlüsselwörter: Kortikosteroide, Endometritis, Phagozytose, polymorphkernige Neutrophile, Pferd, Reproduktion

Introduction

Endometritis is the third most commonly reported medical problem of adult horses, and the problem continues to be one of the most economically important problems in equine reproductive management as it is a major cause of subfertility in the mare (*Traub-Dargatz* et al. 1991, *Watson* 2000). Currently recognized as a multifactorial disease, breeding-induced endometritis has been examined from a variety of perspectives including: classification (acute, chronic, active, subclinical, post-partum); categorization (sexually transmitted diseases, chronic infectious endometritis, breeding-induced endometritis); predisposing factors (mare's age, parity, reproductive conformation, impaired innate uterine defense mechanisms); and etiology (bacterial, fungal, semen) (*Troedsson* 1999, *Hurtgen* 2006, *Stout* 2008). During the mating process a variety of antigenic agents are introduced into the uterus. Natural service results most notably in the introduction of sperm, seminal plasma, and bacteria, while artificial insemination may intro-

duce semen extenders as well. The introduction of these antigenic agents results in the activation of the complement cascade characterized by an intraluminal influx of fluid containing predominantly polymorphonuclear neutrophils (PMNs). The inflammation, along with coordinated contractions of the myometrium, is the mechanism by which the uterus attempts to rid itself of non-viable spermatozoa and other contaminants introduced during the mating process (*Asbury* et al. 1982, *Troedsson* et al. 1998, *Troedsson* 1999, *Watson* 2000, *Portus* et al. 2005, *Troedsson* 2006). This process begins within 30 min after breeding and resolves within 24-36 hours. The inflammation is considered a pathological process if it is not resolved within 36-48 hours (*Troedsson* 1999).

Diagnosis of persistent endometritis in the mare has primarily relied upon the ultrasonographic detection (volume and characterization) of intra-uterine fluid (*Liu* et al. 2008). Culture, cytology, and histopathology of uterine specimens have also been utilized to diagnose or predict persistent breeding-induced endometritis (*Card* 2005, *Nielsen* 2005, *Liu* et al. 2008). Studies have shown that intraluminal fluid accumulation greater than 2 cm during estrus is a strong indicator that the mare is susceptible to persistent breeding-induced endometritis (*Brinsko* et al. 2003). Furthermore, if the inflammation does not clear by 5 days after breeding/ovulation, the effects on the uterine environment are not compatible with embryonic survival (*Watson* 2000, *Troedsson* 2006, *Liu* et al. 2008).

A multitude of strategies have been suggested for the treatment of persistent endometritis. Treatment strategies have ranged from post-breeding infusion of antibiotics, ecbolics, uterine lavage, electro-acupuncture, and immunostimulants like Proprionibacterium acnes (*Rohrbach* et al. 2007, *Liu* et al. 2008). While there is currently no "gold standard" for the treatment of persistent breeding-induced endometritis in the mare, mechanical removal of intraluminal uterine fluid via uterine lavage and administration of ecbolic agents continue to be the treatments of choice due to the proven efficacy and safety of these methods (*Liu* et al. 2008). These strategies address the impaired myometrial function, which has been documented as the major predisposing factor to the accumulation of intrauterine fluid and the development of persistent endometritis in the mare (*Troedsson* et al. 1991, *Troedsson* et al. 1993, *LeBlanc* et al. 1994).

Recent research has investigated the efficacy of corticosteroids for the treatment of mares susceptible to breeding-induced endometritis. Administration of corticosteroids around the time of ovulation/breeding improved pregnancy rates (frozen and fresh semen) and uterine lavage fluid character on susceptible mares, as measured by a decrease in uterine edema/fluid accumulation and improved uterine fluid turbidity (*Bucca* et al. 2008, *Papa* et al. 2008). Additionally, no deleterious effects were noted on embryological survival and development (*Bucca* et al. 2008). However, the mechanism by which corticosteroids alter the development of breeding-induced endometritis is not fully understood. Potential side effects may result from an immunosuppressive effect of corticosteroids on polymorphonuclear neutrophil (PMN) function. Impaired PMN-function has been implemented in the pathophysiology of persistent endometritis. PMN chemotaxis and phagocytosis were both impaired in mares susceptible to endometritis due to an altered uterine environment (*Troedsson* et al. 1993).

The aim of this study was to investigate if a commonly used corticosteroid (dexamethasone) altered the ability of PMNs to phagocytose bacteria in vitro.

Methods and Materials

The test group consisted of seven mares, with no history of chronic uterine infections, each of whom served as their own control. Each mare received serial transrectal ovarian and uterine ultrasound examinations to monitor follicular development and uterine edema. Due to a history of laminitis during the previous year, one of the mares was removed from the study prior to sample collection. When a mare's ovary contained a pre-ovulatory follicle measuring between 30 and 35 mm in diameter with the presence of grade 2 uterine edema (on a scale of 1 to 3), a 30 mL jugular blood sample was collected into vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing sodium heparin as anticoagulant. Immediately following collection of this sample, a 50 mg dose of dexamethasone (2mg/ml, AgriLabs, St. Josephs, MO, USA) was administered intravenously in the jugular vein. A second sample of blood was collected from the jugular vein 24 hours later using the same technique.

Blood samples were centrifuged at 1000 x g for 10 minutes and the plasma removed. The buffy coat was mixed with an isotonic saline solution (0.9 g/mL), layered on lymphocyte separation medium (Organo Teknika, Durham, NC), and centrifuged at 1000 x g for 20 minutes at 25°C. The PMN-rich buffy coat was collected from the pellet underneath the lymphocyte preparation medium. Distilled water (6 mL) was added to the suspension in order to lyse the red blood cells, and 3 mL of a 2.7% v/v NaCl solution was added after 45 seconds to the sample before centrifugation at 1000 x g for 5 minutes at 25°C. The PMN-pellet was finally resuspended in HBSS (Hanks Buffered Salt Solution) to a final concentration of 1 x 10^6 in 0.8 mL in 1 x PBS 2ith 10% FCS (pH 7.0) as previously described by *Dahms* and *Troedsson* (*Dahms* et al. 2002).

All samples were analyzed at the University of Florida ICBR Flow Cytometry Core Laboratory (Gainesville, FI, USA). Predexamethasone and post-dexamethasone samples were prepared in triplicates using the pHrodo™ E. coli BioParticles® Kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The pHrodo™ E. coli BioParticles® conjugates provided by this kit are inactivated Escherichia coli which are fluorogenic particles for the detection of phagocytic ingestion. Concentrated white blood cells without bioparticles served as negative controls. Cells were analyzed on an LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and a gate was selected for polymorphonuclear neutrophils based on cell size (FSC-A) and density of granularity (SSC-A). Nucleated phagocytes were distinguished from debris by gating on the granulocyte population using forward and side scatter properties. The pHrodo™ fluorescent signal was detected in the channel normally used for PE (Blue D: 575/26 BP filter) with 488nm laser excitation. The percentage of positive cells represented those that had phagocytosed the pHrodo™ E. coli BioParticles® and were measured against negative controls. Data were analyzed using a paired t-test (Statistix 9, Tallahassee, FL, USA). Data is expressed as mean + SD. A p-value equal to or less than 0.05 was considered statistically significant

Results

None of the mares developed adverse reactions to the treatment and all mares ovulated during the estrous period studied. A gate (P1) was selected on the FSC-SSC histogram to include equine polymorphonuclear neutrophils (figure 1). This region was used for gating to measure phagocytosis. There was no difference in the percentage of peripheral-blood derived PMNs

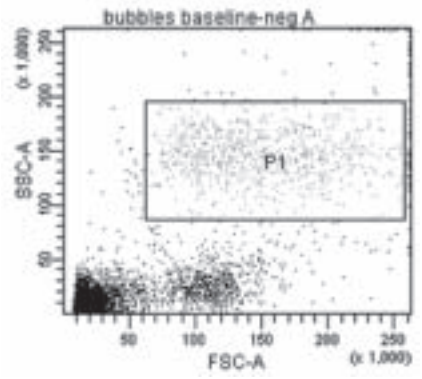

Fig. 1 Scatterplot showing the gate (P1) selected for polymorphonuclear neutrophils based on cell size (FSC-A) and density/granularity (SSC-A) using the LSR II flowcytometer (BD Biosciences) on a whole blood sample.

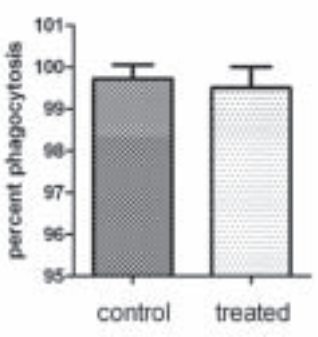

Fig. 2a Phagocytosis of blood derived PMNs before and after treatment with 50 mg dexamethasone. Treatment did not affect the phagocytosing ability of PMNs (99.7+0.34 vs. 99.5+0.51).

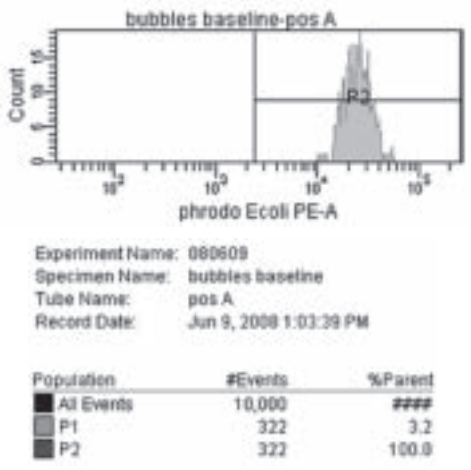

Fig. 2b Representative data collected from a sample obtained pre-dexamethasone administration. The histogram shows the fluorescence of neutrophils after phagocytosis of E.coli conjugated to pHrodo™ dye. Neutrophils isolated from that particular mare displayed 100% phagocytosis as can be seen in the result table.

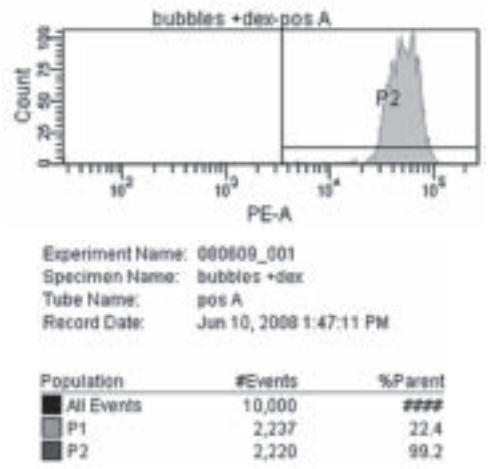

Fig. 2c Representative data collected from a sample obtained post-dexamethasone administration. The histogram shows the fluorescence of neutrophils after phagocytosis of E.coli conjugated to pHrodo™ dye. Neutrophils isolated from that particular mare displayed 99.2% phagocytosis as can be seen in the result table.

that phagocytosed bacteria in vitro between samples obtained pre- and post-dexamethasone treatment (99.7+0.34 vs. 99.5+0.51, respectively; P>0.10) (Figure 2a-c).

Discussion

The use of corticosteroids on the modulation of persistent post-breeding endometritis has been suggested to be a safe and effective treatment strategy for increasing fertility rates in susceptible mares (*Dell'Aqua* et al. 2006, *Bucca* et al. 2008, *Papa* et al. 2008). As endometritis is a major economic problem to the equine industry, with one study finding that approximately 15% of Thoroughbred broodmares being susceptible to the development of persistent breeding-induced endometritis, there is an incentive to investigate more efficacious treatment strategies (*Zent* et al. 1998).

Dexamethasone administered at 50 mg IV (an anti-inflammatory dose at approximately 0.1 mg/kg for a 500 kg mare) has the simplicity of a single IV injection that can be administered in field conditions in the peri-ovulatory period (within 24 hours prior to breeding), with no reported deleterious impact on ovulation (*Bucca* et al. 2008). However, it is important to recognize that dexamethasone is a potent glucocorticoid and consideration should be made of the potential side effects of this drug when administered systemically at such a high dose. Corticosteroids are immunosuppressive in that they alter leukocyte function, are cidal to lymphocytes, and are suppressive to a number of inflammatory mediators (*Troedsson* et al. 1998). PMNs are an essential part of the normal physiological response of the uterus post-breeding to rid the lumen of non-viable spermatozoa and bacteria. In order to accomplish this, neutrophils must be capable of normal chemotaxis and phagocytosis. The PMNs examined in this study were blood-derived. It has been documented that there is no significant difference in the phagocytotic function of PMNs derived from peripheral blood versus uterine-derived PMNs (*Asbury* et al. 1982, *Troedsson* et al. 1993). The results of this study demonstrate that even at a high dose of

dexamethasone, PMNs are not affected in their ability to phagocytose bacteria. *Dell'Aqua* et al. (2006) described the adverse effect of corticosteroid treatment on PMN function by demonstrating a significantly decreased ability of PMNs to reduce nitroblue tetrazoleum (NBT), a test that measures the respiratory oxidative burst, which is an important aspect of the innate immunity (killing capacity) of PMNs (*Dell'Aqua* et al. 2006). We did not evaluate the killing capacity of PMNs, but rather the ability of PMNs to phagocytose bacteria. Since the proposed mechanism of uterine clearance after breeding includes a fine tuned interaction between PMN-phagocytosis and uterine contractility, activation of PMNs and phagocytosis may be more important than the killing of antigens in this particular case (*Troedsson* 2006).

The risk of immunosuppression is not the only potential negative effect associated with the use of corticosteroids to treat post-breeding endometritis. High systemic doses of dexamethasone should be used with extreme caution in mares with a history of laminitis. One mare was removed from the treatment group due to a history of laminitis and concerns that she was again showing mild clinical signs. These mares are at an increased risk for developing glucocorticoid-associated laminitis, the pathogenesis of which still remains controversial. The risk of developing de novo laminitis due to systemic administration of glucocorticoids is debatable, but the gross morphological appearance of a glucocorticoids-affected hoof closely resembles that of a chronically foundered hoof (*Johnson* et al. 2002).

While most strategies for the treatment of persistent breeding-induced endometritis continues to focus on mechanical clearance of accumulated fluid via multiple uterine lavages and administration of oxytocin, corticosteroids are increasingly used as an adjunctive therapy. Clinically, corticosteroids have been suggested to reduce uterine edema, intraluminal fluid accumulation, and have also been reported to improve fertility in selected mares with persistent breeding-induced endometritis (*Dell'Aqua* et al. 2006, *Bucca* et al. 2008, *Papa* et al. 2008). We did not investigate the efficacy of treatment with dexamethasone on any of these parameters. However, results from this study suggest that a one-time treatment with 50 mg dexamethasone prior to ovulation has no effect on the function of PMNs to allow phagocytosis of sperm and bacteria following breeding.

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