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Persistent post-breeding endometritis: effect of corticosteroid treatment on the number of protein bands from uterine endometrial fluid of susceptible mares

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Summary

The aim of this study is to compare the number of endometrial fluid protein bands (protein profile) of mares susceptible to persistent post-breeding endometritis (PPBE), infected or non-infected with bacteria Streptococcos zooepidemicus, treated with an steroidal anti-inflammatory drug with the ones from non-treated mares. This study was conducted from January 2008 to March of 2009. During estrus, pure endometrial fluid of 16 cyclic Warmblood mares was recovered by the use of vaginal tampons for specimen collection. Four groups were constituted: G1 - Control, G2 - treated with 20mg isoflupredone acetate, every 12h, for 3 days, G3 - experimental infection with Streptococcus zooepidemicus and G4 - experimental infection with Streptococcus zooepidemicus and treated with corticosteroid treatment (same as in G2). Samples were processed and submitted to two-dimensional electrophoresis technique according to *O'Farrel* (1977), modified by *Rodnight* et al. (1988). Electrophoresis gels were scanned and analyzed to determine the number of endometrial protein bands. Endometrial electrophoresis gels showed 33 protein bands in G1, 54 in G2, 51 in G3 and 72 in G4. Protein spots ranged from 15 to 105 kDa molecular weights and pH 4.3 to 10.0 isolelectric points. An increase in the number of protein bands was observed in the two corticosteroid treated groups (G2 and G4). Treatment with steroidal anti-inflammatory drug isoflupredone can alter the number of endometrial fluid protein profile of estrus mares susceptible to PPBE, by an increase in the number of protein bands.

Keywords: Reproduction, endometritis; corticosteroid; equine; endometrial fluid; proteins; electrophoresis

Die nach der Belegung persistierende Endometritis: Zum Effekt einer Kortikosteroidbehandlung auf die Anzahl der Proteinbanden in der endometrialen Flüssigkeit bei empfänglichen Stuten

Ziel der Studie ist es, die Anzahl der Proteinbanden (Proteinprofil) im Uterussekret von Stuten vergleichen, die empfänglich sind für eine nach der Belegung persistierende Endometritis, infiziert bzw. nicht infiziert wurden mit Streptococcus zooepidemicus, und mit einem steroidalen Antiphlogistikum behandelt wurden oder unbehandelt blieben. Die Studie wurde von Januar 2008 bis März 2009 durchgeführt. Während der Rosse erfolgte die Gewinnung reinen Uterussekrets von 16 zyklischen Stuten mithilfe eines Vaginaltampons. Vier Gruppen wurden gebildet: G1: Kontrolle; G2: Behandlung mit 20mg Isoflupredonazetat, täglich über 3 Tage; G3: experimentelle Infektion mit Streptococcus zooepidemicus; G4: experimentelle Infektion mit Streptococcus zooepidemicus und Kortikosteroidbehandlung (wie G2). Die Proben wurden aufgearbeitet und eine zweidimensionale Elektrophorese nach *O Farrel* (1977), modifiziert nach *Rodnight* et al. (1988) durchgeführt. Die Elektrophoresegele wurden gescannt und analysiert, um die Anzahl der Proteinbanden zu bestimmen. Die endometrialen Elektrophoresegele zeigten in G1 33, in G2 54, in G3 51 und G4 72 Proteinbanden. Die Proteinspots lagen zwischen 15 und 105 kDa Molekulargewicht und bei einem isoelektrischen Punkt zwischen pH 4,3 und 10,0. Ein Anstieg der Proteinbandenanzahl wurde in den beiden Gruppen mit Kortikosteroidbehandlung (G2 und G4) beobachtet. Die Behandlung mit dem steroidalen Antiphlogistikum Isoflupredon führt zu einer Veränderung des Proteinprofils im Uterussekret bei Stuten, die empfänglich sind für eine nach der Belegung persistierende Endometritis durch einen Anstieg der Anzahl der Proteinbanden.

Schlüsselwörter: Reproduktion, Endometritis, Kortikosteroid, Pferd, Uterussekret, Proteine, Elektrophorese

Introduction

Endometritis is a normal physiological event that occurs after mating (*Watson* 2000) due to a response induced by semen deposited into the mare uterine lumen (*Kotilainen* et al. 1994, *Fiala* et al. 2007). This inflammatory reaction removes excessive semen and bacterial contamination inoculated at the time of breeding (*Kotilainen* et al. 1994, *Troedsson* et al. 1995, *Fiala* et al. 2007).

However, if inflammation persists, the resulting environment is not compatible with the establishment of pregnancy (*Watson* 2000). Mares that fail to clear the inflammation within the first 36 hours after mating and accumulate fluid in the uterine lumen (*LeBlanc* 2003) are classified as susceptible and are believed to have an impaired physical clearance of the uterus. Accumulation of uterine fluid during estrus was associated with compositional changes in the uterine secretions. The mean total protein concentration in the uterine secretion of mares with IUF was one-third of that found in mares without this condition. This presumably indicates that uterine fluid is composed of both glandular secretions and of a transudate (*Reilas* 2001). It has been described that corticosteroid treatment, before breeding, increases the pregnancy rates of susceptible mares (*Dell'Aqua Jr.* 2006). The aim of this study is to compare the number of endometrial fluid protein bands (protein profile) of mares susceptible to PPBE, infected or non-infected with bacte-

ria Streptococcos zooepidemicus, treated with a steroidal anti-inflammatory drug, with the ones from non-treated mares.

Materials and Methods

The study was conducted from January to March of 2008/2009. A total of 16 cyclic susceptible Warmblood mares, aged between 4 and 30 years, from an experimental herd entered the study. Animals were kept in fields, pastured and supplemented with oats and hay.

Mare's selection

Mares were examined for reproductive soundness, including evaluation of perineal conformation, transrectal palpation and ultrasonographic examinations of the reproductive tract. Only clinically normal mares were used. Mares were examined by means of palpation and ultrasonography per rectum, daily, in order to evaluate follicular growth, grade of uterine edema and presence of intrauterine fluid accumulation (IUF). Mares were classified as susceptible to PPBE according to historical data of previous studies. Mares were considered susceptible when IUF was detected by ultrasonographic examination of the uterus 48 hours after an induced uterine inflammation caused by semen deposition after insemination.

Treatment and experimental infection

After animal selection, the experiment began when a pre-ovulatory follicle (> 35 mm) and a characteristic ultrasonographic image of estrus uterus were observed. All mares underwent the four groups that were constituted and had an interval of one estrus between treatments. Groups were: G1 (n=8) Control: first estrus of the experiment, mares did not receive any treatment. G2 (n=8) Treated: third estrus, mares were administered with 20 mg isoflupredone acetate, every 12h, for 3 consecutive days. G3 (n=8) Infected: fifth estrus, mares were inoculated with 1 x 10^9 Streptococcus zooepidemicus and G4 (n=4) Infected + Treated: seventh estrus, mares were inoculated with 1 x 10^9 Streptococcus zooepidemicus and received corticosteroid treatment, same as in G2. Bacteria were inoculated at the second day of treatment. Experimental infection was performed by the infusion of a bacterial culture suspended in 20 ml saline solution from an insemination pipette into the uterus. All mares were treated with uterine lavage and local infusion of antibiotics until confirmed free of uterine inflammation. Additionally, it is relevant to report that in some occasions, at sample collection, insufficient volume was recovered because some mares did not produce enough endometrial fluid. These samples were not considered in the study. Also, one mare developed permanent endometritis after infection. The endometritis did not respond to medical treatment until the mare entered seasonal anestrus, therefore this mare was carried out from the experiment.

Sample collection and processing

Samples were collected 12h latter by the use of a cotton tampon (mini OBâ – Johnson & Johnson) aseptically inserted into the uterus. The technique used for sample collection was a modification of the procedure described by *Reilas* (2001). A 50 cm umbilical tape was attached to the tampon, which was then passed through the cervix into the uterus using a modified doubleglove technique. The distal part of a rectal glove was cut off from the wrist to make a plastic tube. The gloved hand with the tampon was placed inside the tube, and then closed by gathering its end with one's fingers. The gloved hand was set free from the plastic tube before reaching the cervix. The tampon was kept in the uterus for 30 min and withdrawn from the uterine lumen protected by a gloved hand. After tampon removal, each tampon was placed inside a 20 ml syringe, and the absorbed fluid was squeezed out into a sterile plastic tube. An aliquot of 2.0 ml of endometrial secretion was centrifuged at 1500 x g for 15-20 minutes. The supernatant was transferred into cryovials and stored in liquid nitrogen until assay.

Electrophoresis

Frozen samples were thawed, recentrifuged at 10.000 × g for 60 min at 4°C and a 50 μL aliquot of the supernatant was taken and transferred into cryovials for storage at -80°C. Protein concentration was assessed according to *Lowry* et al. (1951) using bovine serum albumin (1 mg/ml) as a standard. Endometrial samples from two mares had insufficient protein content and were not considered suitable to perform electrophoresis. Endometrial secretion samples were subjected (in duplicate) to the two-dimensional gel electrophoresis technique described by *O'Farrel* (1977) and modified by *Rodnight* (1988). Gels were immersed in a solution of 0.15% Comassie brilliant blue R-250 (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 53% methanol, 7% acetic acid and water, and were stained overnight. The gels were destained in a mixture of 50% methanol, 7% acetic acid and water, with a minimum of five solution changes per gel. Destained gels were equilibrated in a mixture of 50% methanol, 1% glycerol and water, for 2 h. The gels were then placed between two cellophane sheets until dry. After drying, gels were scanned (Hewlet-Packard 6100C, Palo Alto, CA, USA) and analyzed using a software (Optiquant Acquisition & Analysis, version 02.00, Canberra, Australia) to determine the number of endometrial protein bands.

Results

Endometrial samples from 16 mares were collected and a total of 40 gels from susceptible mares were used. Protein spots ranged from 15 to 105 kDa molecular weight and isolelectric point (Ip) from 4.3 to 10.0 (Figure 1). Endometrial electrophoresis gels showed 33 protein bands in G1, 54 in G2, 51 in G3 and 72 in G4.

Discussion

An increase of protein bands was observed in this study, in the two corticosteroid treated groups (G2 and G4). This increase may be explained by the presence of anti-inflammatory proteins (such as some anti-inflammatory cytokines), caused by the well-known potent corticosteroid anti-inflammatory action. *Malschitzky* et al. (2008) observed higher optic density and frequency in 12 and 8 protein bands, respectively, in samples from susceptible mares, when compared to resistant mares. In 11 spots was possible to see a relationship with some proteins that have already been reported in horses, from which 6 may be involved in the inflammatory process. *Fumoso* et al. (2003) reported that susceptible mares in estrus presented higher levels of mRNA

expression for 4 pro-inflammatory cytokines (IL-1 , TNF- , IL-6 and IL-8) before breeding, when compared to resistant mares. The presence and concentration of nitric oxide (NO), 13 hours after artificial insemination was observed by *Alghambdi* and *Troedsson* (2002), which demonstrated that total NO was significantly higher in susceptible than resistant mares. Nitric oxide is a main mediator of smooth muscle relaxation in different organs, including the uterus. Therefore, high levels of NO in the uterine secretion of susceptible

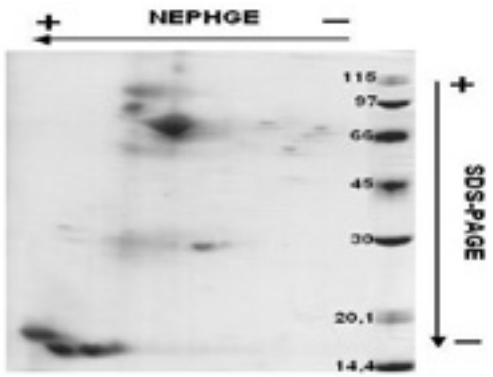

Fig. 1 Example of a two-dimensional polyacrylamide gel of endometrial secretion proteins, from Control group. Two-dimensional 12% SDS-PAGE gel stained with Comassie blue. The horizontal arrow on the top shows the direction of the non-equilibrated pH gradient (NEPHGE) from the basic end (+) to the acid end (-), in the first dimension. The molecular weight marker (ladder) is to the right.
Beispiel eines zweidimensionalen Polyacrylamidgels von endometrialen sekretorischen Proteinen aus der Kontrollgruppe. Das zweidimensionale SDS-PAGE-Gel wurde mit Comassie-Blau gefärbt. Der horizontale oben liegende Pfeil zeigt die Richtung des equilibrierten pH-Gradienten (NEPHGE) vom sauren (+) zum basischen (-) Ende in der ersten Dimension. Rechts ist aufsteigend das Molekulargewicht aufgezeigt.

mares may be a cause or an effect of their susceptibility. Resistant mares are capable to completely eliminate inflammatory products as soon as they are generated, while susceptible mares fail to spontaneously do so, because of their pendulous uteri suspended deeply in the abdominal cavity, resulting in an accumulation of NO (*Alghambdi* and *Troedsson* 2002). Consequently, the myometrium fails to contract and inflammatory products accumulate acting as a continuous stimulus to persistent endometritis. The use of prednisolone close to the time of ovulation, resulted in a higher pregnancy rate of susceptible mares compared to non-treated mares (*Dell'aqua Jr.* et al. 2006). Corticosteroids promote a reduction in the expression of several inflammatory cytokines, such as IL-1 , TNF- , IL-4, IL-3, IL-5 and IL-8, thus reducing inflammation. Also, the administration of corticosteroids reduces the levels of NO and enzymes responsible for the synthesis of prostaglandins and leukotrienes and decreases the activity of adhesion molecules, which reduces the migration of leucocytes from blood vessels (*Janeway* et al. 1999).

Another conjecture is that some of the proteins present in the endometrial fluid after corticosteroid treatment do not have specific roles during the inflammatory process, but other functions not clear to us yet. Nevertheless, anti-inflammatory cytokines were expected to arise to control pro-inflammatory cytokines expression in the uterine environment of susceptible mares, in an attempt to bring balance to their impaired immunological system.

In conclusion, treatment with steroidal anti-inflammatory drug isoflupredone can alter the number of endometrial fluid protein profile of mares susceptible to PPBE, during estrus, promoting an increase in the number of protein bands. Further studies are necessary to identify inflammatory proteins and to elucidate their role, associated, if ever, to corticosteroid treatment.

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