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Uterine nitric oxide levels and isofluopredone treatment effect in mares susceptible to persistent post-breeding endometritis

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Abstract

Transient endometritis is a normal consequence of breeding and results from uterine contamination with both semen and bacteria. The modulation of the inflammatory response with the use of isoflupredone has been proposed as efficient for the treatment of endometritis by increasing pregnancy rates. The aim of the current study was to determine the effects of isoflupredone on nitric oxide (NO) levels in uterine samples from mares susceptible to persistent postbreeding endometritis, presenting or not the infectious process. Seven consecutive estrous cycles were induced in 11 mares, being the first one used as control (no treatment). All mares were submitted to the following four treatments: treatment 1: control, treatment 2: glucocorticoid (GC) treatment (20 mg isoflupredone acetate) every 12 h, for three consecutive days, treatment 3: infected treatment (intrauterine infusion of $1x10^9$ CFU/ml *Streptococcus equi* subsp. *zooepidemicus*), treatment 4: combination of GC + infected treatment (infusion of bacteria 24 h after the first GC treatment). At 12 h after the end of each treatment, uterine samples were collected by flushing and NO was determined. After nitrate reduction, total nitrite was determined by spectrophotometer. No significant differences on nitric oxide concentration were verified by analysis of variance in the different experimental groups. It is concluded that the use of isoflupredone did not alter the nitric oxide concentration in uterine flushing's from susceptible mares 12 h after treatment.

Keywords: bacterial infection, endometritis, equine, glucocorticoid, NO.

Introduction

Transient endometritis is a normal consequence of breeding and results from uterine contamination with both semen (Katila *et al*., 1995) and bacteria (Williamson *et al*., 1984). *Streptococcus equi* subsp. *zooepidemicus* is the pathogen most commonly isolated from the uterus from mares with endometritis (Ferreiro *et al*., 1986). Normal mares eliminate bacteria, sperm and inflammatory by-products rapidly, in 24-48 h (Katila, 2008). However, in approximately 10-15% of Thoroughbred mares this uterine clearance system fails (Zent *et al.*, 1998) and the normally transient inflammation becomes persistent endometritis. These mares are called susceptible to uterine infections (Katila, 2008) causing a substantial economic loss due to low fertility (Troedsson, 2011), and presenting three times more embryonic death rates than in normal mares (Malschitzky *et al*., 2003).

Increasing evidence shows that differences in the mechanical drainage of the uterus provide the best explanation for susceptibility to uterine infections (Katila, 2008). Susceptible mares presented reduced myometrial activity (Troedsson *et al*., 1993), delayed physical clearance (LeBlanc *et al*., 1994), and defects in myometrial function (Rigby *et al*., 2001). Mucus secretion increases in mares with delayed uterine clearance and bacterial endometritis (Causey *et al*., 2000). Resistant and susceptible mares have differences in protein composition of their endometrial fluid (Malchitzky *et al*., 2008) and susceptible mares had an increased intrauterine nitric oxide (NO) production compared to resistant mares (Woodward *et al*., 2013).

Nitric oxide (NO) is a free gaseous radical (Frean *et al*., 1997) with a very short half-life (D'Acquisto *et al*., 1997) of 3 to 5 sec (Rodeberg *et al*., 1995). This gas causes smooth muscle relaxation (D'Acquisto *et al*., 1997), including the uterus (Bani *et al*., 1999). NO synthesis occurs with the oxidation of L-arginine (Rodeberg *et al*., 1995) by the action of the enzyme nitric oxide synthase (NOS). The enzyme NOS converts the terminal guanidine group of L-arginine in NO (Rodeberg *et al*., 1995). There are at least three isoforms of the enzyme: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). While nNOS and eNOS are calcium-dependent, iNOS is calcium-independent and produces large amounts of NO (Cameron and Campbell, 1998). Alghamdi *et al*. (2005) reported the presence of the iNOS expression in uterine biopsies and of NO in uterine secretions of mares resistant and susceptible to endometritis, with higher concentrations being found in susceptible mares. Although it is not clear whether the higher NO is a cause or a result of delayed uterine clearance, the difference between these two groups suggests a possible role for NO in myometrial contractility.

Glucocorticoids are well-recognized anti-inflammatory and immunomodulatory agents (Rasmussen *et al*., 1998). The modulation of the inflammatory response has been proposed as an efficient method for the treatment of endometritis by increasing pregnancy rates with the use of isoflupredone (Dell'Acqua *et al*., 2006; Papa *et al*., 2008) and decreasing uterine fluid accumulation (Bucca *et al*., 2008) with dexamethasone. However, isoflupredone and dexamethasone affected the proteomic profile of the

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endometrial fluid, although the altered proteins are different (Arlas *et al*., 2014). It has been demonstrated that glucocorticoids inhibit NO production in many cell types and reduce iNOS mRNA levels (Shinoda *et al*., 2003). Our hypothesis is that the use of isoflupredone reduces uterine NO levels.

The aim of the current study was to determine the effects of isoflupredone, in the presence and absence of a uterine infectious process, on NO levels in uterine samples from mares susceptible to persistent postbreeding endometritis.

Material and Methods

Mares

Eleven mares (4 to 30 y old) of various breeds, susceptible to endometritis, with endometrial status varying from IIb (n = 8) and III (n = 3; Kenney and Doig, 1986), cycling and clinically healthy, were used. Susceptibility was determined after an artificial insemination with 500 x 10^6 sperm diluted in skim milk (final volume, 20 ml) according to Malschitzky *et al*. (2008). Those mares with an intrauterine fluid accumulation >15 mm in diameter (based on ultrasonographic examination of the uterus 36 to 48 h after AI), were classified as susceptible. The mares were part of an experimental herd and were maintained in an open field, supplemented with oats and alfalfa hay, with *ad libitum* access to water.

Reproductive management

Mares were given 5 mg prostaglandin F2α IM (PGF2α; Lutalyse®, Pfizer Saúde Animal, Paulínia, SP, Brazil), at 14 d intervals and estrus was confirmed by transrectal palpation and ultrasonographic examination of the reproductive tract. Once estrus was confirmed (ovarian follicle >35 mm in diameter and marked uterine edema), mares were subjected to four treatments (done consecutively, at every second estrus period). To ensure that all mares were in estrus at sample collection, they were routinely examined by transrectal palpation and ultrasonographic examination of the reproductive tract at 12 h intervals to detect ovulation. If a mare ovulated during treatments, those data were not used.

Before starting each treatment, clinical examinations of the genital tract and endometrial cytology, using a guarded swab, was performed on all mares to ensure that there was no pre-existing endometritis. Only mares without cytological evidence of endometritis (absence of PMNs in the slide at 400X), were used.

Control treatment (control)

Mares did not receive any treatment.

Glucocorticoid treatment (GC)

Mares were given 20 mg isoflupredone acetate IM (Predef®, Pfizer, Saúde Animal), every 12 h, for three consecutive days, after the mares were confirmed in estrus (Dell'Acqua *et al*., 2006).

Experimental infection (infected)

Streptococcus *equi* subsp. *zooepidemicus* (*S. zooepidemicus*) was isolated from a mare with clinical signs of endometritis. After isolation, bacteria were cultured in brain heart infusion (BHI) for 24 h. Glycerol was added to the bacteria in BHI bouillon, placed in 2.0 ml vials (Eppendorf do Brasil, São Paulo, SP, Brazil) which were put directly into a -20°C freezer. Before infusion, bacteria were thawed in a water-bath at 37°C, cultured in BHI for 24 h (37°C) and seeded onto 100 mm blood-agar dishes (20-30 dishes). Dishes were incubated at 37°C for 24 h to allow bacterial growth. Dishes were washed with PBS and bacterial colonies were carefully removed. The resulting bacterial suspension was then filtered to remove agar particles and re-suspended in PBS to a final concentration of 1 x 10^9 CFU/ml. The suspension was placed in 20 ml vials and kept at 5°C until use. An insemination pipette was used to infuse the suspension into the uterus.

Glucocorticoid + experimental infection (GC + infected)

Glucocorticoid and *S. zooepidemicus* infusion were performed as described above, with bacterial cultures being infused 24 h after the first GC application.

Experimental design

All mares were submitted to the four treatments and between each treatment they had at least one spontaneous estrous cycle. Since a new element was added for every next treatment, treatment order was as described by Wolf *et al*. (2012), to ensure that the new element would not affect the next treatment. Treatment 1: control, treatment 2: glucocorticoid (GC), treatment 3: experimental infection (infected) and treatment 4: glucocorticoid + experimental infection (GC + infected). Before all transvaginal manipulations, the mare's tail was wrapped and the perineal region cleaned with water, neutral detergent and degermant solution (Laboriodine®, Segmenta, Ribeirão Preto, Brazil) and dried with a paper towel. To ensure that inflammation was absent before starting the next treatment, at the end of the latest sample collection, mares were treated by uterine washing and intrauterine infusion of 10 x 10^6 IU penicillin (Novapen; Marcolab, São João do Paraíso, MG, Brazil) for 5 days. Mares were monitored based on the absence of neutrophils in a uterine swab and the absence of uterine fluid accumulation.

Sample collection

Endometrial flushings were collected from mares detected in estrus (ovarian follicle >35 mm in diameter and marked uterine edema) 12 h after the end of each treatment (GC and GC + Infected groups) or infection (infected group). Control treatment mares

were collected 12 h after estrus detection. Ovulated mares were excluded from the experiment. For flushing recovery, 100 ml of lactated Ringer solution was infused through the cervix with a Foley catheter (2w30cc24F, Rüsch, Germany). The fluid was distributed to the uterine body and horns by transrectal massage, and recovered by gravity aided by transrectal manipulation of the uterus. Flushings were centrifuged (1.500 x *g* for 20 min) to remove all existent cells and the supernatant stored in a freezer at -80°C, until use.

Nitric oxide determination

In order to accurately compare the NO concentration between groups, all samples were frozen sequentially until the collection was completed then analyzed at the same time. Uterine flushing samples were thawed for NO determination by an available commercial kit (Catalogue n° ADI-917-010, Stressgen®), following manufacturer recommendations. Nitrate was converted to nitrite, by the enzyme Nitrate Reductase. After nitrate reduction, total nitrite was determined by spectrophotometer after the addition of Griess reagent to the samples and reading the color absorbance at 540 nm, then calculating the concentration using a standard curve (R^2 = 0.99). Samples were run in duplicate. Intra-assay coefficient of variation was 2.4%. Nitric oxide levels were determined in µmol/l. Outliers were defined as ± 2 SD from the mean and removed (1 of 54 data point, 1.8%) from statistical analyses.

Statistical analysis

NO concentration data in different treatments were submitted to a nonparametric Kruskal-Wallis Test, with significance set at 5%.

Results

Intrauterine infusion of *S. zooepidemicus* provoked clinical endometritis in all mares, characterized by an accumulation of intrauterine fluid (over 15 mm, as determined by transrectal ultrasonography) before sample collection. No mares used presented evidence of endometritis by cytology nor ovulated before the end of the treatments. NO concentration means did not show difference among treatments (P = 0.22) and are described in Table 1.

Table 1. Means and standard error of NO concentration from uterine flushings in the four treatments (Control, Corticotherapy treatment - GC, Infected and Corticotherapy treatment + experimental infection - Infected + GC) from 11 mares.

Treatments	NO means (µmol/L) ± standard error
Control	46.5[a] ± 9.2
GC	68.0[a] ± 26.4
Infected	22.1[a] ± 6.7
Infected + GC	17.3[a] ± 5.6

[a]The same letters in the column represent no significant difference (P = 0.22).

Discussion

Glucocorticoids are widely used due to their anti-inflammatory and immunodepressive effects (Fang *et al*., 2007), however pro-inflammatory effects have been attributed to these agents and discrepancies have emerged (Franchimont *et al*., 1999). Some studies demonstrated that glucocorticoids enhance NO production (Yukawa *et al*., 2005), while others expressed that they inhibit iNOS expression (D'Acquisto *et al*., 1997; Pudrith *et al*., 2010), decreasing NO production. However, these studies examined different isoforms of NOS, which are related to the diverse functions of NO attributed to its physical and chemical properties and to the range of cells in which it is synthesized (Cameron and Campbell, 1998); therefore, their results cannot be compared. These controversial effects indicate a complex action of glucocorticoids, probably depending on the context in which it is inserted.

Results from the present study demonstrated that isoflupredone therapy did not alter NO concentration in uterine flushings collected in the presence or absence of uterine infection. The modulation of the inflammatory response by isoflupredone has been proposed as efficient for the treatment of endometritis by increasing pregnancy rates (Dell'Acqua *et al*., 2006; Papa *et al*., 2008). Isoflupredone act through an immunomodulatory action, inhibiting and stimulating acute phase proteins (APP) in the equine endometrium, mainly when applied in the presence of an inflammatory stimulus, such as infection (Wolf *et al*., 2012). The isoflupredone therapy increases the presence of apolipoprotein A-1 (ApoA1) in the endometrial fluid after an infection (Wolf *et al*., 2012). Abundant levels of ApoA-1 have an inhibitory capacity of this APP on production of proinflammatory cytokine IL-1β (Hyka *et al*., 2001) which is an inducer of iNOS (Persichini *et al*., 2006). In the same way the IL1β mRNA expression in uterine fluid observed after the use of dexamethasone in mares was lower when compared with control (Woodward *et al*., 2014). However, no influences in the production of NO were observed. Probably the NO production in susceptible mares after isoflupredone treatment may be regulated by mechanisms other than the IL1 pathway similar to the described after dexamethasone treatments (Woodward *et al*. 2013).

In spite of the proteomic differences in endometrial fluid (Arlas *et al*., 2014), isoflupredone and dexamethasone have similar timing peak, varying from 1 to 2 h (Cross *et al*., 2011). This quick peak can probably explain the no detection by Fioratti *et al*. (2010) of differences in NO between control mares and treated with a single administration of dexamethasone 10 and 26 h before sample collection. In the present experiment uterine fluid was collected 12 h after the last treatment when isoflupredone action was probably low. Using dexamethasone Christoffersen *et al*. (2012) observed an increase in gene expression of the proinflammatory cytokine IL-1β (inducer of iNOS) immediately (3 h) after *E. coli* inoculation. However, no

effect on the anti-inflammatory cytokine was detected 6 h after breeding. Probably during 3 to 6 h the cytokine would have been expressed differently, potentially leading to the decrease in IL1β observed 6 h after breeding (Woodward *et al*., 2014). Similar effect can occur with isoflupredone.

Consumption of NO by extravascular parenchymal cells directly depends on oxygen concentration. Conditions of relative hypoxia increase the amount of NO, and the closer NO is to cells, the faster it is removed from the solution. If susceptible mares have an increase in intrauterine fluid as a result of inflammation, then the increased volume in susceptible mares permits for a longer biological life in the uterus (Woodward *et al*., 2013), as NO needs to travel further to reach the cells (Thomas *et al*., 2001). Nevertheless, the bacterial infection provoked in this study did not alter the NO concentration when compared with the control mares. Probably in spite of intrauterine fluid and the further travel of NO to the cells, some other source of O_2 is present.

It is concluded that the use of isoflupredone did not alter the nitric oxide concentration in uterine flushings from susceptible mares 12 h after treatment.

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Bioethics and biossecurity committee approval

All procedures performed were approved by the Ethics Committee on Animal Experiments of the Universidade Federal do Rio Grande do Sul (CEUA-UFRGS, project number 22017) in September 19th 2012.

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