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Effects of pentoxifylline supplementation to semen extender on post-breeding inflammation response assessed by endometrial cytology and vascular perfusion in mares

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ABSTRACT

The aim was to evaluate effects of addition of pentoxifylline to skimmed milk semen extender on uterine inflammatory response. Thirty-six estrous cycles of 15 mares were randomly divided into five groups for artificial insemination (AI): Control: mimicking the AI procedure ($n = 7$); Extender: deposition of skimmed milk based extender ($n = 7$); Extender + PTX: skimmed milk based extender plus pentoxifylline (7.18 mM; $n = 8$); Semen: semen diluted with extender without pentoxifylline ($n = 7$), and Semen + PTX: semen diluted with extender containing pentoxifylline ($n = 7$). Mares in estrus were examined by trans-rectal palpation and using ultrasonography, and ovulation was induced. Uterine hemodynamics were assessed immediately before ovulation induction (T-30), immediately before AI (T0), 2 (T2), 6 (T6), 12 (T12), 24 (T24) and 48 (T48) h after AI. Endometrial samples were collected 6 h after AI, and slides were stained and examined to determine percentage of PMN. Pentoxifylline had no additional effect on vascular perfusion. There was a major inflammatory response with pentoxifylline treatment that was greater than that of the control group. In the group treated with Extender + PTX, there were more PMN (57.98 ± 9.42%) than in the group treated with Extender (20.20 ± 6.63%) and in the Semen + PTX group more PMN (82.84 ± 5.71%) than in the Semen-treated group (47.83 ± 10.61%). These findings indicate the addition of pentoxifylline does not stimulate blood flow; however, it induces a greater immune defense response because more neutrophils migrate to the uterine lumen.

1. Introduction

Endometritis is one the major causes of infertility in mares, and the third most common disease after colic and respiratory disorders (Troedsson, 1999; Card, 2005). The most common form of endometritis is induced post-breeding (Troedsson, 1997). The inflammatory process induced post-breeding by semen and microorganisms is necessary for the maintenance of an uterine

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environment conducive for embryo development. Some mares, however, are considered susceptible to endometritis and are unable to overcome the inflammatory process at the appropriate time, thus developing persistent post-breeding endometritis, resulting in a large rate of embryonic loss (Troedsson, 1999, 2014; Fedorka et al., 2018).

Pentoxifylline is a xanthine used to treat peripheral disorders to reduce deformity of red blood cells, decrease blood viscosity, and prevent thrombus formation, through functioning as a competitive and non-selective inhibitor of phosphodiesterase (Banihani et al., 2017). Pentoxifylline is thought to be involved in these regulatory functions via two modes of action: as a rheological agent, improving blood flow to compromised tissues, and as an immunomodulatory agent (Baumgartner, 2007; Banihani et al., 2017). These actions lead to prevention of the production of tumor necrosis factor (TNF) by inhibiting phagocytosis, production of reactive oxygen species, actions of proteolytic enzymes produced by macrophages and granulocytes, and inflammation (Perelló et al., 2017). Pentoxifylline is a potent stimulator of sperm motility (Banihani and Abu-Alhayjaa, 2016) and functions to increase values for sperm kinetic characteristics and the number of spermatozoa having hyper-activated motility (Calogero et al., 1998; Marques et al., 2002; Esteves et al., 2007; Tsunoda et al., 2015). Recently, pentoxifylline has been used to identify viable sperm in asthenozoospermic patients and to increase sperm motility prior to the use of ICSI (Navas et al., 2017).

Pentoxifylline also improves bacterial clearance by decreasing bacterial colonization, thereby potentially decreasing tissue damage caused by neutrophils (Samlaska and Winfield, 1994; Baumgartner, 2007). There are also some studies of the relation between pentoxifylline and blood flow. Short-term treatment with pentoxifylline via oral administration led an increased testicular blood flow (Pozor et al., 2011), but when a therapeutic dose was orally administered to pregnant mares, it did not alter values for uterine artery blood flow variables (Bailey et al., 2012).

Endometrial inflammation causes changes in uterine perfusion and can be assessed using Doppler ultrasonography to quantify blood flow by spectral or color flow mode. The resistance index (RI) is the parameter evaluated using spectral mode and vascularization score (VS) assessments by using color flow mode ultrasonography (Bollwein et al., 2003a). Another technique often used to evaluate endometrial inflammation is through the detection of neutrophils on exfoliative endometrial cytology (Card, 2005). Uterine cytology assessments provide for gaining direct evidence of extent of uterine inflammation (Causey, 2006). The use of the cytobrush technique is a simple approach for recovery of endometrial samples that is easy, consistent, and rapid for field use in mares (Cocchia et al., 2012).

The purpose of the present study was to examine the inflammatory response of the uterus caused by the addition of pentoxifylline to semen extender by utilizing two techniques, Doppler ultrasonography and exfoliative endometrial cytology.

2. Materials and methods

This experiment was conducted in “Estância Sonho Meu” in Piedade city, São Paulo state, Brazil, at 23° south latitude and 47° west longitude, altitude between 750–1227 m above sea level. The procedures used to conduct this experiment were consistent with Ethical Principles in Animal Research adopted by the “Ethics Committee on Animal Use” of the School of Veterinary Medicine and Animal Science of University of São Paulo, protocol number CEUA 2315/2011.

2.1. Animal selection

Animals used for the study included 15 mares of the Margalarga breed, between 4 and 12 years of age. Animals were fed twice daily *Lolium multiflorum*, and were provided mineral supplementation and water *ad libitum*. Candidate mares for the study were previously submitted to gynecological examination, including evaluation of perineal and vulvar conformation, transrectal palpation and ultrasonic examination of the genital tract, a vaginal speculum examination and cytological evaluation. All mares selected were clinically healthy, had normal estrous cycles and had no previous history of persistent post-breeding endometritis. Inflammatory responses were determined based on the percentage of neutrophils present in a sample with an on Card (2005) classification (< 5%, noninflammatory; 5% to 15%, mild inflammation; 15% to 30%, moderate inflammation; and > 30% severe inflammation), and mares > 5% of PMN were excluded from the experiment. Importantly, only reproductively healthy mares were used in the study.

2.2. Semen evaluation and preparation of insemination dose

Ten semen batches previously cryopreserved (100×10^6 spermatozoa/0.5 mL straw) from a unique stallion were analyzed for use in the experiment. Two frozen semen straws were thawed (37 °C/30 s), semen was placed in a microcentrifuge tube (heated at 37 °C) and homogenized. The sperm movement was evaluated using the Computer-Assisted Sperm Analysis (CASA), as described by Nascimento et al. (2008). Integrity of plasma and acrosomal membranes and mitochondrial membrane potential was analyzed by the association fluorescent probes: propidium iodide (PI), Hoechst 33342 (H342), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) adapted from Nascimento et al. (2008) and Celeghini et al. (2010). Sperm morphology was analyzed using a differential interference contrast (DIC) microscopy, with cells classified using the procedures described by Brito (2007), and chromatin denaturation assessed using the technique of staining with toluidine blue described by Beletti et al. (2005).

Frozen-thawed semen batches had a mean and standard error of 42.80 ± 4.36% total motility, 23.20 ± 3.88% progressive motility, 29.60 ± 1.99% intact plasma membranes, intact acrosomes and high mitochondrial membrane potential, 14.00 ± 0.90% abnormal sperm and 94.09 ± 0.55% intact chromatin. To prepare the insemination dose, skimmed milk-based extender (Botusemen®, Botupharma- Botucatu, SP, Brazil) was aliquoted to two treatment groups, one without any addition (Extender,

Table 1
Mean ± standard error of the sperm characteristics from semen batches used for artificial insemination after thawing and dilution in skim milk extender (SM Extender) or skim milk extender plus pentoxifylline (SM Extender + PTX, at 7.18 mM).

Sperm characteristics (%)	SM Extender	SM Extender + PTX
Total Motility	45.48 ± 2.32	46.87 ± 1.93
Progressive Motility	31.00 ± 1.75	33.77 ± 1.65
PMI[a]	41.37 ± 1.50	41.10 ± 1.48
AMI[b]	95.72 ± 0.52	94.81 ± 0.68
HMMP[c]	61.48 ± 2.16	61.44 ± 2.03
IPIAH[d]	41.03 ± 1.51	40.32 ± 1.48
Total of abnormal sperm	12.87 ± 1.15	11.62 ± 0.71
AT I[e]	94.11 ± 0.54	94.07 ± 0.60
AT II[f]	5.09 ± 0.50	5.06 ± 0.57
AT III[g]	0.79 ± 0.10	0.86 ± 0.09

[a] PMI: plasma membrane integrity.
[b] AMI: acrosome membrane integrity.
[c] HMMP: greater mitochondrial membrane potential.
[d] IPIAH: intact plasma membrane, intact acrosome and greater mitochondrial membrane potential.
[e] ATI: cells without chromatin decondensation.
[f] ATII: intermediate decondensation.
[g] ATIII: high decondensation; Sperm characteristics of SM Extender and SM Extender + PTX were similar ($P > 0.05$).

pH = 6.7–7.0, osmolarity = 340–380 mOsm/L) and the other with pentoxifylline added (Extender + PTX, pH = 6.7–7.2, osmolarity = 340–380 mOsm/L). Pentoxifylline (P-1784, Sigma-Aldrich) was added so there was a final concentration of 7.18 mM pentoxifylline (0.119 g on 100 mL), using the procedures described by Guasti et al. (2013). Both extenders were pre-heated at 37 °C to prevent cold shock injury in sperm cells.

After extenders were prepared, the insemination dose was made using 10 semen straws (0.5 mL, 200 × 10^6 spermatozoa/mL) thawed in a water bath at 37 °C for 30 s and then diluted with 25 mL of extender to a final volume of 30 mL (33 × 10^6 spermatozoa/mL). There is a description in Table 1 of sperm characteristics after thawing and dilution in skim milk extender with or without pentoxifylline.

2.3. Experimental groups

Thirty-six estrous cycles of 15 mares were randomly distributed into five groups for artificial insemination (AI): Control group: no deposition of semen or extender, mimicking the AI procedure ($n = 7$); SM Extender group: deposition of skimmed milk based extender ($n = 7$); SM Extender + PTX group: deposition of skimmed milk based extender plus pentoxifylline ($n = 8$); Semen group: deposition of semen diluted with extender without pentoxifylline ($n = 7$), and Semen + PTX group: deposition of semen diluted with extender containing pentoxifylline ($n = 7$).

Treatment schedules were randomized. Estrous cycles were assigned alternately, and all mares had all treatments administered; thus, each mare was her own control for the different treatments. In addition, mares had an estrous cycle between cycles in which treatment occurred when there were no treatments imposed.

2.4. Follicular development, ovulation detection and treatment

Mares in estrus were examined daily using trans-rectal palpation and ultrasonic examination until it was possible to detect a dominant follicle measuring ≥35 mm in diameter and evident endometrial folds (edema). At this time, 2,500 IU of human chorionic gonadotropin (hCG, Vetecor®) were administered intravenously.

After 30 h of ovulation induction (hCG administration), there were ultrasonic examinations every 6 h so that insemination of mares occurred as close as possible subsequent to the time of ovulation. The ultrasonic procedures used to perform all the evaluations was Doppler Ultrasonography (M5Vet, Mindray Medical International Limited, China) with transrectal linear probe (6.5 MHz).

As soon as there was determination that ovulation had occurred by ultrasonography, there was treatment administrations to mares (AI mimicking, deposition of extender or diluted semen).

2.5. Assessment of endometrial inflammatory response

2.5.1. Vascular perfusion of the endometrium

Analyses of color flow mode of the left and right uterine horns and spectral mode of the left and right uterine arteries were performed at seven predetermined times: immediately before ovulation induction (T-30), immediately before AI (TAI), 2 h (T2), 6 h (T6), 12 h (T12), 24 h (T24) and 48 h (T48) after AI.

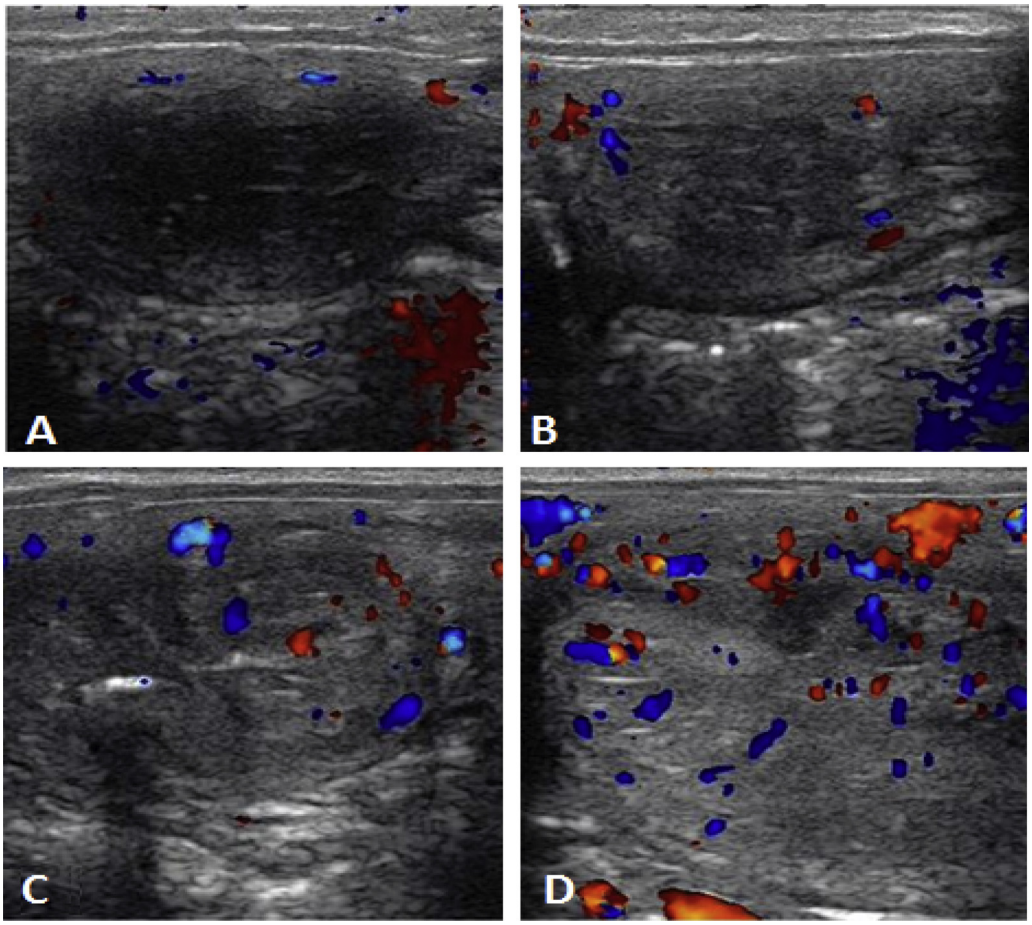

Fig. 1. Sonograms of equine uterine horn in transversal section, by color Doppler equipment (M5Vet, Mindray) to vascular scoring (VS) system for subjective vascularization of mesometrium; Considering from less vascularized (A) to more vascularized (D); Score 1 (A), Score 2 (B), Score 3 (C) and Score 4 (D).

The uterine artery was located using the procedures described by Bollwein et al. (2000) and there was visualization of its branching from the external iliac artery and following of its course to the crossing point with the deep circumflex ilium artery. This area was defined as the region of interest and a pulsed Doppler range gate was placed across the vessel. The examination was performed using the procedures described by Oliveira et al. (2014). The extent of local perfusion or blood flow of the uterine horn was estimated subjectively using the color-flow films. The evaluation of vascular score (VS) was made by use of a scoring system quantification from 1 (lesser perfusion) to 4 (greater perfusion) according to Ginther (2007). Film evaluations were made as described by Oliveira et al. (2014). This approach for color Doppler evaluation has appeal for both clinical and research purposes because it focuses directly on the tissue or structure of interest, as depicted in Fig. 1. For spectral mode, at least nine similar waves of the cardiac cycle was obtained, and data were collected. There was a subsequent evaluation of these waves so that for every three similar waves, the resistance index (RI) value of the medium wave was selected and added to the values of the other two medium waves of the next estrous cycle when these data were obtained. For statistical analysis, there was averaging of the values from the three selected waves. All assessments of vascular perfusion of the endometrium were performed by the same evaluator, and were conducted in a double blind approach for treatment and timing.

2.5.2. Endometrial cytology

Endometrial samples for cytology were collected using the cytobrush technique. The covered, lubricated instrument was passed through the vagina and advanced trough the cervix into the uterine body, at which point the outer tube was retracted far enough to expose the cytobrush. Endometrial cytology samples were collected rotating the cytobrush in a clockwise direction while in contact with the uterine wall. The cytobrush was retracted into the outer tube prior to removal from the uterus. Slides for cytology were prepared by gently rolling the cytobrush onto a glass microscope slide and air-dried.

The samples placed on cytological slides were stained using a commercial stain (Panótico Rápido® - Laborclin LTDA. Paraná, Brazil), air-dried and examined using microscopy (Nikon, Eclipse E200, Tokyo, Japan) at 400× magnification. Cells (n = 300) on each slide were examined and classified as endometrial epithelial cells (cubic or columnar cells with basal round nucleus) or

Table 2
Means ± standard error of resistance index (RI, 0–1) of uterine arteries; Effect of infusion of groups: with no deposition of semen, or extender, mimicking the AI procedure (Control), after infusion of skim milk extender (Extender) or skim milk extender plus pentoxifylline (Extender + PTX), and semen diluted in skim milk extender (Semen) or semen diluted in skim milk extender plus pentoxifylline (Semen + PTX), at T30 (immediately before ovulation induction), TIA (immediately before infusion), and 2 (T2), 6 (T6), 12 (T12), 24 (T24), and 48 (T48) hours after infusion.

Time related to Treatment	Control	Extender	Extender + PTX	Semen	Semen + PTX	Mean of Time
T-30	076 ± 0.01^{a,b}	0.75 ± 0.01^{b}	0.75 ± 0.01	0.75 ± 0.01^{a,b}	0.76 ± 0.01	0.75 ± 0.007^{b}
TAI	0.76 ± 0.01^{a,b}	0.75 ± 0.01^{b}	0.78 ± 0.01	0.78 ± 0.02^{a,b}	0.76 ± 0.01	0.77 ± 0.007^{ab}
T2	0.78 ± 0.01^{a,b}	0.78 ± 0.01^{a,b}	0.77 ± 0.01	0.74 ± 0.01^{b}	0.78 ± 0.01	0.76 ± 0.006^{ab}
T6	0.73 ± 0.02^{b}	0.75 ± 0.01^{b}	0.76 ± 0.02	0.76 ± 0.02^{a,b}	0.75 ± 0.01	0.76 ± 0.007^{ab}
T12	0.80 ± 0.02^{a}	0.78 ± 0.01^{a,b}	0.77 ± 0.01	0.77 ± 0.02^{a,b}	0.77 ± 0.02	0.77 ± 0.008^{ab}
T24	0.79 ± 0.01^{a,b}	0.79 ± 0.01^{a}	0.76 ± 0.02	0.77 ± 0.02^{a,b}	0.77 ± 0.01	0.77 ± 0.008^{ab}
T48	0.77 ± 0.02^{a,b}	0.77 ± 0.01^{a,b}	0.78 ± 0.01	0.80 ± 0.01^{a}	0.78 ± 0.01	0.78 ± 0.007^{a}
Mean of Treatment	0.77 ± 0.006	0.76 ± 0.006	0.77 ± 0.006	0.77 ± 0.007	0.77 ± 0.005	

^{a,b}Means with different superscripts within each column differ ($P < 0.05$).

neutrophils (polymorphonucleated cells, PMNs) using a method of classification that was adapted from Card (2005). For the comparison of the groups, the percentage values of PMN 6 h subsequent to AI were considered. All evaluations of endometrial cytology were performed by the same evaluator that was blind with regard to treatment assignments.

3. Statistical analysis

All data were evaluated using SAS System for Windows (Version 9.3; SAS Institute Inc., Cary, NC, USA). Data obtained were previously assessed for normality of residues (Shapiro-Wilk test) and homogeneity of variances (Bartlett's test). When any of the tests were significant ($P < 0.05$), data were transformed and/or removal of outliers were performed with subsequent reassessment. For none of the data collected was there need to conduct a non-parametric statistical analyses. There were comparisons between groups using the combined procedure (PROC MIXED) of SAS with the "repeated" command for treatment (Control, Extender, Extender +PTX, Semen and Semen+PTX) and time (T-30, TAI, T2, T6, T12, T24, and T48), in a 5 x 7 factorial treatment arrangement for analysis of the interaction of treatment x time, and the main effect when there was no interaction. Data from endometrial cytology assessments were evaluated using an ANOVA. All means were compared using the Tukey's test. Data are presented as mean ± mean standard error (SEM), except as otherwise specified. The significance level was 5% ($P \leq 0.05$).

4. Results

4.1. Vascular perfusion of the endometrium

Effects of the treatment (Control, Extender, Extender + PTX, Semen and Semen + PTX) were considered for each analysis time (T-30, TAI, T2, T6, T12, T24, T48) for RI (Table 2) and VS (Table 3). There was no difference ($P > 0.05$) between treatment groups, and pentoxifylline did not have any additional effect on endometrial vascular perfusion after infusion. When comparing the effect of treatments at all times of sample collection, the resistance index (RI), at T48 was greater ($P \leq 0.05$) than at T-30, which means there was a lesser perfusion at 2 days after infusion. For VS, however, at T48 it was greater and different ($P \leq 0.05$) than that at T24.

For RI, there was time effect ($P \leq 0.05$) for the Control, Extender-treated and Semen-treated groups. In the Control group, the RI value was greater at T12 (0.80 ± 0.02) than at T6 (0.73 ± 0.02). For the Extender-treated group, the RI value was greater at T24 (0.79 ± 0.01) than T-30, TAI and T6 (0.75 ± 0.01 for all of these). In the Semen-treated group, the RI was greater ($P \leq 0.05$) at T48

Table 3
Means ± standard error of vascularity score (VS, 1–4) of uterine horns; Effect of infusion of groups: with no deposition of semen, or extender, just mimicking the AI procedure (Control), after infusion of skim milk extender (Extender) or skim milk extender plus pentoxifylline (Extender + PTX), and semen diluted in skim milk extender (Semen) or semen diluted in skim milk extender plus pentoxifylline (Semen + PTX), at T-30 (immediately before ovulation induction), TIA (immediately before infusion), and 2 (T2), 6 (T6), 12 (T12), 24 (T24), and 48 (T48) hours after infusion.

Time related to Treatment	Control	Extender	Extender + PTX	Semen	Semen + PTX	Mean of Time
T-30	3.37 ± 0.19^{aA}	2.86 ± 0.19^{a,bB}	3.03 ± 0.35^{aAB}	3.07 ± 0.27^{AB}	2.87 ± 0.30^{AB}	2.97 ± 0.12^{ab}
TAI	3.12 ± 0.30^{a,b}	2.86 ± 0.22^{a,b}	2.71 ± 0.33 ^{a,b}	2.79 ± 0.33	2.53 ± 0.15	2.77 ± 0.11^{ab}
T2	2.50 ± 0.37^{b}	2.37 ± 0.24^{b}	2.78 ± 0.32^{a,b}	3.00 ± 0.33	2.67 ± 0.11	2.79 ± 0.12^{ab}
T6	2.93 ± 0.18^{a,b}	2.75 ± 0.18^{a,b}	2.71 ± 0.21^{a,b}	3.14 ± 0.29	2.91 ± 0.23	2.87 ± 0.11^{ab}
T12	2.87 ± 0.41^{a,b}	2.65 ± 0.24^{a,b}	2.54 ± 0.30^{b}	3.21 ± 0.30	3.14 ± 0.27	2.91 ± 0.12^{ab}
T24	2.92 ± 0.24^{a,b}	2.70 ± 0.19^{a,b}	2.50 ± 0.28^{b}	2.64 ± 0.28	2.50 ± 0.26	2.68 ± 0.11^{b}
T48	3.17 ± 0.20^{a,b}	3.06 ± 0.18^{a}	2.85 ± 0.28^{a,b}	3.10 ± 0.18	2.95 ± 0.43	3.02 ± 0.11^{a}
Mean of Treatment	2.98 ± 0.10	2.75 ± 0.09	2.82 ± 0.10	2.98 ± 0.10	2.78 ± 0.09	

^{a,b}Means with different lowercase letters in the same column indicate difference between times ($P < 0.05$) ^{A,B}Means with different capital letters on the same line indicate difference between treatments ($P < 0.05$).

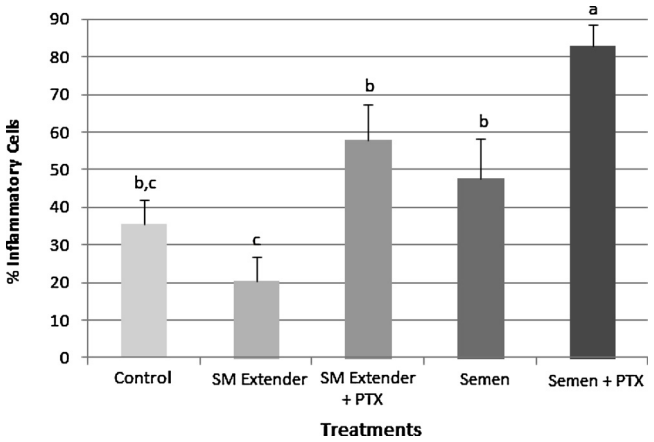

Fig. 2. Means and standard error of percentage of polymorphonuclear (PMN) cells found in mares after 6 h from treatment; Control: no deposition of semen, or extender, just mimicking the AI procedure; SM Extender: infusion of skim milk extender; SM Extender + PTX: skimmed milk extender plus pentoxifylline; Semen: semen diluted in skimmed milk extender; Semen + PTX: semen diluted in skim milk extender plus pentoxifylline; Different letters on the bars indicate a difference ($P < 0.05$).

(0.80 ± 0.01 for both) than at T2 (0.74 ± 0.01).

For vascular perfusion by scoring system quantitation, there was a time effect ($P \leq 0.05$) for the Control group, and Extender with and without pentoxifylline treatment groups. There was also a difference ($P \leq 0.05$) between the Control group which had a greater vascularity score than Extender-treated group at T0.

The Control group had a greater vascular score (0–4) at T30 (3.37 ± 0.19) than at T2 (2.50 ± 0.37). The vascular score for the Extender-treated group was only greater ($P \leq 0.05$) at T48 (3.06 ± 0.18) than T2 (2.37 ± 0.24). For the Skim Milk Extender + PTX-treated group, only at T-30 was there a greater ($P \leq 0.05$) perfusion (3.03 ± 0.35) than at T12 and T24 (2.54 ± 0.30 and 2.50 ± 0.28). For the other groups, there were no statistical differences between time of analysis.

4.2. Endometrial cytology

The results for percentage of inflammatory cells related to endometrial cytology are depicted in Fig. 2. It is clear that Extender + PTX (57.89 ± 9.42%) and Semen + PTX (82.84 ± 5.71%) had a major inflammatory induced response when compared to the Extender (20.20 ± 6.63%) and Semen (47.83 ± 10.61%) treaments.

5. Discussion

In this experiment, there was analysis of the effect of extender, pentoxifylline and semen on the uterine inflammatory response until 48 h after infusion to characterize which factors were responsible for variations in blood flow or neutrophil migration.

5.1. Vascular perfusion of the endometrium

There has been reported to be a positive relationship between genital blood perfusion and fertility (Bollwein et al., 2004). An adequate blood supply is required for hormone signaling, uterine contractility, placentation, and fetal-endometrial interactions (Zooler et al., 2016). Furthermore, in this experiment, the rheological properties of pentoxifylline (Baumgartner, 2007) was not evident, because the results for intrauterine infusion did not indicate there was a positive effect, neither in the group treated with skim milk extender only, nor the group treated with semen diluted in skim milk extender. In addition, Bollwein et al. (2003a) reported infusion with skim milk semen extender had no effect on uterine blood flow; however, within 1 h after infusion of seminal plasma or raw semen, there was an increase in the time-averaged mean velocity (TAMV) values for both uterine arteries ($P \leq 0.05$). It must be considered that in this previous study (Bollwein et al., 2003a) there was detection of changes in blood flow by evaluating the TAMV, different from that which occurred in the present study, where the evaluations were made through resistance index (RI) values. Silva et al. (2005) observed that TAMV was greater and RI less in the uterine arteries of pregnant mares compared to those of non-pregnant mares. The RI is frequently used, and has a negative relationship between the extent of resistance in the tissues and the extent of vascular perfusion - the greater the resistance, or RI, the lesser the perfusion, whereas TAMV is an average of the maximum values during the time of a cardiac cycle, termed the time-averaged mean velocity (Ginther and Matthew, 2004).

There is another arterial pressure measurement, the pulsatility index (PI), an expression of the extent of the difference between the peak systolic velocity (PSV) and end diastolic velocity (EDV) of the blood pulse in the vessel. The RI and PI are highly correlated ($r > 0.9$) and usually only one is necessary (Ginther and Matthew, 2004). For example, the effect from orally administered pentoxifylline was a decrease in both RI and PI, which resulted in a decrease in vascular resistance of the testicular artery of stallions, increasing vascular perfusion (Pozor et al., 2011). From this evaluation, results of the present study were compared with those of

studies where there was use of the PI for this evaluation. Thus, Bollwein et al. (2002) observed a time trend in changes of uterine blood flow resistance during the estrous cycle, where there were greater PI values on day 0 (ovulation) and 1, followed by a decrease until day 5. This sequence of changes was also evident in the present study where vascularity score increased between Days 1 to 2, which may be related to the effect of the increase of progesterone in plasma due to preparation of the uterus for the embryo which generally occurs on Day 5. The RI data from the present study, however, are not consistent with this change in blood flow dynamics.

There are several studies where there was evaluation of the optimal concentration of pentoxifylline on semen samples. Centola et al. (1995) observed that 2.5 mg/mL may be the optimal beneficial concentration, whereas, a concentration of > 5 mg/mL may be detrimental to sperm viability. In another study, there was an improvement of sperm motility only when semen was diluted at a 1 mM pentoxifylline concentration (Blanes et al., 2004). There was an increase in percentage of hyperactive spermatozoa, and greater progressive motility when there was dilutions of pentnoxifylline at 3.5 mM (Gradil and Ball, 2000; Marques et al., 2002; Ortgies et al., 2012). The infusion of pentoxifylline at a concentration of 7.18 mM improved motility characteristics of recently recovered epididymal sperm and had no deleterious effect on plasma membrane integrity and freezing capacity of stallion epididymal sperm (Guasti et al., 2013). Similar to the present study, however, treatment with pentoxifylline did not result in an acute increase in uterine blood flow, and the concentration of 7.18 mM may not be enough to effect uterine vascular perfusion in mares. Relatively greater concentrations, however, could be deleterious to sperm viability.

When there was oral administration of pentoxifylline at 17 mg/kg, there was no increase in uterine artery blood flow. This finding indicates the effects of endometriosis were not overcome by pentoxifylline treatment. Long-term pentoxifylline treatment appears to lead to an increase in placental vascular resistance which could be potentially harmful (Ousey et al., 2010).

Because there are individual variations in blood flow between older multiparous mares that have a relatively greater resistance to uterine blood flow than younger primiparous mares (Bollwein et al., 2003b), it is possible that the technique and variable used to determine blood flow could have affected the results in each of these studies. There is a need to standardize research methodology and Doppler parameters to enhance the applicability of the results of various studies and the use of this technique.

5.2. Endometrial cytology

The results of the present study clearly indicate treatment with pentoxifylline affects inflammatory reactions. The group treated with extender plus pentoxifylline (57.98%) had 2.87 times more intrauterine PMN migration than the group with extender only (20.20%). An immunomodulatory agent, pentoxifylline and its metabolites, appear to increase the rate of neutrophil migration and have a protective effect against infection (Samlaska and Winfield, 1994). The results of the present study indicate pentoxifylline may have different effects on migration, depending on its concentration (Elferink et al., 1997). This suggests that the addition of pentoxifylline induces an intense inflammatory response. A weaker insult to the endometrium induces a longer residual inflammation (Nikolakopoulos and Watson, 2000) whereas a greater stimulation of defense mechanisms promotes more rapid endometrial recovery (Fiala et al., 2007).

There was the same effect in the present study in the groups where semen was diluted with an extender containing or not containing pentoxifylline, whereas with the extender containing pentoxifylline (82.84%) there was 1.7 times greater numbers of PMN than when there was treatment with semen without pentoxifylline (47.83%). In the present study, when the groups treated with semen were compared to groups treated with extender only, treatment with semen induced an intense inflammatory reaction. This happens because the presence of spermatozoa is the major factor responsible for the inflammatory reaction (Nikolakopoulos and Watson, 2000). Spermatozoa have a chemotactic effect on equine neutrophils (Dell'Aqua et al., 2006). The spermatozoa that contributes to fertilization of the oocyte are present in the uterine lumen for 4 h after artificial insemination, at which time these cells become a target for the PMNs through complement activation (Troedsson et al., 2001).

Results of several experiments, therefore, confirm that there is a greater inflammatory reaction when semen is present, and there is a greater reaction when frozen semen is used for insemination. Mares bred with frozen/thawed semen develop a marked persistent inflammation that has been attributed to result from the removal of seminal plasma during the process of cryopreservation. The seminal plasma has a modulatory function in the inflammatory reaction by suppressing complement activation, PMN-chemotaxis and phagocytosis *in vitro* (Troedsson et al., 1998; Card, 2005). Consistent with the results of Kotilainen et al. (1994), inseminations with frozen/thawed semen in a small volume where sperm are highly concentrated results in a greater uterine response than inseminations with larger volumes where the sperm concentrations are less. Results of Elferink et al. (1997) indicate that greater sperm numbers or concentration probably induce a greater chemotactic response by PMNs, resulting in a more rapid and efficient phagocytosis of sperm and bacteria.

6. Conclusion

Pentoxifylline treatments in the present study did not have additional effects on blood flow as detected using Doppler ultrasonography. Pentoxifylline treatments did stimulate greater defense mechanisms because there was an increased neutrophil migration to the uterine lumen as early as 6 h after infusion, which may promote more rapid endometrial recovery.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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