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High concentrations of myeloperoxidase in the equine uterus as an indicator of endometritis

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ABSTRACT

Intraluminal fluid and excessive abnormal hyperedema are regularly used for the diagnosis of endometritis in the mare, which is routinely confirmed by the presence of neutrophils on endometrial smears. Studies show a relation between neutrophils and myeloperoxidase (MPO), an enzyme contained in and released by neutrophils during degranulation or after cell lysis. This enzyme has been found in many fluids and tissues, and associated with different inflammatory pathologies in the horse. The aims of this study were to assess the presence and concentration of MPO in the equine uterus, and to investigate its relation with neutrophils, and other clinical signs of endometritis. Mares ($n = 51$) were evaluated for the presence of intraluminal fluid and excessive endometrial edema before breeding, and a small volume lavage and cytology samples were obtained. From 69 cycles, supernatant of the uterine flushes was analyzed with a specific equine MPO ELISA assay to measure MPO concentration. Cytology samples were used for the diagnosis of endometritis. Myeloperoxidase was present in the uterus of all estrus mares in highly variable concentrations. Myeloperoxidase concentrations were significantly ($P < 0.05$) higher in samples with positive cytologies and in the presence of intraluminal fluid. Occasionally, some samples with negative cytologies showed high MPO concentration, but the opposite was never observed. Cycles presenting hyperedema weren't associated with high concentration of MPO, intraluminal fluid, or positive cytology, making it a poor diagnostic tool of endometritis.

1. Introduction

Breeding mares results in a transient physiological inflammation of the endometrium [1–3]. This occurs as a response to semen, seminal plasma, extender, and/or bacteria present within the uterine lumen, but other factors including anatomical abnormalities and uterine degeneration can also contribute to its development [4]. Failure of this inflammation to resolve majorly affects the mare's fertility [1,5–8].

Activation of various inflammatory mediators increases vascular permeability, induces the influx of serum proteins and immunoglobulins (Ig) into the uterine lumen [9,10], and migration of polymorphonuclear neutrophils [9–12]. Clinical signs such as intraluminal fluid [4,13] and to a lesser extent, the presence of an abnormal quantity in edema of the endometrial folds, so called hyperedema [7,14], are used as practical tools to diagnose the pathology. The presence of inflammatory cells, mainly neutrophils within the endometrial lumen or wall can be used as a marker of endometritis [10,11,15,16]. Uterine cytology and biopsy are commonly performed to confirm a suspicion of the condition [17], whereas uterine culture may identify the eventual bacteria involved.

A better understanding of the mechanisms and processes involved in this pathology is necessary to improve the management of susceptible mares, and increase their pregnancy

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rate. Various inflammatory markers of the uterine environment have already been investigated [18–20]. In the horse, myeloperoxidase (MPO) has been found in many fluids and tissues, and associated with different inflammatory pathologies [21,22]. Myeloperoxidase is a prooxidant enzyme contained in and released by neutrophils during degranulation or after cell lysis [23,24]. In the field of equine reproduction, MPO has been mainly studied in sperm [25–28], but to our knowledge, presence of the enzyme and its significance in the uterus of the mare has not been investigated yet.

The objective of this study was to assess the presence of MPO in equine uterine lumen during estrus, to measure its concentrations, and to determine its relation with endometritis and its associated clinical signs.

2. Materials and methods

2.1. Mares and samples collection

The study was performed on mares presented for insemination with fresh or frozen semen, at Linalux-MLS Equine Reproduction Center between April 2011 and January 2013, without interfering with the normal monitoring and insemination procedure. All samples were obtained before breeding during a complete routine reproductive examination. A total of 51 Warmblood, Arabian, Lusitanian, Quarter Horse, and Draft Horse mares, aged from 5 to 23 years (mean: 13.4) were included, and 69 cycles were exploited. Pregnancy diagnosis was routinely performed at Day 14 with ultrasonography.

Mares were regularly scanned until a follicle greater than 35 mm was observed, when all samples and data were collected. Swabs and uterine lavages were obtained from all mares. After a vulvar scrub, a uterine cotton swab (Equivet; Kruuse, Marselv, Denmark) or a double-guarded uterine Cytology Brush (Minitüb, Tiefenbach, Germany) was passed per vagina into the uterus, and an endometrial cytology sample was obtained. Cytologic slides were air dried and stained with Diff-Quick (RAL, Martillac, France).

Using a sterile double-sleeve technique and a sterile insemination pipette (Minitüb), a low-volume (60 mL) uterine lavage was performed with Ringer's lactate. After a uterus massage the fluid was reaspirated into EDTA and/or dry tubes, and samples were centrifuged at 600× g for 10 minutes immediately. For technical reasons, some samples were collected in EDTA (n = 26) and some others (n = 38) in dry tubes. To validate the assay for both tubes, five samples were collected in EDTA and dry tubes. Supernatants were collected in 2 mL tubes, and stored at −20 °C until MPO analysis. Pellets were smeared on a glass slide, air dried and stained with Diff-Quick for cytologic examination.

2.2. Intraluminal fluid and edema grade

The quantity of intraluminal fluid was measured using ultrasonography. More than 1 cm of liquid was scored as abnormal for statistical analysis.

Edema was graded with a subjective scoring system slightly modified from a previous description [29]. Grade 0 was scored in the absence of edema; grade I when uterine folds were difficult to identify; grade II when some of the endometrial folds could be identified and the cervix had a fish-bone appearance; grade III when endometrial folds were easily identified with hyperechoic borders and hypoechoic centers (cartwheel); and grade IV for mares with "hyperedema", where endometrial folds were abnormally thick, and the normal architecture of the cartwheel was lost.

2.3. Cytology

A minimum of 10 fields were evaluated microscopically (400×) by the same examiner. The number of neutrophils per field was used to evaluate the degree of inflammation. For statistical analysis, an average of one or more neutrophil per field at 400× magnification was considered a sign of inflammation as previously described [7,30].

2.4. Myeloperoxidase analysis

Concentration of MPO was determined by a commercial ELISA (Bioptis SA, Liège, Belgium) developed by Frank, et al. [24] The primary antibody, rabbit IgG against MPO, was coated onto microplate wells (Cliniplate EB; Thermo Labsystems, Helsinki, Finland). Equine MPO standard (0, 1.015, 2.03, 4.06, 8.12, 16.25, 32.5, and 65 ng/mL) and low-volume uterine flush supernatants diluted 50× and 200× were added (100 μL) into the wells, and microplates were incubated overnight at 4 °C. After the plates were washed with 0.9% NaCl solution containing 0.1% Tween 20 (Sigma Chemical Company, St. Louis, MO, USA) the immobilized antibody–antigen complexes were incubated for 2 hours at 37 °C with the secondary antibody, guinea pig IgG against equine MPO labeled with alkaline phosphatase. After another washing, phosphatase activity was determined by incubation for 30 minutes at 37 °C in the dark with a paranitrophenyl phosphate stabilized solution. The reaction was stopped with 2.5 M NaOH and the absorbance at 405 nm read with the Multiscan Ascent Plate Reader (Thermo Labsystems). The absorbance was directly proportional to the activity of alkaline phosphatase bound to the secondary antibody against MPO and thus to the concentration of MPO immunocaptured from the sample. Each sample was assayed at two dilutions (50× and 200×), and when a concentration of MPO was obtained at both dilutions, the mean was calculated and taken into consideration. The mean value of coefficient of variation (CV) intraassays was 14.92 ± 13.15%, and 74% of the samples have CV lower than 14.92%. The CV greater than 20% corresponds to samples situated at the lower and upper limit of detection of the calibration curve. Despite a double dilution (50× and 200×) to obtain values for low and high concentration samples, some remained out of calibration curve, and they were marked as higher than 7000 ng/mL (i.e., the upper limit of detection).

2.5. Statistical analysis

For discontinuous data such as the presence of uterine liquid, hyperedema, pregnancy, and cytology, Fisher's test was used to determine the significance of contingency table. Normal distribution of parameters was tested with Kolmogorov–Smirnov test. As values were non-normally

distributed, results are expressed in medians. The MPO medians were compared between groups with Mann–Whitney test. For the comparison of results on EDTA with dry tubes, a Wilcoxon matched-pairs signed-rank test, which is a nonparametric test was used. Statistical significance was established at P-value less than 0.05.

3. Results

3.1. Hyperedema, intraluminal fluid, and cytology

A total of 69 cycles were studied. Distribution of cytology results, presence of edema and intraluminal fluid for these cycles is summarized in Table 1.

A strongly significant association between the presence of free fluid and the cytology result (P = 0.0003) was observed. No other statistical relation was found between the other parameters.

3.2. Myeloperoxidase concentration

When comparing the MPO concentrations from samples collected in EDTA with dry tubes (n = 5 for each method), there was no significant difference in MPO concentration detected (P = 0.4375). Therefore, the results of the study with samples collected in EDTA (n = 26) and dry tubes (n = 38) were combined for data analysis. Myeloperoxidase was detected in all uterine flushes. The concentration of the enzyme varied between cycles, with values ranging from 10.06 to greater than 7000 ng/mL, the upper detection limit of the assay. The overall median concentration of MPO was 1617.860 ng/mL. Results of MPO (median) concentration in relation with the different parameters studied are given in Table 2.

Generally, mares with endometritis had higher MPO concentrations in uterine supernatants (P = 0.0087) than those from mares with negative cytology. Occasionally, some negative cytology results were associated with a high concentration of MPO, but the contrary (small quantities of MPO in uterine fluids from mares with an endometritis) was not observed.

Significantly higher concentrations of MPO were observed in mares presenting an abnormal accumulation of free fluid (P = 0.0367). Of 10 cycles, where more than 1 cm of fluid was observed, only one was associated with a low MPO concentration. However, samples considered free of liquid had variable MPO concentrations.

MPO concentrations in the uterine flush of mares presenting a hyperedema during estrus were highly variable, but showed no statistical difference compared with mares with a normal degree of edema, from grade I to III (P = 0.6382).

Table 1
Distribution of cytology results, presence of edema, and intraluminal fluid for the studied cycles.

	Cytology+	Cytology−	Fluid+	Fluid−
Hyperedema	2 (3%)	19 (28%)	3 (4%)	18 (26%)
Normal edema	6 (9%)	42 (61%)	7 (10%)	41 (59%)
Fluid+	6 (9%)	4 (6%)		
Fluid−	4 (6%)	55 (80%)		

Table 2
Myeloperoxidase median concentrations (ng/mL) in uterine lavage according to the clinical parameters studied.

	Positive	Negative
Cytology	7000[a] (899.335–>7000)	1128.7[b] (10.060–>7000)
Hyperedema	1121.9 (10.060–>7000)	1617.9 (13.93–>7000)
Uterine-free fluid	7000[c] (50.815–>7000)	1436.8[d] (10.060–>7000)
Pregnancy	1099.6 (21.055–>7000)	2552.3 (13.93–>7000)

Different superscripts within lines indicate statistical difference (P < 0.05).

3.3. Pregnancy

No association between pregnancy diagnosis and either the presence of fluid, hyperedema, or positive cytology results was observed. Although, nonpregnant cycles (54.2%) presented higher levels of MPO concentration, no statistical difference was observed (P = 0.0917).

4. Discussion

Ultrasonographic detection of intraluminal fluid has been reported to be an accurate indicator of endometritis [13,31], which is supported by the association between fluid and the presence of inflammatory cells in the present study. Intraluminal fluid observation is routinely used in practice as a tool to diagnose endometritis [32]. However, the diagnosis cannot solely rely on the presence of this clinical sign, as some mares will show intraluminal fluid without the presence of inflammation [4,33]. Similarly, the presence of excessive endometrial edema postbreeding has been considered as a clinical sign of endometrial inflammation [7,14].

An increase of the uterine blood perfusion occurs in mares under the influence of estrogens, resulting in an increase of endometrial edema. In most cases, appearance and disappearance of estrus-associated endometrial edema is progressive [34,35], but a percentage of mares present a grade of edema without concordance with the moment of the cycle or estrogen plasma concentration [14,29]. Hyperedema is characterized by visualization of thickened and prominent endometrial folds that have a hyperechoic border and marked hypoechogenicity at the center [14]. Excessive edema has been described as an indicative of uterine pathology when present during the normal estrus period [29], and associated positive cytologies have been found in 56.9% of cases in a study [14]. The study considered mares as having excessive edema either when it was observed at the time of breeding, or when normal edema had failed to regress 48 hours after breeding. Positive cytologies in that study were thus related to postmating endometritis. In our study, only 3% of mares presenting hyperedema were found positive for cytology, and no association was shown between those parameters. Part of this discrepancy may be attributed to false negative results for cytology [7,17,36]. However, because data on cytology and degree of edema were obtained, when a follicle of 35 mm was present (i.e., before breeding), our results suggest that hyperedema in early estrus is not related to

endometritis. Although preexisting endometritis explaining hyperedema before breeding should be considered, noninflammatory causes may account for hyperedema. An impaired uterine drainage because of cervical incompetence [37], excessive vascular response to estrogens, a lymphatic pathology or an altered myoelectrical activity [7,12], may also be responsible for this excessive edema.

One study has associated endometrial hyperedema and the presence of uterine fluid [14]. However, this was not observed in our experiment, and has not been confirmed by further similar reports in the literature. It may be hypothesized that observation of intraluminal fluid in the presence of excessive edema depends on the severity of drainage deficiency inducing the latter.

Results of samples collected on EDTA and dry tubes were considered indifferently, as MPO concentration of divided samples showed no significant difference. Although EDTA has been described as the best condition to maintain stable MPO concentration in blood samples by preventing blood coagulation leading to the stimulation of neutrophils, subsequent degranulation, and partial destruction [38], it can be assumed that the very nature of our samples (uterine flush vs. blood), and immediate centrifugation in our experiment prevented significant further neutrophils degranulation and alteration of measured MPO concentrations.

Myeloperoxidase was detected in uterine flushes from all estrus mares suggesting a physiological MPO presence during this phase of the cycle. To our knowledge, this is the first report about the presence of free MPO in the uterine lumen of mares. Myeloperoxidase concentrations observed in the equine uterus are highly variable. Minimal values were lower than plasma concentrations of healthy horses (169.7 ng/mL) [21], and maximal values were higher than bronchoalveolar lavage content of pathologic horses (500 ng/mL) [21] and plasma concentrations from horses with large intestine strangulated pathology (1169.46 ng/mL) [22]. Myeloperoxidase in equine frozen semen has been reported to be quite high (19050 ng/mL) [26], however, as no further dilution was performed to obtain a definite value, and high concentrations were recorded as above 7000 ng/mL, MPO in the uterine lumen in our study cannot be compared with those results.

Mares with a positive cytology had a significantly higher MPO concentration. This relation is not surprising as MPO, being contained in and release by neutrophils [23], is involved in the inflammatory reaction. Some individual mares that were negative for cytology also showed high concentrations of MPO. False negative results are mainly associated with the use of cotton swabs [39,40], and may explain these cases. Some pathogens seem to induce a limited acute neutrophilic response [36], and in chronic endometritis, neutrophils are present in deeper layers of the endometrium explaining negative swabs. A similar approach with uterine biopsies, which are accepted as a "gold standard" [17] for the diagnosis of endometritis, should be used to alleviate this limitation.

Alternatively, these levels of MPO might be the remaining trace of previous endometrial inflammation as a recent article has suggested a potential delayed release of MPO by noninflammatory cells after capture [41,42].

As already described, presence of intraluminal fluid is a tool for the diagnosis of endometritis [32]. A statistically significant ($P = 0.0367$) relation between fluid accumulation and MPO concentration was observed, confirming that both are markers of endometrial inflammation. Conversely, hyperedema was not related to positive cytology, intraluminal fluid, or MPO concentration in our study, suggesting it should not be considered as an indicator of endometritis. The relation between pathologic uterine condition and MPO concentration needs to be further studied to establish a threshold between physiological and pathologic MPO concentrations in uterine flushes.

Pregnancy diagnosis was not affected by any of the studied parameters. All mares of the study were part of a commercial breeding program, and benefited from intense management and treatments, possibly improving pregnancy rate despite uterine condition.

4.1. Conclusions

As a conclusion, this is the first report of MPO presence within the uterine lumen in the mare. Myeloperoxidase was observed in all estrus mares, suggesting a physiological role during that phase of the cycle. Presence of MPO during diestrus and anestrus still needs to be investigated.

High concentrations of MPO and intraluminal fluid accumulation indicate endometrial inflammation, but excessive edema does not. Hyperedema should only be seen as a marker of an underlying disorder interfering with edema resorption (e.g., decreased lymphatic drainage), potentially contributing to the further development of endometritis. Further work to establish the threshold between physiological and pathologic uterine concentrations of MPO in the mare is still needed to make it a practical tool in breeding management.

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