Dose-dependent inhibition of uterine contractility by nitric oxide: A potential mechanism underlying persistent breeding-induced endometritis in the mare

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ABSTRACT

Nitric oxide (NO) may have a role in persistent breeding-induced endometritis in mares through an inhibitory effect on uterine contractility. The objectives of this study were to test the effect of NO on spontaneous uterine contractility in vitro and to evaluate whether this effect varied between the longitudinal and circular muscle layers of the uterus. Reproductive tracts were collected from eight euthanized non-pregnant mares (age 4–19 years; body weight 405–530 kg). Transrectal examination of the reproductive tract was performed before euthanasia to evaluate stage of the estrous cycle and presence of any apparent abnormality. After euthanasia, one uterine tissue sample was collected for histological evaluation and four full-thickness uterine tissue strips (10–12 mm x 2 mm), two parallel to each muscle layer, were excised for in vitro contractility evaluation. Strips were suspended in tissue chambers containing Krebs–Henseleit solution, with continuous aeration (95% O2–5% CO2; pH 7.4) at 37 °C. After equilibration, spontaneous contractility was recorded (pre-treatment) and strips excised in each direction were randomly allocated to each of two groups: 1) SNAP (S-nitroso-N-acetylpenicillamine, an NO donor); or 2) NAP (N-acetyl-D-penicillamine, vehicle and time-matched control). These were treated at 15 min intervals with increasing concentrations (10^-7 M to 10^-3 M) of SNAP and NAP, respectively. Contractility data was recorded throughout the experiment. An interaction effect of group-by-concentration was observed (P < 0.0001). The mean contractility after treatment with 10^-4 M and 10^-3 M SNAP were significantly lower than the pre-treatment contractility and the mean contractility after treatment with lower SNAP concentrations. In contrast, contractility did not change significantly in the NAP treated controls. The effect of treatment on uterine contractility was not influenced by age or weight of the mare, stage of estrous cycle, uterine histology grade, or muscle layer. Secondary findings included significant main effects of stage of estrous cycle (increased contractility in estrus compared to diestrus), uterine histology grade (decreased contractility in grade IIB compared to grade I) and age (decreased contractility in mares aged > 8 years compared to mares aged ≤ 8 years). In conclusion, results of this study indicate that NO has a dose-dependent inhibitory effect on spontaneous uterine contractility irrespective of the muscle layer in the mare.

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1. Introduction

A mild transient endometritis which occurs after breeding in mares is a normal, physiological response and does not warrant any treatment [1]. In contrast, persistent breeding-induced endometritis (PBIE), where inflammation and intra-uterine fluid retention persist, has a significant negative impact on fertility. Considered as
a major reproductive problem in the mare [2], PBIE has been reported in about 15% of 746 estrous cycles in a Thoroughbred broodmare population [3] and in as many as 43% of 552 estrous cycles in a mixed population of mares [4]. In the latter study, mares with PBIE had a lower pregnancy rate compared to normal mares (49% versus 62%). It has been suggested that the reduced pregnancy rate in mares with PBIE could result either from a direct negative effect on embryonic survival or indirectly from premature luteolysis due to increased prostaglandin F2alpha (PGF2α) production [5].

Mares show variability in their susceptibility to persistent endometritis. In one study, some mares were able to efficiently resolve endometritis after experimental bacterial inoculation of the uterus while others were not [6]. More recent studies using intramuscular infusion of live [7] or killed [8] spermatozoa have reported similar findings. It has been demonstrated that susceptible mares exhibit delayed uterine clearance [9,10], which is believed to be a major factor in the development of persistent endometritis [11]. Using multiple site electromyography recordings of uterine activity, Troedsson et al. [12] showed that susceptible mares displayed impaired myometrial activity, characterized by reduced frequency, duration, and intensity of myometrial contractions. Mechanisms underlying the reduced myometrial activity in susceptible mares are still not completely understood. A possible mechanism was proposed by Rigby et al. [13] who, using an in-vitro model to measure isometric tension generated by longitudinal and circular uterine muscle strips in response to potassium chloride, oxytocin, and PGF2α, showed that susceptible mares have an intrinsic contractile defect of the myometrium. Interestingly, this contractile defect did not result from altered regulation of intracellular calcium ion concentration. The impaired uterine clearance could be restored by using ecbolic agents [14], leading Alghamdi et al. [7] to suggest that the reduced myoelectrical activity in susceptible mares represents an inhibition of contractility or induced relaxation rather than an intrinsic inability to contract.

A possible role of the nitric oxide system in the development of persistent endometritis has been suggested by Alghamdi et al. [7] and Woodward et al. [8] based on their findings that susceptible mares have higher amounts of nitric oxide (NO) in uterine secretions [7,8] and greater endometrial expression of inducible nitric oxide synthase (iNOS) after insemination [7,9]. Nitric oxide is synthesized in the body from L-arginine by the enzyme nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and iNOS (reviewed in Khan et al. [15]). Two of the isoforms, eNOS and iNOS, have been shown to be expressed in the equine endometrium [7,16,17]. The eNOS isoform is constitutively expressed and regulates vascular function whereas iNOS expression is typically upregulated during an inflammatory process [18]. In a study involving collection of uterine secretions and endometrial biopsy samples 13 h after insemination, it was observed that susceptible mares have higher amounts of NO in uterine secretions and greater expression of iNOS in the endometrium than resistant mares [7]. Higher amounts of uterine NO in untreated susceptible mares compared to resistant mares and a significantly greater increase in uterine NO production at 6 and 12 h post-insemination in susceptible mares were reported in a more recent study [8]. Considering the well-established relaxant effect of NO on smooth muscle tissues in general, and the previously documented relaxant effects on myometrium in other species such as rat [19], monkey [20], and human [21], it seems reasonable to speculate that NO may reduce uterine clearance in susceptible mares through an inhibitory effect on uterine contractility, leading to the development of persistent endometritis. The only documented evidence that NO may have an inhibitory effect on uterine contractility in the mare is the reported inability of myometrial tissue in vitro to respond to electrical stimulus in the presence of NO [22]. To our knowledge, the effect of different NO concentrations on spontaneous uterine contractility in the mare has not been investigated.

The objectives of this study were to test the effect of NO on spontaneous uterine contractility in-vitro and to evaluate whether this effect varied between the longitudinal and circular muscle layers of the uterus. It was hypothesized that NO would have a dose-dependent inhibitory effect on spontaneous uterine contractility irrespective of the muscle layer.

2. Materials and methods

All animal procedures in this study were conducted in accordance with the guidelines of the University of Guelph Animal Care Committee and conformed to the recommendations of the Canadian Council on Animal Care. The method of collection and processing of uterine tissue samples and the basic protocol for measuring uterine contractility in this study were similar to those used previously by Hirshbrunner et al. [23] for investigating spontaneous uterine contractility in the mare.

2.1. Collection and transportation of uterine tissue

Reproductive tracts were collected from clinically healthy non-pregnant light breed mares (N = 8) within 30 min of euthanasia using a standard protocol involving an intravenous overdose of pentobarbital sodium (Euthansol, Merck Animal Health Intervet Canada Corp, Kirkland, QC, Canada) at the Ontario Veterinary College, University of Guelph. The mares ranged in age from 4 to 19 years and in body weight from 405 to 530 kg. Transrectal palpation and ultrasonography of the reproductive tract were performed prior to euthanasia to evaluate stage of the estrous cycle and presence of any apparent abnormality. A mare was considered to be in estrus if she had a relaxed cervix, uterine edema, at least one follicle with diameter greater than or equal to 35 mm and no corpus luteum (CL) and in diestrus if she had a firm cervix, tonic uterus, no uterine edema and a CL. After euthanasia, the reproductive tract was visually examined for any gross abnormality and a tissue sample was collected from the base of the right uterine horn for histological evaluation. An 8–9 cm long, full thickness circumferential segment close to the base of the right uterine horn was excised and transported within 15 min to the muscle contractility laboratory in a flask containing Krebs-Henseleit solution (KHS) consisting of (in mM): NaCl, 118; KCl, 4.75; CaCl2, 2.54; MgSO4.7H2O, 1.18; NaHCO3, 24.8; KH2PO4, 1.18; Glucose, 10. All chemicals used in the preparation of KHS were purchased from Fisher Scientific, Waltham, MA, USA. The solution was kept at room temperature and pre-aerated with 95% O2–5% CO2 mixture to reach a pH of 7.3–7.4.

2.2. Preparation of uterine tissue strips and experimental protocol

The circumferential uterine segment was incised along its longitudinal axis and pinned flat in a dissecting dish containing Krebs-Henseleit solution continuously aerated with 95% O2–5% CO2. Full thickness uterine tissue strips of about 10–12 mm length and 2 mm width were dissected, using a custom designed scalpel with two parallel blades. Two strips were excised parallel to the direction of longitudinal muscle fibers and two strips were excised parallel to the circular muscle layers. Each strip was tied at the ends with 5.0 gauge suture silk and suspended in an individual organ bath containing 10 mL of warm (37°C) Krebs-Henseleit solution continuously aerated with 95% O2–5% CO2. The strips were attached to a fixed point at one end and an isometric force transducer (model FT03, Grass Medical Instruments, Quincy, MA, USA) at the other.
After a 15 min equilibration, 1 g tension was applied, followed 15 min later by another 1 g tension. Fifteen min after application of the second 1 g tension, spontaneous contractility data was recorded for 30 min in order to determine the pre-treatment contractility of the uterine tissue strips. The strips excised in each direction were then randomly allocated to each of two groups: 1) SNAP (S-nitroso-N-acetylpenicillamine, an NO donor; Sigma Aldrich, St. Louis, MO, USA); or 2) NAP (N-acetyl-o-penicillamine, vehicle and time-matched control; Sigma Aldrich, St. Louis, MO, USA). These were treated at 15 min intervals with increasing concentrations (10^-7 M to 10^-3 M) of SNAP and NAP, respectively. The experimental protocol is illustrated in Fig. 1. Contractility data was recorded throughout the treatment period and until 15 min after application of the last treatment. The data was recorded using the MP100WSW data acquisition system and AcqKnowledge 3 software (Biopac Systems Inc., Goleta, CA, USA) on a computer (ASUSTeK Computer Inc.). After each experimental run, length and weight of the tissue strips were measured.

2.3. Contractility data analysis

From the recorded data, cumulative area under the curve (AUC) during the 30 min pretreatment period and cumulative AUC during the 15 min period following each treatment were calculated using an in-built measurement function of the AcqKnowledge 3 software. The pretreatment cumulative AUC was divided by 2 to adjust it to 15 min. Cross-sectional area of the uterine tissue strips was calculated using the formula area = mass/(density × length) and a tissue density value of 1.056 g/cm³ [23]. The AUC values were then normalized by dividing them by the corresponding cross-sectional area. These normalized AUC values were used as a measure of contractility (dependent variable) in the statistical analyses.

2.4. Histological evaluation

Uterine tissue samples were fixed in 10% neutral-buffered formalin and submitted to the Animal Health Laboratory at the University of Guelph. The samples were processed by routine paraffin embedding followed by sectioning at 4–5 μm and staining with Hematoxylin and Eosin (H&E). Histological evaluation of the samples was performed in accordance with the grading scheme of Kenney and Doig [24] by a reproductive and board-certified pathologist (RAF) who was blinded to the contractility data.

2.5. Statistical analyses

Statistical analyses were performed using SAS software (version 9.3, SAS Institute, Inc., Cary, NC, USA). A linear mixed-effects model procedure (PROC MIXED) was used. Various models that included the main effects and interactions of mare age, body weight, stage of estrous cycle, uterine histology grade, muscle layer, group and concentration were fitted and tested using the Akaike Information Criterion (AIC) method. Various correlation error structures offered by the statistical software were applied to account for a potential autocorrelation between repeated measurements. The error structures included autoregressive 1, autoregressive heterogenous 1, toepplitz and banded toepplitz 2 through 7 as well as the heterogenous versions, and so-called unstructured and the banded versions unstructured 2 through 7. The application of unstructured correlation error structure yielded the lowest AIC (the best fit model). To assess the ANOVA assumptions, residual analyses were performed. The residuals were formally tested for normality by means of Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling tests. The residuals were also plotted against the predictive values and explanatory variables used in the model to identify any outliers, unequal variance, or other issues that would require further investigation. Assumptions of normality were satisfied by logarithmic transformation of the data. Post hoc analysis was performed using Least Significant Difference (LSD) test with the Bonferroni correction for multiple comparisons. Results are presented as back-transformed least squares means (LS-means) and 95% confidence intervals (CIs).

3. Results

Of the eight mares used in this study, three were classified to be in estrus and five in diestrus. No apparent abnormalities were detected in any of the mares either on transrectal examination of the reproductive tract before euthanasia or visual examination of the reproductive tract after euthanasia. Based on histological examination, one mare was classified as having a grade I uterus (normal), five mares were classified as having grade IIA uteri with mild inflammatory changes) and two mares as having grade IIB uteri (with moderate inflammatory changes). A total of 32 uterine tissue strips were used in the in-vitro contractility experiment. One strip excised parallel to the circular muscle layer and allocated to the NAP (control) group lost the tie at one end during the experiment and was excluded from the data analysis.

![Fig. 1](image-url) A schematic diagram of the experimental protocol used in this study to test the effect of nitric oxide on uterine contractility (Abbreviations: SNAP, S-nitroso-N-acetylpenicillamine; NAP, N-acetyl-o-penicillamine).
An interaction effect of group-by-concentration on uterine contractility was observed ($P < 0.0001$). The mean contractility after treatment with $10^{-4}$ M and $10^{-3}$ M SNAP were significantly lower than the mean pre-treatment contractility and the mean contractility after treatment with lower SNAP concentrations (Fig. 2a). In contrast, contractility did not change significantly in the NAP treated controls (Fig. 2b). The effect of treatment on uterine contractility was not influenced by the age or weight of the mare, stage of estrous cycle, uterine histology grade, or muscle layer (interaction of group with each of these factors was non-significant; $P > 0.05$).

Other findings included significant main effects of age ($P < 0.0001$), stage of estrous cycle ($P < 0.0001$), and uterine histology grade ($P = 0.0066$). The mean spontaneous contractility of uterine strips from mares greater than 8 years old (LS-mean 351.63; 95% CI 330.65, 372.61; $n = 114$ observations; $N = 5$ mares) was significantly lower than the corresponding value in mares aged 8 years or younger (LS-mean 975.12; 95% CI 923.76, 1026.50; $n = 72$ observations; $N = 3$ mares). The negative effect of age on uterine contractility was also evident on a correlation analysis between age and uterine contractility (Spearman’s correlation coefficient of $-0.616$, $P < 0.01$). The mean contractility was significantly greater in estrus (LS-mean 703.11; 95% CI 615.92, 790.30; $n = 72$ observations; $N = 3$ mares) than in diestrus (LS-mean 514.83; 95% CI 459.58, 570.08; $n = 114$ observations; $N = 5$ mares). With respect to the uterine histology, significantly greater uterine contractility was observed in grade I (LS-mean 812.34; 95% CI 543.98, 1213.09; $n = 24$ observations; $N = 1$ mare) compared to grade IIb (LS-mean

![Fig. 2. Contractility (LS-means and 95% CIs area under the curve) of uterine tissue strips before (pretreatment) and after treatment with different concentrations ($10^{-3}$ M to $10^{-4}$ M) of SNAP (a) or NAP (b). Each mean in the SNAP and NAP groups represents an average of 16 and 15 observations, respectively ($N = 8$ mares). Different letters indicate significant differences ($P < 0.05$).](image-url)
365.74; 95% CI 272.97, 490.06; n = 42 observations; N = 2 mares). However, the mean AUC of grade IIA (LS-mean 504.63; 95% CI 422.15, 603.22; n = 120 observations; N = 5 mares) was not different from either of the other two grades (I and IIB).

4. Discussion

The results of this study support the hypothesis that NO has a dose-dependent inhibitory effect on spontaneous uterine contractility irrespective of the muscle layer. These findings, taken together with the earlier observations that mares susceptible to PBIE have greater amounts of uterine NO [7,8], suggest a role of NO in the development of PBIE in mares. It is interesting that the inhibitory effect of NO on uterine contractility did not vary with age and uterine histology grade, as indicated by the non-significant interactions of group with age and uterine histology grade. It has been demonstrated previously that older mares and mares with higher uterine histology grades (IIB and III) are more susceptible to PBIE [25]. The absence of a group-by-age or a group-by-uterine biopsy grade interaction in the present study suggests that the greater susceptibility to PBIE in these mares may be due to higher uterine NO concentrations rather than increased uterine sensitivity to NO. However, further studies with mares of different ages and all four uterine histology grades are required to directly test these hypotheses. Similarly, the inhibitory effect of NO on uterine contractility was not influenced by stage of the estrous cycle based on observation of a non-significant group-by-stage interaction. While this suggests an absence of differential sensitivity of the uterine musculature to NO during different stages of the estrous cycle, it does not preclude a role of NO in regulating uterine contractility during the estrous cycle. There is evidence of a differential expression of NO isoforms across different stages of the estrous cycle [16,26,27]. However, studies on differences in the actual uterine NO concentrations between estrus and diestrus in the mare are lacking.

Studies on in-vitro uterine contractility in the mare have reported a wide range of percentages (0%–91.2%) of uterine tissue strips that showed spontaneous contractility [13,23,28,29]. Possible reasons for this great variability, such as use of anesthetized versus euthanized mares, individual variation and differences in survivability of tissue ex vivo, number of active receptors and muscle activity, have been proposed previously [23,29]. The higher percentage (100%) of uterine tissue strips demonstrating spontaneous contractility after placement in the organ baths in the present study may be partly due to the establishment of a very consistent protocol of tissue handling. Training in the uterine tissue handling procedures was undertaken and the experimental protocol was optimized using nine ovine and two porcine uteri prior to commencement of the study. Ovine and porcine uteri were used for training and optimization instead of equine uteri because of a greater local availability of ovine and porcine reproductive tracts.

The significant main effects of mare age and uterine histology grade on uterine contractility and the significant negative association of increasing age with uterine contractility observed in this study are consistent with the previously reported influence of age and uterine histology score on susceptibility of mares to PBIE [25]. The main effect of age on uterine contractility was tested in the present study with age as a continuous variable. The categorization of age was performed afterwards in order to compare the results with those of an earlier study [25] in which age was used as a categorical variable in the statistical analyses. Similarly, the analysis of correlation between age and uterine contractility was performed afterwards to further evaluate the main effect. Future studies with larger sample sizes at the mare level are needed to investigate the effects of age and uterine histology grade on uterine contractility in mares and the mechanisms underlying these effects. The significant main effect of stage of estrous cycle on uterine contractility (greater contractility during estrus as compared to diestrus) makes biological sense considering the functions of the uterus during the different stages of the estrous cycle. Greater contractility during estrus would aid spermatozoal transport to the site of fertilization and uterine clearance of dead spermatozoa and inflammatory debris after insemination [30]. Due to the small number of true biological replicates in the present study, the secondary findings related to effects of age, stage of estrous cycle and, especially, uterine histology grade on uterine contractility should be interpreted with caution.

An apparent limitation of this study is the use of an NO donor (SNAP) instead of authentic NO. However, the use of NO donors instead of NO is a very common and accepted practice in both in-vitro and in-vivo studies investigating the effects of NO on different biological processes. It is widely known that NO gas is difficult to handle, requires complete exclusion of oxygen to prevent its oxidation to nitrogen dioxide, involves a complex process during preparation of different concentrations and is highly unstable in solution [31,32]. When using NO donors, there is a possibility that the observed effect might be partly or fully due to breakdown products other than NO. To test this possibility, similar concentrations of NAP, the other breakdown product of SNAP besides NO, were applied to control uterine tissue strips in this study. The absence of a significant effect on uterine contractility at all the tested NAP concentrations rules out the possibility that the inhibitory effect observed in the SNAP treated group could have resulted from breakdown products of SNAP other than NO.

In conclusion, results of this study indicate that NO has a dose-dependent inhibitory effect on spontaneous uterine contractility irrespective of the muscle layer in the mare. The presence of increased NO concentrations in the uteri of mares susceptible to PBIE coupled with our findings that NO decreases uterine contractility constitutes a potential mechanism underlying development of PBIE in the mare.

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