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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 × 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% O_2–5% CO_2; 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.

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

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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 intrauterine 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]. Nitric oxide is synthesized in the body from L-arginine by the enzyme nitric oxide synthase that has three isoforms: endothelial 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 Hirsbrunner 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 nonpregnant 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; $CaCl_2$, 2.54; $MgSO_4$, 1.18; $NaHCO_3$, 24.8; KH_2PO_4, 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% O_2–5% CO_2 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% O_2–5% CO_2. 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 fibers. 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% O_2–5% CO_2. 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.